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Current developments in modelling the tumour microenvironment *in vitro*: Incorporation of biochemical and physical gradients

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ABSTRACT

Tumour cell proliferation, metabolism and treatment response depend on the dynamic interaction of the tumour cells with other cellular components and physicochemical gradients present in the tumour microenvironment. Traditional experimental approaches used to investigate the dynamic tumour tissue face a number of limitations, such as lack of biological relevance for the tumour microenvironment and the difficulty to precisely control fluctuating internal conditions, for example in oxygen and nutrients. The arrival of advanced *in vitro* models represents an alternative approach for modelling the tumour microenvironment using cutting-edge technologies, such as microfabrication. Advanced model systems provide a promising platform for modelling the physiochemical conditions of the tumour microenvironment in a well-controlled manner. Amongst others, advanced *in vitro* models aim to recreate gradients of oxygen, nutrients and endogenous chemokines, and cell proliferation. Furthermore, the establishment of mechanical cues within such models, e.g., flow and extracellular matrix properties that influence cellular behaviour, are active research areas. These model systems aim to maintain tumour cells in an environment that resembles *in vivo* conditions. A prominent example of such a system is the microfluidic tumour-on-chip model, which aims to precisely control the local chemical and physical environment that surrounds the tumour cells. In addition, these models also have the potential to recapitulate environmental conditions in isolation or in combination. This enables the analysis of the dynamic interactions between different conditions and their potentially synergistic effects on tumour cells. In this review, we will discuss the various gradients present within the tumour microenvironment and the effects they exert on tumour cells. We will further highlight the challenges and limitations of traditional experimental models in modelling these gradients. We will outline recent achievements in advanced *in vitro* models with a particular focus on tumour-on-chip systems. We will also discuss the future of these models in cancer research and their contribution to developing more biologically relevant models for cancer research.

1. Introduction

In recent years, views on cancer as a disease have shifted considerably. Cancer was long seen as a cellular disease, defined by events within the genome of tumour cells. However, due to our increasing knowledge, cancer is now regarded as a complex tissue that encompasses interactions between malignant and non-malignant cells as well as their surroundings (Bizzarri and Cucina, 2014). As a result, cancer research increasingly focuses on a deeper understanding of the wider tumour microenvironment (TME) and its role in tumour progression and treatment resistance (Tsai et al., 2014). An overview of the TME is provided in Fig. 1 and will be discussed in more detail below. The TME is exceptionally complex, containing a heterogeneous population of cells: both cancerous and various non-cancerous cell types (Balkwill et al., 2012). The latter are so-called stromal cells, which are recruited to the tumour site (Kidd et al., 2012). Stromal cells is an umbrella term describing cells from the immune system, endothelial cells and fibroblasts (Hanahan and Weinberg, 2011) (see Fig. 1). All these different cell types interact with each other, affecting cellular processes, such as proliferation, invasion and angiogenesis (Cirri and Chiarugi, 2011). In addition, stromal cells secrete chemokines and growth factors that play an integral role in tumour cell metastasis and response to chemotherapeutics (Conze et al., 2001). As such, tumour invasion and migration through the extracellular matrix (ECM), representing the non-cellular part of the TME, is highly influenced by the dynamic interactions between tumour cells and their stromal counterpart (Lu et al., 2012; Guo

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and Deng, 2018). The ECM consists of proteins, glycoproteins, and polysaccharides, providing biochemical cues as well as structural support and are essential for regulating cellular proliferation and migration (Walker et al., 2018; Hynes, 2009) (see Fig. 1). The biochemical properties of the ECM, such as the cell adhesion sites and degradable elements that are present throughout the ECM, allow tumour cells to interact with their environment directly. These signals regulate gene expression and may influence cellular behaviour (Hynes, 2009). They also enable tumour cells to remodel the ECM in response to certain cues, including a low oxygen environment or acidosis. In addition, the ECM rigidity, porosity, spatial orientation and overall physical properties affect the tumour cells' ability to transverse through the ECM and invade other tissues (Hartman et al., 2017). The ECM can also function as a physical barrier for drug penetration and, consequently, influences therapeutic efficacy (Minchinton and Tannock, 2006). Overall, with their dynamic functions and interaction within the TME, both stromal cells and ECM regulate cancer cell behaviour and exposure to drugs (Guerra et al., 2017; Plava et al., 2019). Another key characteristic of the TME is the presence of molecular gradients, such as oxygen, nutrients, but also administered therapeutics (Helmlinger et al., 1997; Carmona-Fontaine et al., 2017; Tredan et al., 2007) (see Fig. 1). These gradients develop mainly as a result of the disrupted homeostatic balance between tissue growth and blood vessel formation in solid tumours, where rapidly proliferating tumour cells in the TME trigger development of regions that have a limited supply of oxygen and nutrients (see Fig. 1). Molecular gradients are important influencers of the behaviour of cancer cells, affecting cell responses in terms of metabolism, proliferation, viability and drug sensitivity (Cairns et al., 2011) (see Fig. 1). Moreover, these microenvironmental gradients do not exist in isolation but may exhibit synergistic effects on tumour cells. For example, tumour cell migration and invasion are simultaneously influenced by oxygen, nutrients, pH, and the chemical concentration gradient of chemokines and growth factors (Zhou et al., 2017; Lewis et al., 2016; Muller et al., 2001). Therefore, it is essential to understand the dynamic interplay between these gradients and subsequent effects on cellular behaviour.

This review paper will discuss the molecular gradients present within the TME, their effects on cancer cell behaviour, and treatment resistance. We will elaborate on various experimental approaches to establish gradients for investigative purposes in cancer research. We will highlight the latest developments in TME modelling, focussing on advanced *in vitro* model systems that can recapitulate the cellular architecture of solid tumours. Here, the use of innovative engineering approaches and microfluidics to enable control of the cellular, chemical, and physical parameters surrounding tumour cells in a manner that resembles their native microenvironment will be discussed. We will examine how advanced models are used to gain deeper insights into the mechanisms of tumour progression, metastasis and chemotherapeutic resistance. Lastly, we will discuss the future of advanced *in vitro* models in cancer research and examine the different challenges and limitations that must be addressed before these models can be fully integrated in cancer research.

2. Microenvironmental gradients in solid tumours

2.1. Gradients of oxygen and pH

Oxygen is one of the most important regulators of cellular function (Fathollahipour et al., 2018). Levels vary within the human body. The oxygen levels in the brain average at 4.5%, whereas those in the lung alveoli measure around 14.5%. On average, healthy tissue has oxygen levels of around 5%. However, in certain diseases, of which cancer is a prime example, oxygen levels can be severely compromised, falling below the normal, physiological levels. Levels in cancer typically vary between 0.01 and 4.2% oxygen, and can show regional and temporal fluctuations within the TME (Jagannathan et al., 2016; McKeown, 2014). These regions with low levels of oxygen are known as hypoxia. The variability in oxygenation within solid tumours occurs as a result of the uncontrolled proliferation of tumour cells and the resulting abnormal vasculature (Carmeliet and Jain, 2000; Eales et al., 2016). A chronic form of hypoxia develops as the available vasculature continuously fails to provide an adequate oxygen supply to the tumour. This is a consequence of the increased intercapillary distance between the

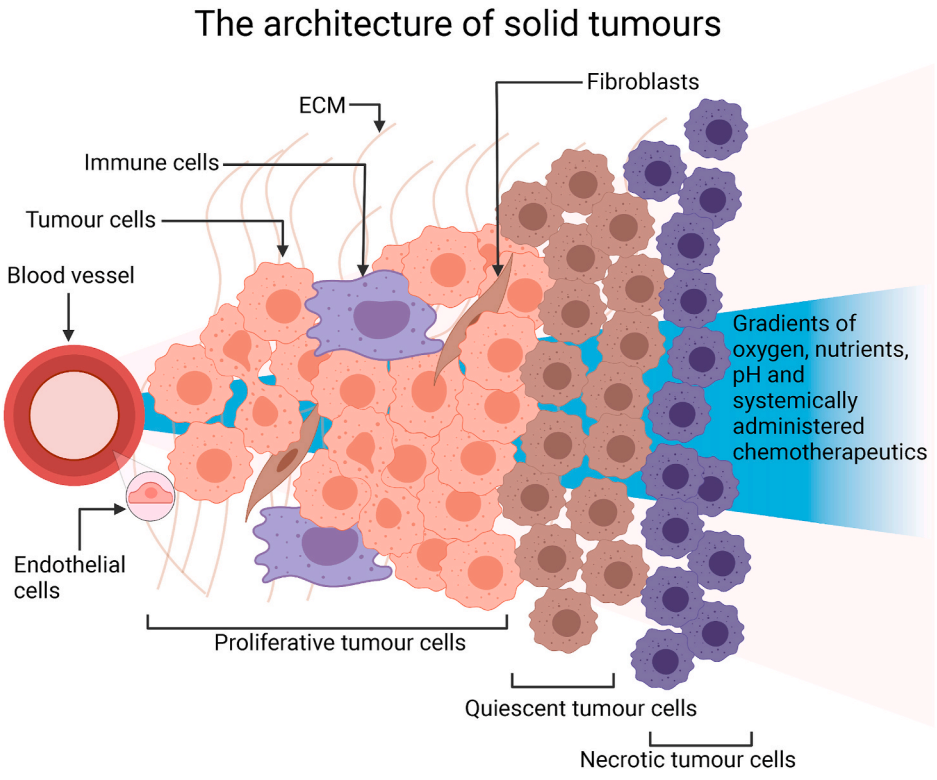

Fig. 1. The architecture of solid tumours. Solid tumours are heterogeneous both spatially and temporally. Within the tumour microenvironment (TME), tumour cells co-exist with stromal cells such as endothelial cells, immune cells and cancer-associated fibroblasts and together with the extracellular matrix (ECM), they constitute the cellular and non-cellular part of the TME. The microenvironment is also characterised by physical and chemical gradients, e.g. oxygen, nutrients, pH and biochemicals. These gradients develop due to the leaky vasculature of solid tumours in addition to the high proliferative ability of tumour cells. Tumour cells that reside near blood vessels typically have sufficient nutrients to proliferate, forming a proliferative ring around the vasculature. Cells further from the blood supply adapt their metabolic activity to the limited nutrients. These cells enter a state of cellular arrest, where they decrease or even completely stop their proliferation. This leads to populations of quiescent tumour cells. Furthermore, severe deprivation of oxygen and nutrients for prolonged periods of time leads to necrotic regions within the tumour niche.

vasculature and tumour cells being beyond the diffusion capabilities of oxygen (Michiels et al., 2016). This type of hypoxia is often associated with the formation of necrotic cores. Nevertheless, tumours are able to generate supporting vasculature via hypoxia-induced angiogenesis (Nussenbaum and Herman, 2010). However, the newly formed vessels are often disorganised and collapse easily (Michiels et al., 2016). This, in addition to vascular occlusion by cell aggregates, can result in dynamic changes in oxygen levels within the tumour (Jagannathan et al., 2016; Nussenbaum and Herman, 2010). As a result, so-called acute or intermittent hypoxia can arise, in which episodes of perfusion are alternated with oxygen deficiency (Dewhirst, 2009). Regardless of the hypoxia type, the severity of oxygen deficiency varies within the TME and displays gradient patterns (see Fig. 1). Tumour cells near the vasculature will have the largest amount of oxygen at their disposal. Due to the high metabolic demand of these cells, combined with the diffusion limit of oxygen, oxygen availability will steeply decline. Tumour cells located more distant from the blood vessels will have inadequate amounts of oxygen and as the tumour grows, the oxygen gradient may aggravate. On the other hand, spatiotemporal fluctuations in oxygen levels occur due to the intermittent perfusion cycles in the vasculature. The alternation between periods of hypoxia and reoxygenation was observed in patients and animal models (Trotter et al., 1989; Pigott et al., 1996). Both forms of hypoxia contribute to modifying cellular responses, as we will describe below (Saxena and Jolly, 2019).

Oxygen is recognised as an essential regulator of cellular processes in solid tumours. These processes can change dramatically to accommodate the decline in oxygen supply found in oxygen gradients (Michiels, 2004). The resulting tumour cell response is mediated via different signalling pathways. One of the most prominent pathways functions via the hypoxia-inducible factors (HIFs) (Denko, 2008). HIFs are transcription factors that are expressed in most cells in response to oxygen deprivation (Semenza and Wang, 1992). The best characterised isomer of this family is HIF-1α. Via activation of HIF-1α, oxygen gradients can promote tumour progression and metastasis (Eales et al., 2016). Several *in vivo* and *in vitro* studies show that tumour cells migrate from hypoxic regions toward regions with higher oxygen levels. For instance, when sarcoma cells, derived from a mouse model, were maintained in an oxygen gradient between 0.1% and 6%, they showed higher tendencies to migrate from hypoxic regions toward well-oxygenated ones (Lewis et al., 2016). Low oxygen levels increase the migratory potential of tumour cells by influencing epithelial-to-mesenchymal transition (EMT) (Muz et al., 2015a). EMT is a migratory process where tumour cells lose their cell-cell and cell-matrix adhesions. Hypoxia induces the expression of critical transcription factors that directly promote EMT initiation, such as Slug, Snail and Twist (Zhang et al., 2015a; Luo et al., 2011). Another important role hypoxia plays in tumour metastasis is the remodelling of the tumour ECM, enabling tumour cell invasion. Collagen is a major constituent of the tumour ECM (Hofbauer et al., 2003). Hypoxia regulates the expression of several procollagen proteins responsible for the hydroxylation and deposition of type 1 collagen (Gilkes et al., 2014). Cyclic hypoxia also affects tumour cell invasion and metastasis. For example, mice bearing KHT tumours were exposed to low oxygen conditions between 5 and 7% O_2 on daily intervals during tumour growth to recreate the effect of chronic and cyclic hypoxia. The results from the study showed that intermittent cycles of hypoxia and reoxygenation significantly increased the spontaneous metastasis formation of KHT murine tumours in secondary locations when compared to chronic hypoxia (Cairns et al., 2001). In addition, cyclic hypoxia was found to influence EMT directly. In MDA-MB-231 cells, cyclic exposure with 1% O_2 significantly induced the expression of the mesenchymal marker vimentin when compared to continuous hypoxia. Cyclic hypoxia also significantly increased the migration speed of MDA-MB-231 cells (Liu et al., 2017). In another study, intermittent hypoxia induced the expression of different mesenchymal markers, such as Snail and N-cadherin and decreased the expression of the epithelial markers ZO-1 and E-cadherin (Gupta et al., 2011). Low oxygen levels also induced genetic changes in tumour cells, resulting in a more aggressive and invasive phenotype with an increased ability to metastasise (Graham et al., 1999). This was evident when Waldenstrom macroglobulinemia cells were cultured under hypoxic conditions of 1% O_2 and injected into mice. These cells spread faster towards secondary organs than the cells initially cultured in atmospheric oxygen (21% O_2) (Muz et al., 2015b).

Low oxygen environments, either cyclic or chronic, can directly influence the tumour cell response to chemotherapeutics. For example, hypoxia enables tumour cells to tolerate a higher dosage of chemotherapeutic agents by inhibiting pro-apoptotic signalling pathways such as tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) (Rohwer and Cramer, 2011; Pei et al., 2010). TRAIL functions as an apoptosis-inducing cytokine that, upon activation, leads to cellular death. This makes TRAIL an interesting candidate for chemotherapeutics that can directly induce TRAIL action (Thorburn, 2007). However, tumour cell adaptation to hypoxia and activation of HIF-1α reduces TRAIL expression and allows tumour cells to tolerate higher levels of chemotherapeutic injury. Similarly, HIF-1α suppresses the expression of the p53 gene. In harsh microenvironmental conditions, such as hypoxia, p53 protein expression decreases in order to protect cells against apoptosis and enable their survival (Lakin and Jackson, 1999). Consequently, the efficacy of chemotherapeutics that induce the p53 apoptotic pathway is considerably reduced in the hypoxic TME (Rohwer et al., 2010). HIF-1α also promotes DNA repair mechanisms in tumour cells (Logsdon et al., 2016; Wrann et al., 2013). As DNA damage is one of the main mechanisms behind chemotherapeutic action, hypoxia reduces the efficacy of multiple chemotherapeutic agents (Yang et al., 2014; Feng et al., 2016). Biopsies from head-and-neck squamous cell carcinomas (HNSCC) showed a direct correlation between HIFs and treatment resistance. Samples with a higher expression of HIFs were more resistant to carboplatin than the samples with lower expression of HIFs (Koukourakis et al., 2002). To further demonstrate the crucial role of hypoxia in treatment resistance, the inactivation of HIF-1α in mouse embryonic fibroblasts increased their sensitivity to carboplatin and etoposide (Unruh et al., 2003). Furthermore, hypoxia affects the delivery of chemotherapeutics and their penetration into the tumour mass. Overexpression of HIF-1α activates multidrug resistance gene 1 (MDR1) (Comerford et al., 2002). MDR1 encodes the membrane resident P-glycoprotein (P-gp), an ATP binding cassette transporter that acts as a drug efflux pump (Hodges et al., 2011). Hypoxic activation of P-gp in mice was found to decrease the intracellular concentration of cytotoxic chemotherapeutics inside tumour cells by increased efflux (Gottesman et al., 2002). HIF-1 mediated activation of P-gp was observed in multiple cancer types, including colon cancer, gastric and breast cancer. Overall, hypoxia increases the resistance of tumour cells toward chemotherapy.

Tumour cells can adapt their metabolic activities to meet the insufficient oxygen supply and limited nutrients. One primary example is the reliance of tumour cells on glycolysis for energy production. HIF-1α induces glycolysis, the oxygen-independent glucose metabolic pathway, instead of the oxygen-dependent tricarboxylic acid (TCA) pathway (Al Tameemi et al., 2019). HIF-1α increases the glucose uptake rate through the induction of Glucose Transporter 1 (GLUT-1) and GLUT-3 (Maxwell et al., 2001). Once inside the tumour cell, glucose has several metabolic fates. However, HIF-1α induces phosphofructokinase 1 and aldolase, leading to the breakdown of glucose into pyruvate. In healthy tissue, pyruvate enters the mitochondria, oxidizing it to CO_2 and H_2O through oxidative phosphorylation. However, in hypoxic tumour cells, HIF-1α downregulates mitochondrial function, and as a result, pyruvate accumulates in the cytoplasm. HIF-1α also regulates pyruvate breakdown via lactate dehydrogenase A (LDHA), by increasing the expression and activity of LDHA. This in turn converts pyruvate to lactic acid, which is then released into the extracellular space (Eales et al., 2016). The increased efflux of lactic acid into the extracellular space reduces the pH in some parts of the TME. While the intracellular pH in tumour cells remains similar to normal cells, the extracellular pH varies and can fall to 6.2 (Boedtkjer and Pedersen, 2020). As a result, some parts of the TME

exhibit a pronounced gradient in pH. Similar to the oxygen gradient, the development of a pH gradient can drastically increase the invasive capacity of tumour cells and promote their metastasis. Several *in vitro* studies have shown that exposure of tumour cells to acidic pH increases the expression of metastasis promoting proteins, such as the matrix metalloproteinases (MMPs), MMP-2 and MMP-9 (Rohani et al., 2019). MMPs are pH sensitive, and they are activated in an acidic environment. MMPs are an important regulator of the metastatic process of tumour cells (Lu et al., 2012). They can degrade ECM proteins and remodel the ECM, resulting in the release of chemokines, growth and angiogenic factors, facilitating tumour cell metastasis, angiogenesis, and inflammation (Park et al., 1999; Mott and Werb, 2004). In addition, the acidic TME reduces the cellular uptake of weak base chemotherapeutic agents, such as mitoxantrone, and negatively influences their efficacy (Vukovic and Tannock, 1997). The acidic microenvironment can ionise these agents, leading to their accumulation in the extracellular space outside of their target areas, a process often called ion trapping (Mahoney et al., 2003). As such, the acidic microenvironment creates a physiological barrier for cellular uptake of weakly basic chemotherapeutics that accumulate in the intracellular space. In addition, chemotherapeutic activity of some agents is pH-dependent in terms of cellular uptake, and hence reduction of the TME pH would reduce their efficacy and eventually contributes to treatment failure (Wojtkowiak et al., 2011).

2.2. Gradients in cell proliferation

The TME is also characterised by the presence of cells with varying degrees of proliferative potential (Loddo et al., 2009). The cellular population within solid tumours is typically classified into proliferative, quiescent, and necrotic cells (see Fig. 1). During tumour growth, tumour cells divide rapidly, consuming oxygen and nutrients to maintain their proliferative status. Similar to oxygen, due to the disorganised vasculature of solid tumours, tumour cells that reside further from blood vessels have limited access to a steady supply of nutrients and oxygen and thus are restrained in their proliferation and metabolism. As a result, a necrotic zone forms where tumour cells are considered dead due to nutrient starvation and oxygen shortage (Gatenby and Gillies, 2004). In between the proliferative rim and the necrotic zone are cells that enter a quiescent state. In this intermediate layer, tumour cells adapt to declining nutrients and limited oxygen levels by altering their metabolic activities to minimise nutrient and oxygen consumption. As a result, a subpopulation of quiescent tumour cells arises. Quiescent cells have been observed in many types of cancer, such as liver and pancreatic cancer, breast cancer, acute myeloid leukaemia, melanoma, and glioblastoma (Zhu et al., 2017). The exact mechanisms and signalling pathways through which cells enter quiescence are not fully defined. Likely, the stress induced in the TME by hypoxia and acidosis is the main contributor (Smallbone et al., 2007). In general, cellular quiescence is a reversible state of dormant cells that may re-enter the cell cycle in response to certain stimuli. These stimuli include changes in the extrinsic microenvironment of quiescent cells, such as restoration of nutrients or changes in oxygen supply (Nakamura-Ishizu et al., 2014; Tannock, 1968). However, if nutrients and oxygen supply are restricted for prolonged periods, these quiescent regions would develop into necrotic zones. The presence of this proliferation gradient may suggest that within the heterogeneous TME of solid tumours, a viability gradient might exist. As nutrients and oxygen decline through the tumour niche, tumour cells become restricted in their viability.

Quiescent cells are characterised by their ability to reversibly arrest proliferation, escape chemotherapeutics and self-renew (Kim and Tannock, 2005; Subramaniam et al., 2014). As they have greater cell repair capacity than proliferating cells, quiescent tumour cells are less susceptible to cellular stress and toxicity (Masunaga et al., 2013; Pei et al., 2017). Cellular quiescence in the heterogeneous cellular population of solid tumours enables tumour cell survival after treatment, allowing for the repopulation of solid tumours (Kim and Tannock, 2005). Since most chemotherapeutic agents target actively proliferating cells, quiescent tumour cells naturally display an inherent resistance toward standard chemotherapy due to their cellular arrest in the G0 phase. As such, quiescent tumour cells represent a natural barrier to treatment success (Quayle et al., 2018). Repeated dosing of chemotherapeutics over longer periods of time has been suggested to play a role in quiescent tumour cell persistence and tumour repopulation. Following exposure, chemotherapeutics kill proliferative cells around blood vessels, enabling reoxygenation and restoring the nutrient supply to quiescent cells (Saggar and Tannock, 2015). For example, in breast cancer patients, quiescent tumour cells were found to resist chemotherapy and have a higher prevalence in patients with progressive disease than patients with primary tumours (Quayle et al., 2018). In between these intervals, quiescent cells can resume their proliferation and repopulate the tumour. In addition, repeated dosing can also induce quiescence in tumour cells, thereby reducing the efficacy of further treatment (Pei et al., 2017; Dey-Guha et al., 2011). An *in vitro* study showed that chemotherapeutics could directly promote quiescence in solid tumours. When breast cancer cells were exposed to tamoxifen, a proliferation inhibitor in hormone-sensitive breast cancer, expression of $p27^{Kip1}$ in cancer cells was induced, resulting in cell cycle arrest and treatment resistance (Carroll et al., 2003). Therefore, due to their location and inherent resistance to chemotherapeutics, quiescent cells present a challenge to treatment success.

2.3. Gradients of soluble factors and therapeutics

In parallel to gradients of oxygen and nutrients, local chemical concentration gradients of soluble endogenous factors and systemically administrated chemotherapeutics are also present in the TME. During cancer progression, tumour cells secrete chemokines to attract immune cells, fibroblasts and other stromal cells toward the tumour mass (Roussos et al., 2011). Once recruited, these cells secrete their own chemokines and growth factors, creating local gradients thereof. These stimulate the directional migration of tumour cells and drive metastasis. In response to an endogenous chemical gradient such as chemokines and growth factors, the directional cellular motility is often described as chemotaxis (Roussos et al., 2011). One of the most important and well-defined chemokines is the stromal cell-derived factor 1 (CXCL12) and its chemokine receptor (CXCR4). In breast cancer, the expression of CXCR4 is translated into increased invasiveness and metastasis of cancer cells (Muller et al., 2001). Interestingly, CXCL12 is mainly expressed and secreted by pericytes, which are perivascular cells that, once recruited to the tumour site, contribute to tumour growth and metastasis by inducing breast cancer cell chemotaxis (Muller et al., 2001; Ribeiro and Okamoto, 2015; Attwell et al., 2016). Other studies have shown that the CXCL12 gradient also mediates tumour cell invasion and metastasis in pancreatic cancer as well as ovarian cancer (Scotton et al., 2002; Koshiba et al., 2000). In addition, epidermal growth factor (EGF) also plays an integral role in directing tumour cell metastasis. Tumour-associated macrophages are responsible for the production of the EGF gradient, which initiates tumour cell migration through the tumour stroma (Wyckoff et al., 2004). Other stromal cells also create local chemokine gradients in the TME. For example, several *in vitro* studies showed the presence of fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), transforming growth factor β (TGF-β) and vascular endothelial growth factor (VEGF), which can all directly influence tumour cell chemotaxis (Roussos et al., 2011). These findings reveal the continuous and dynamic interaction between tumour and stromal cells present in the TME. Tumour cells can also initiate their own chemical gradients by degrading chemoattractants present in the TME. Muinonen-Martin et al. showed that increased invasiveness and metastasis of melanoma cells is mediated by the presence of a local chemokine gradient created by tumour cells. Tumour cells were shown to break down the lipid agonist lysophosphatidic acid (LPA), supplemented in the culture medium, creating an outward gradient of LPA. LPA is a pluripotent lipid mediator that

regulates growth, motility and differentiation in tumour cells. LPA acts as a potent chemoattractant for melanoma cells in all tumour stages, and accordingly, tumour cells facilitate directional migration in response to the LPA gradient (Muinonen-Martin et al., 2014).

In addition to gradients of endogenous molecules, systemically administered therapeutics also display gradients in the local concentration (Minchinton and Tannock, 2006; Lankelma et al., 1999). In part, this is due to the diffusion limit of therapeutics through the tumour tissue as a result of inadequate vasculature. The high proliferative ability of tumour cells and the consequent overexpression of pro-angiogenic factors promote the formation of new vasculature via angiogenesis. The new vasculature is disorganised, extremely heterogeneous with collapsed or blunt-ended vessels and anatomically different from normal blood vessels. The absence of supporting structures such as basement membranes and perivascular cells, in addition to the poorly aligned endothelial cells results in porous blood vessels that are leaky and more permeable (Kalyane et al., 2019; Wu, 2021). This enhanced permeability of tumour vasculature leads to an increased drug concentration in cells near blood vessels, whereas cells further from the blood supply experience lower concentrations (Durand, 2001). *In vivo* tracking of doxorubicin in a xenograft model showed a gradient where a higher drug concentration is detected in the periphery of the tumour. A steep decline in doxorubicin is seen further going into the tumour mass (Lankelma et al., 1999). As such, the limited distribution of chemotherapeutic agents in the tumour niche substantially increases tumour cell resistance.

Overall, microenvironmental gradients are important determinants for cell behaviour, affecting cancer cell proliferation, migration, metabolism and tumour response to treatment. As they are a consequence of a disorganised architecture of solid tumours, all these gradients are coupled and co-exist. Therefore, it is essential to analyse the close interplay between these gradients and how they influence cellular processes.

3. Gradients in the TME modelled in pre-clinical systems

The TME of solid tumours shows a continuous and dynamic interaction between tumour cells, stromal cells, ECM, and molecular gradients. Such a dynamic tissue needs dedicated and purpose-built *in vitro* model systems in order to study it in a manner that is relevant for human disease. Traditional model systems are unable to fully provide this. For example, animal models are less versatile in controlling molecular gradients. Gradients present within xenografted tumour models can be controlled in their magnitude only to a limited extent, for example, by varying tumour size, oxygen levels to which the animals are exposed, or by reducing the blood flow to the tumour (De Bruycker et al., 2018). In addition, the translation of these models to the human situation is hampered by the reported low predictive ability (Mak et al., 2014). There are crucial differences in genetic, metabolic and cellular responses between humans and animal models, affecting the suitability of animals as a representative model (Schuh, 2004). *In vivo* animal models also remain inadequate when systematically investigating the interaction of the various components of the TME. While patient-derived xenografts can better recapitulate the complexity of human conditions than *in vitro* models, the microenvironment within laboratory animals differs from the original patient microenvironment regarding ECM, cellular interactions, and the immune system as it is inevitably of animal origin (Pompili et al., 2016; Kondo, 2020). In Fig. 2A, we provide an overview

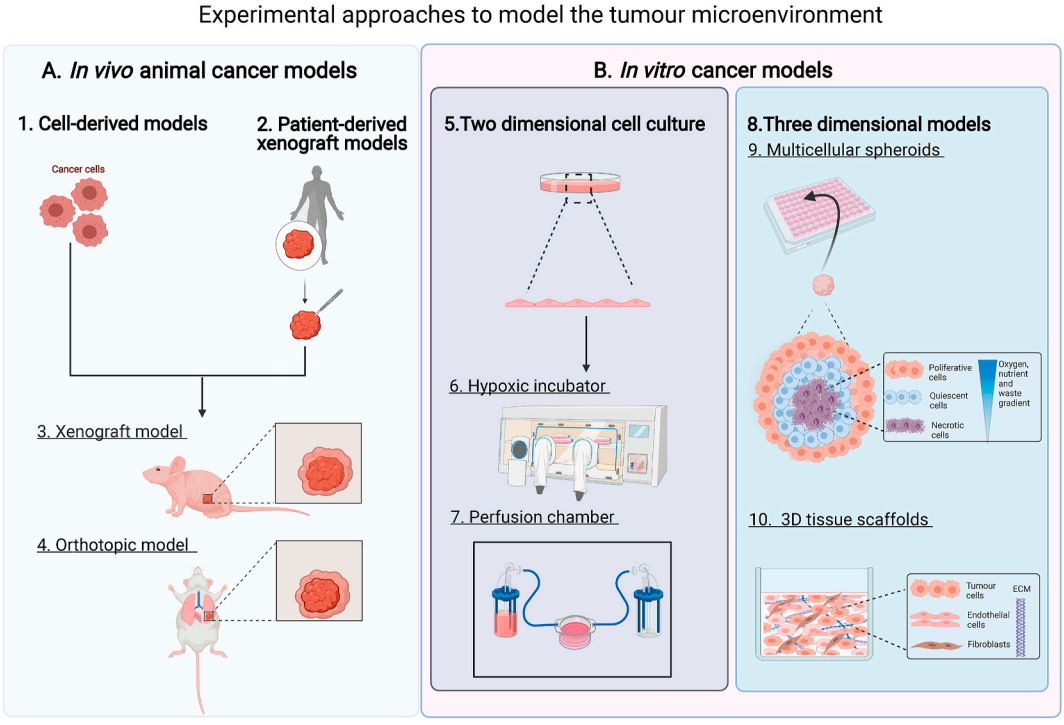

Fig. 2. Experimental approaches to model the tumour microenvironment. Typical approaches rely on A) *In vivo* animal models to create an experimental model that can capture the complexity of the TME. Animal models are either derived from (1) cancer cell lines or from primary patient material in (2) patient-derived xenografts (PDXs). Cancer cells and human tumour material can be injected or transplanted subcutaneously in immunocompromised animals to generate a (3) xenograft model or injected at the original tumour site to create an (4) orthotopic model. B) *In vitro* approaches include (5) Two-dimensional (2D) cell culture. These models mainly rely on growing cells on flat surfaces at atmospheric oxygen levels of 21%. In order to mimic the physicochemical gradients of the TME, tools such as (6) hypoxic incubators and (7) perfusion chambers to generate the oxygen conditions of human tumours can be utilized. (8) Three-dimensional (3D) model systems can inherently mimic parts of the TME. (9) Tumour spheroids are characterised by the presence of a proliferation gradient with proliferative and quiescent cells, and when spheroids grow large enough, a necrotic core develops. The cellular architecture of multicellular spheroids creates oxygen and nutrient gradients. (10) 3D tissue scaffolds, where tumour cells can be co-cultured with stromal cells such as endothelial cells or fibroblasts in a 3D hydrogel that can resemble the native ECM *in vivo*.

of *in vivo* animal models.

At the other end of the spectrum, monolayer cell cultures represent one of the simplest *in vitro* models, typically consisting of a single cell type (see Fig. 2B, number 5). These two-dimensional (2D) models offer the potential to fully control incorporated microenvironmental conditions in their designs. However, they tend to control one condition in the TME at a time to study associated effects on tumour progression or treatment efficacy. This does not fully reflect the complex interplay within the microenvironment found *in vivo*. Precisely replicating and controlling gradients from the TME *in vitro* remains challenging. For example, monolayer cell cultures are often maintained in the regular atmospheric pO_2 of approximately 21% O_2, whereas the median O_2 level of breast cancer, for instance, is 3.6% O_2 (Jagannathan et al., 2016; Das et al., 2008). Overall, the level of oxygenation is important as changes in oxygen tension from physiological levels to the complete absence of oxygen trigger different biological responses. Recent technological advances have allowed the development of more accurate methods to mimic the oxygen levels of the TME. Hypoxic incubators and perfusion chambers are often used to recreate the effect of hypoxia on cellular processes in solid tumours (see Fig. 2B, number 6 and 7). Hypoxic incubators are cell culture incubators that maintain and grow cells at the desired oxygen concentration (Liu et al., 2021) (see Fig. 2B, number 6). On the other hand, perfusion chambers can control the oxygen levels in the cellular compartment by limiting oxygen diffusion from the atmosphere and introducing a maintenance medium with pre-defined oxygen concentration (Brennan et al., 2014) (see Fig. 2B, number 7). These tools have provided the opportunity to study the effect of hypoxic conditions in great detail. However, they typically lack spatiotemporal variations in oxygen, which does not replicate the oxygen gradients that are present *in vivo*. As a result, the role of the oxygen gradient in the TME remains underexplored. Furthermore, the experimental setup of monolayer cultures requires cells to adhere to a flat surface and thus lack three-dimensional growth properties. Adapting to 3D cell culture models provides more physiologically relevant models as they are significantly different from 2D cell culture systems in terms of protein expression, proliferation profiles, and therapy sensitivity (Griffith and Swartz, 2006; Yamada and Cukierman, 2007). Investigation of the other gradients present in the TME, and their overall integration requires similar strategies.

3.1. Shifting towards three-dimensional cell culture models

The introduction of three-dimensional (3D) cell culture models, such as multicellular tumour spheroids and 3D tissue scaffolds, have provided the opportunity to cultivate tumour cells in more physiologically relevant microenvironments (see Fig. 2B, number 8). Multicellular tumour spheroids, for example, display many characteristics found in the TME, such as oxygen and nutrient gradients, proliferation gradients accompanied by a quiescent region and acidosis (Nunes et al., 2019; Nagelkerke et al., 2013) (see Fig. 2B, number 9). When grown large enough, a necrotic core becomes apparent. Spheroids can also reproduce part of the TMEs cellular heterogeneity by co-culturing tumour cells with stromal cells such as endothelial cells, fibroblasts and immune cells. 3D cell models can also recapitulate vital interactions in the TME, such as cell-cell and cell-ECM interactions. In 3D tissue scaffolds, where tumour cells are suspended in a hydrogel matrix, tumour cells can reside in an environment similar to the native ECM *in vivo* (Loh and Choong, 2013) (see Fig. 2B, number 10). This bioengineered tissue allows tumour cell proliferation and migration in addition to the exchange of nutrients, chemokines and oxygen. In this regard, 3D cell culture models can capture the cellular adaptation of tumour cells to the conditions in the TME and, more importantly, study the fate of therapeutics inside the tumour. Compared to monolayer models, cell growth in 3D promotes changes in gene expression patterns and drug response (Stock et al., 2016). For instance, doxorubicin showed higher cytotoxic activity against MCF-7 when cultured in monolayer than 3D spheroids. MCF-7 spheroids showed an 80-fold increased resistance to doxorubicin than cells in monolayer (Gong et al., 2015). In addition, when MCF-7 cells were cultured in monolayer systems, cells showed behavioural patterns that are different from those witnessed *in vivo*. The expression levels of the glucose transporter GLUT-1 were two-fold lower in MCF-7 when cultured in monolayer compared to *in vivo*, suggesting that monolayer culture systems show different metabolic activity to that seen *in vivo* (Pereira et al., 2017).

Nonetheless, neither spheroids nor 3D tissue scaffolds can fully recreate the complex 3D architecture of solid tumours. They lack the tissue-tissue interface that is crucial for several key processes in tumour progression, such as immune cell recruitment. Moreover, both models lack the ability to fully control the extent of the molecular gradients present within their cellular microenvironment. In addition, the absence of vasculature within these models means that cells do not experience the mechanical cues that influence tumour cells *in vivo* (Guan et al., 2015; Heldin et al., 2004). A further challenge is to perform investigative assays in spheroids. Real-time analysis and direct visualisation of cells in the microenvironment and/or tracking drug effects is difficult, especially as spheroids become larger (Ding, 1997; le Roux et al., 2008). Furthermore, the limited investigative capacity of spheroids presents another challenge (Ding, 1997; le Roux et al., 2008; Holub et al., 2020). Multiple experimental interventions were developed to enhance the investigative capability of spheroids, such as sequential trypsinisation and disaggregation at different time periods to allow the collection of cells from the different cell layers of the spheroid (McMahon et al., 2012). However, none of these approaches provides a rapid method to collect cells at a spatially defined oxygen concentration. As such, accurate estimates of metabolic adaptation to low oxygen levels are difficult in these models. Therefore, a coherent explanation of mechanisms underlying cancer development and treatment resistance relying solely on 3D models as an investigative model is challenging.

3.2. Advanced in vitro models

Advanced *in vitro* models are the new frontier in cancer research. These model systems aim to replicate the dynamic microenvironment of solid tumours and the molecular gradients found in the TME with great precision and accuracy. Often a bottom-up engineering approach is taken to reproduce the cellular architecture, tissue-tissue interface, and the physiochemical gradients of the TME within these models (Bhatia and Ingber, 2014). This approach also allows advanced models to function as dynamic culture systems with control of the different microenvironmental conditions while simultaneously allowing analysis of how these contribute to cancer progression and treatment efficacy. By integrating microfabrication, microfluidics technology, and tissue engineering approaches, advanced *in vitro* models can exert precise control over the cellular, physical, and chemical parameters of their microenvironment. Microfabrication techniques such as 3D printing, micro-milling, and soft lithography allow these models to recapitulate the complex microstructure of solid tumours (Asano and Shiraishi, 2015; Campillo et al., 2019; Rexius-Hall et al., 2017; Rodenhizer et al., 2016; Wulftange et al., 2019). Advanced models often include channels and membranes to establish a dynamic flow and molecular movement within their designs (Ho et al., 2015; McDonald and Whitesides, 2002; Sosa--Hernandez et al., 2018). In addition to tissue engineering technology, fabrication techniques allow advanced *in vitro* models to adopt the spatial arrangement of the cellular components of the TME (Buchanan et al., 2014; Song and Munn, 2011). Tissue engineering techniques such as bioprinting and the use of biomaterial scaffolds enable advanced *in vitro* models to recreate the cellular 3D architecture of solid tumours, thereby improving their accuracy while investigating the influence of the TME on different cellular processes (Yang et al., 2017). Furthermore, the integration of microfluidics technology has enabled advanced *in vitro* models to mimic the physicochemical gradients of the TME (Mao et al., 2020; Haessler et al., 2012; Hassell et al., 2017). Microfluidics offers the

advantages of spatiotemporal control of both cellular components and fluidic conditions at the microscale. Microfluidics techniques can effectively establish controllable gradients throughout the biological model, such as oxygen and chemical gradients. For example, by limiting oxygen diffusion, advanced models have better control over the hypoxic conditions within their design and can recapitulate the spatial and temporal variations in oxygen levels that occur *in vivo* (Gao et al., 2019; Chen et al., 2011; Koens et al., 2020). Advanced *in vitro* models can also recreate the chemical environment of solid tumours by controlling the diffusion and supply of nutrients as well as introducing growth factors and chemokines (Kim et al., 2013). In addition to oxygen and chemical concentration gradients, the use of microfluidic systems provides precise control of pressure and shear stress. These features have made it possible for advanced *in vitro* models to emerge as a promising tool for scalable gradient-based cellular studies. One representative example of these advanced models is the tumour-on-chip model. Microfluidic tumour-on-chip models are perfused cell culture systems with micro-fabricated structures, populated by tumour and stromal cells, that aim to recreate the interactions and functions that occur in the TME *in vivo*. The use of on-chip technology combined with microfluidics allows tumour-on-chip systems to serve as a flexible, tuneable platform, where experimental manipulation and integration of several investigative assays are also possible. For instance, a microfluidic chip was able to reproduce an invasion assay while maintaining spatial and temporal oxygen control over the chip (Acosta et al., 2014). Microfluidic chips can also integrate sprouting assays to understand the effect of different microenvironmental conditions on angiogenesis and tumour progression (Lam et al., 2018). As a result, advanced *in vitro* models are starting to advance cancer research.

Below we will discuss the approaches advanced *in vitro* models have taken thus far to recreate the different microenvironmental gradients and how these models were used to study key events in cancer progression. We will focus on the analysis of chemotherapeutic distribution and efficacy, chemotaxis, angiogenesis, and tumour cell invasion and migration. First, we will discuss the development of oxygen gradients in advanced models where tumour cells are cultured in an oxygen concentration that resembles the *in vivo* situation. We will further describe the ability of advanced models to recreate the biochemical cues and endogenous chemical concentration gradients that are present within the TME.

3.2.1. *Oxygen gradients in advanced in vitro models*

With advanced *in vitro* models, relevant oxygen levels in stable gradients can be established for cellular studies. Oxygen gradients can be achieved via cellular consumption, diffusion from a gas source through oxygen impermeable materials or chemical reaction via oxygen-generating and oxygen-scavenging chemical reactions (Peng et al., 2013). An overview of these methods is illustrated in Fig. 3A. Via cellular consumption, advanced *in vitro* models can create controllable oxygen gradients by changing the cellular density of their design. The high cellular density and, therefore, high consumption rate leads to an oxygen and nutrient gradient within the cellular compartment (Mehta et al., 2007) (see Fig. 3A, number 1). One of the most prevalent methods to create oxygen gradients within advanced models is by controlling oxygen diffusion from a gas source through a gas permeable material such as polydimethylsiloxane (PDMS). By controlling PDMS thickness in the interface, advanced models can control the amount of oxygen that diffuses to the cellular compartment, creating a stable oxygen gradient (Nam et al., 2020) (see Fig. 3A, number 2). The use of oxygen-generating chemical reactions also allows these models to achieve desirable oxygen levels for cellular studies. Advanced models can create oxygen gradient in the cellular compartment via an oxygen generating reaction such as (H_2O_2 + NaOCl) and an oxygen scavenging reaction (NaOH + pyrogallol) in the channels (Lam et al., 2018; Shih et al., 2019) (see Fig. 3A,

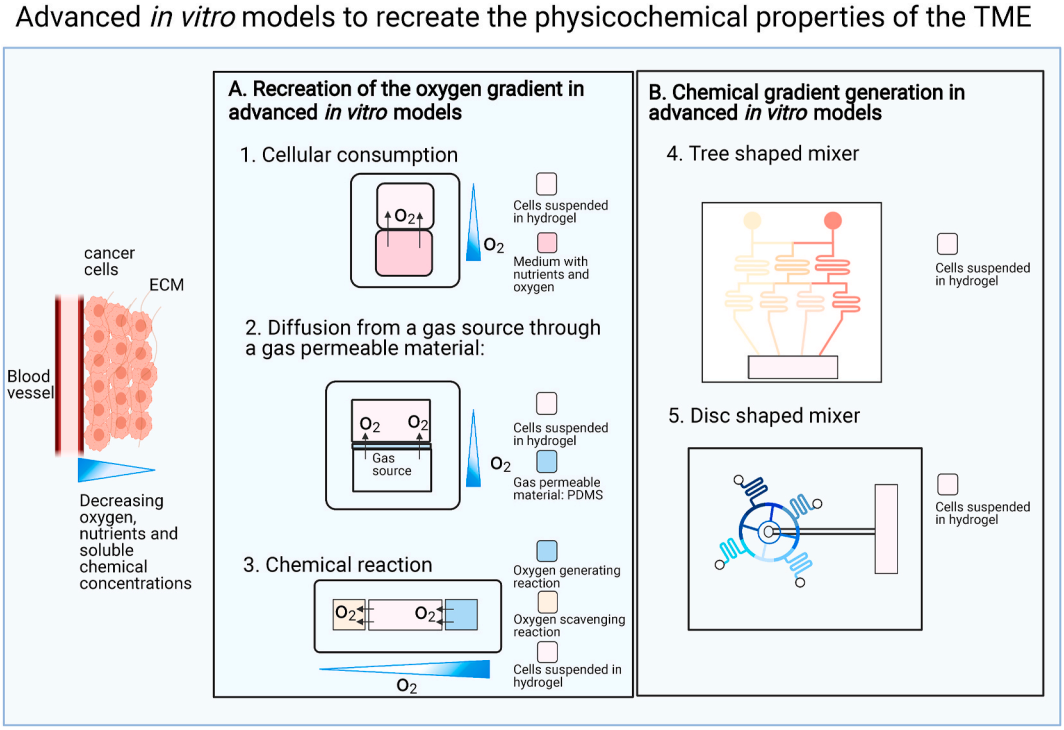

Fig. 3. Advanced *in vitro* models to recreate the physicochemical properties of the TME, such as oxygen and chemical concentration gradients. (A) Several methods have been developed to generate and control the oxygen gradient with advanced *in vitro* models, either via (1) cellular consumption, (2) diffusion from a gas source through a gas permeable material such as polydimethylsiloxane (PDMS) or (3) a chemical reaction where the oxygen gradient is created in the cellular compartment via an oxygen generating reaction. (B) Advanced *in vitro* models can also generate stable chemical concentration gradients by using a chemical concentration generator (CGG), such as (4) tree-shaped mixers and (5) disc-shaped mixers, that can create a chemical concentration gradient by introducing chemicals of different concentrations through different inlets. By thorough mixing and splitting a stable chemical concentration gradient is created.

number 3).

These approaches provide accurate control of oxygen levels throughout the experimental setup and enable advanced models to approach the spatial and temporal variation of oxygen that occur in human cancer *in vivo*. Advanced models also allow for real-time imaging and analysis of the changes induced by oxygen in the cellular compartment. As a result, these models can explore areas in cancer research that were previously challenging. To date, the mechanisms behind tumour cell adaption to the dynamically changing oxygen levels have not been fully elucidated. Few studies have been able to track the effect of oxygen gradient in terms of cellular metabolism, gene expression, protein levels and drug response. Rodenhizer et al. developed a model system to study the effect of oxygen gradients on the cellular metabolism of SKOV-3 ovarian adenocarcinoma cells, which they named TRACER (Rodenhizer et al., 2016). Cells were cultured on collagen layers and rolled like a movie strip to generate a thick 3D tissue. Oxygen and nutrient gradients were created in this design via cellular consumption (see Fig. 3A, number 1). The high cellular density created a declining oxygen concentration throughout the TRACER device, with hypoxic regions in the middle of the roll (Rodenhizer et al., 2016). Within this engineered tissue, collecting cells from different locations at spatially defined oxygen concentrations was possible by simply unrolling the strip. These features have made it possible to analyse the metabolic changes that occur throughout the oxygen gradient as cells were adapting to their hypoxic environment and limited nutrients. In the hypoxic regions of the TRACER, tumour cell reliance on glycolysis was clear, as lactate, hexose and alanine were detected. The reduction of glutathione (GSH) signified hypoxia-induced oxidative stress in the engineered tissue was also present. In addition, the model showed that tumour cell metabolism was highly dependent on HIFs. This was evident by the deregulation of tumour cell metabolism in SKOV-3 shHIF-1 knockout cells (Rodenhizer et al., 2016). In another advanced model, Wulftange et al. have been able to characterise the effect of an oxygen gradient on cellular growth and proliferation (Wulftange et al., 2019). An oxygen gradient between 0 and 3% O_2 was created by regulating oxygen diffusion from a gas source through a PDMS membrane (see Fig. 3A, number 2). Ovarian cancer cells (OVCAR-8) or breast cancer cells (MCF-7) were embedded in Matrigel and cultured inside a bioreactor (Wulftange et al., 2019). The bioreactor consisted of a customised six-well plate with a PDMS membrane acting as an oxygen delivery system. The culture plate was placed above a 3% oxygen source in a chamber maintained at 0% O_2, allowing a 0–3% oxygen gradient to establish. Tumour cells showed higher proliferation rates in gradients when compared to cells grown in a continuous oxygen level of 21% or 3% O_2. RNA analysis showed increased expression levels of genes involved in proliferation when cells were cultured in an oxygen gradient compared to continuous 3% O_2 (Wulftange et al., 2019). Further comparison revealed downregulation in genes involved in metabolism and upregulation of genes that mediate migration and mobility (Wulftange et al., 2019). This study showed that not only accurate representation of oxygen levels is essential for cancer modelling, but also whether oxygen is delivered uniform or as a gradient. These results may not have been achieved without the use of advanced *in vitro* models, demonstrating their capability to analyse the underlying cellular processes in cancer development.

A microfluidic model containing HCT-116 colon cancer cells, similarly revealed that in addition to an oxygen gradient, other microenvironmental gradients such as of nutrients and pH influences tumour cell proliferation and viability. HCT-116 were suspended 3D in a hydrogel and were allowed to create their oxygen gradient via cellular consumption. The medium was supplied at one side of the design. As such, the cells were also able to create their nutrient and pH gradients as waste and metabolic by-products accumulated inside the bioengineered tissue. Immunofluorescence imaging showed the presence of a proliferation gradient within the 3D cell culture, with the distinct proliferative, quiescent, and necrotic regions. Gene expression analysis showed that in the areas with low oxygen concentrations, tumour cells had a lower expression of genes related to proliferation and DNA repair. These cells also had a higher expression of genes related to stress and survival (Ayuso et al., 2019). A more recent report of this microfluidic model unveiled the effect of these microenvironmental gradients on immune cells response inside the TME. A co-culture of MCF-7 breast cancer cells with natural killer (NK) immune cells maintained in an oxygen gradient uncovered how the TME could suppress the immune response by NK cells by regulating the expression of genes involved in their proliferation and activation. For instance, gene analysis showed that at low oxygen and nutrient regions of the device, NK cells downregulated expression of genes associated with proliferation and activation, such as GZMB (Granzyme B) and interleukin-15. Further analysis showed upregulation of stress markers, such as IDO-1 (Indoleamine 2,3-Dioxygenase 1) and VEGF, suggesting that microenvironmental gradients can decrease the immune cell response, allowing tumour cells to escape immune cell surveillance (Ayuso et al., 2021).

In another model, immune cell recruitment towards tumour cells was found to be dependent on the level of oxygenation in the tumour mass (Campillo et al., 2019). The model consisted of a modified transwell system where a differential oxygenation level between the bottom and upper compartment was achieved via oxygen diffusion through a permeable membrane. In the upper compartment, mouse macrophage cells were maintained in oxygen levels between 16 and 19% O_2, while tumour cells were cultured in the bottom compartment at an oxygen level of 1% O_2 (Campillo et al., 2019). Macrophages showed a higher migratory capacity when co-cultured with hypoxic tumour cells in comparison with oxygenated tumour cells. Gene expression analysis showed that both breast cancer and melanoma cells had a higher expression level of VEGFA and PTGS2 genes, suggesting that these two genes might regulate macrophage recruitment via paracrine signalling (Campillo et al., 2019).

Advanced models have also been used to understand the parameters that initiate and contribute to tumour cell migration and invasion. Tumour cell metastasis is a complex, multistep process driven by both the genotype of tumour cells and the extrinsic conditions in the TME (Hunter et al., 2008). Several *in vivo* and *in vitro* studies revealed that metastasis is driven by the dynamic interaction of the different components of the TME, which exhibit a synergistic effect on tumour cell migration and invasion. This was evident when microfluidic models were employed to examine the role of oxygen either individually or in combination with other microenvironmental factors on tumour cell migration. For example, Sleeboom et al. cultured MDA-MB-231 breast cancer cells and their cancer stem cells (CSCs) in an oxygen gradient between <1% and 21% O_2 in a microfluidic chip. The oxygen gradient was maintained by perfusing an oxygen scavenging sodium sulphite (Na_2SO_3) solution through a microchannel adjacent to the cellular compartment containing MDA-MB-231 and their CSCs (see Fig. 3A, number 3). Both cellular populations migrated towards regions with lower oxygen concentrations (Sleeboom et al., 2018). Similarly, the directional migration of A549 lung carcinoma cells was also toward lower oxygen concentration in another microfluidic chip design (Chang et al., 2014). However, when Mosadegh et al. examined the effect of an oxygen gradient in addition to nutrients on A549 cell migration in a paper-based model, the directional migration of tumour cells was toward higher oxygen concentrations. In this model, tumour cells were suspended in hydrogel and placed on paper coated with hydrogel. The gradients were established by oxygen and glucose diffusion through the multilayer layers of the model in addition to cellular consumption. The migration speed of A549 increased significantly when tumour cells were subjected to a nutrient gradient, suggesting that tumour cell migration is also triggered by their metabolic needs (Mosadegh et al., 2015). Takahashi et al. also showed that the formation of acidic metabolites and consequently the development of a pH gradient influences the directional migration of tumour cells. When MDA-MB-231 cells were cultured in an oxygen and pH gradient, tumour cells migrated toward higher

oxygen and pH within their microfluidic design (Takahashi et al., 2020). Similarly, Zhang et al. displayed the close interplay between hypoxia and the acidic pH in initiating SUM-159 migration (Zhang et al., 2015b). When SUM-159 cells were maintained in an oxygen level of 1% and 21% O_2, the migration speed of SUM-159 was higher at low oxygen concentrations when compared to 21% O_2. However, maintaining SUM-159 at 1% O_2 led to reduced extracellular pH. Similar to hypoxia, the acidic pH increased the migratory behaviour of tumour cells (Zhang et al., 2015b). This model system also allowed testing the effect of different pH conditions on cellular migration in a low oxygen environment. When SUM-159 cells were cultured in 1% O_2 at an alkaline and neutral pH, the migration speed was considerably lower than what was previously witnessed in the device. These results suggest that the acidic pH in combination with oxygen gradient can induce tumour cell migration (Zhang et al., 2015b). Furthermore, the ability of this device to manipulate the cellular microenvironment in terms of oxygen and pH provides the opportunity to analyse the effects of different conditions at the same time. Taken together, the studies described above revealed that oxygen could directly affect the directional migration of tumour cells. However, without including the different components of the TME, it is challenging to unravel the mechanisms governing cellular migration and invasion. More importantly, these studies only examined the effect of a uniform oxygen gradient on tumour cell migration. Overall, the rapid proliferative ability of tumour cells in addition to the leaky vasculature of solid tumours leads to the formation of spatial and temporal fluctuations in oxygen tension and intermittent hypoxia inside the TME, which may affect cell migration and invasion differently. As such, Koens et al. used a microfluidic model to show that spatiotemporal fluctuations in oxygen influence the directional movement of tumour cells and increase their migration speed significantly (Koens et al., 2020). For this, MDA-MB-231 cells were suspended in collagen I, and their directional migration was examined under a nutrient and oxygen gradient between 0.3 and 21% O_2. The cellular compartment was flanked with two medium channels that were able to generate a nutrient concentration gradient towards the centre (Koens et al., 2020). In addition, an oxygen gradient between 0.3% and 21% O_2 was generated in the cellular compartment by introducing gas mixtures with predefined oxygen concentrations through gas channels positioned above the medium channels. Temporal changes from atmospheric 21% O_2 to hypoxic 0.3% O_2 were possible within minutes, allowing this device to analyse the effect of both spatial and temporal changes in oxygen on cellular migration. MDA-MB-231 cells migrated from regions with extreme hypoxia toward regions with higher oxygen tension of 5% O_2 (Koens et al., 2020). The microfluidic model also showed that fluctuation between 21% and 0% O_2 significantly increased the migration speed of MDA-MB-231 cells. Acosta et al. used another model to investigate the effect of oxygen gradients and both intermittent and static hypoxia on tumour cell migration and invasion (Acosta et al., 2014). The authors employed a dynamic oxygen control on pancreatic adenocarcinoma (PANC-1) cells by using a modified transwell system that served as an invasion assay. The gradient was created by oxygen diffusion through PDMS from a gas channel on top of the cellular compartment. The device also allowed a long-term observation and maintenance of the cells (Acosta et al., 2014). Compared with cells cultured in normal oxygen levels of 21% O_2, PANC-1 cells cultured in an oxygen gradient from 21% to 1% showed enhanced migration from hypoxic regions toward highly oxygenated ones (Acosta et al., 2014).

The potential of advanced *in vitro* models to serve as a flexible platform for multiple parallel investigative assays whilst maintaining temporal and spatial control of oxygen allowed Lam et al. to adapt a 3D angiogenesis sprouting assay in a microfluidic chip (Lam et al., 2018). The authors specifically designed their system to understand chronic and intermittent hypoxic effects on angiogenesis. The central chamber was seeded with endothelial colony forming cell-derived endothelial cells (ECFC-EC) flanked with two peripheral chambers containing normal human lung fibroblasts (NHLF). Vessel formation in physiological conditions of 5% O_2 was relatively uniform, with vasculature sprouting into the two adjacent compartments. Whereas chronic, moderate hypoxia between 3.2% and 4.6% O_2 showed greater vessel formation compared to 5% O_2. In contrast, intermittent hypoxia where oxygen levels were fluctuating between 1.7% and 4.5% O_2 showed less sprouting vasculature (Lam et al., 2018). NHLF cells under chronic conditions continuously expressed pro-angiogenic factors to encourage enhancement of vessel growth. These findings suggest that fluctuations in oxygen levels and hypoxia highly influence angiogenesis (Lam et al., 2018). This model demonstrates the ability of advanced *in vitro* models to explore angiogenesis and the role of oxygen and the different microenvironmental components play during tumour progression.

As illustrated by the studies described above, advanced *in vitro* models are key to extend our understanding of the cellular process that contribute to tumour progression and metastasis. Advanced *in vitro* models can analyse differential cellular responses to oxygen gradients. Moreover, they highlight the importance of accurate representation of oxygen levels when modelling and studying the TME *in vitro*. Advanced *in vitro* models can incorporate different components of heterogeneous TME simultaneously, for example, nutrient deprivation, pH and biochemical gradients, by introducing soluble factor and chemokine gradients, thereby providing an adequate understanding of the processes behind tumour progression and metastasis as well as chemotherapeutic efficacy.

3.2.2. *The chemical microenvironment in advanced in vitro models*

Using microfluidics technology, advanced *in vitro* models can manipulate the fluidic behaviour and diffusion rates of chemical solutions to recapitulate the biochemical gradients that are normally witnessed *in vivo*. These biochemical cues are known to modulate tumour cell behaviour and are directly involved in tumour cell metastasis and chemotherapeutic responses. Often, chemical concentration gradients are created by using chemical gradient generators (CGG) that make use of the molecular diffusion between concentrated and diluted solutions (see Fig. 3B, numbers 4 and 5). CGGs are designed with a prolonged network of channels with tight junctions, where liquids with different concentrations are introduced through different inlets. Via thorough mixing and splitting, the liquids exchange solute molecules creating a stable and continuous concentration gradient.

One of the earliest microfluidic models determined that the presence of a chemokine gradient regulates the directional migration of tumour cells. The microfluidic chip created multiple concentration gradients of EGF using a CGG (see Fig. 3B). Two EGF concentrations were introduced to the CGG, 0% and 100% EGF. Through the CGG, an EGF concentration range is formed, ranging between 0 and 100% (Saadi et al., 2006). MDA-MB-231 cells showed an increased migratory response to EGF gradient. Their directional movement was toward the highest concentration of EGF (Saadi et al., 2006). However, this study was limited to observations in 2D. Culturing cells in a 3D format could provide a more accurate understanding of the effect of chemokine gradients. To recreate the appropriate environment of tumour cells more closely, Kim et al. examined the effect of EGF and CXCL12 gradients on the directional migration of MDA-MB-231 cells. The cells were suspended in collagen I gel to mimic the 3D environment *in vivo* (Kim et al., 2013). A CXCL12 gradient initiated tumour cell chemotaxis, with MDA-MB-231 cells showing an increased migration toward the higher concentration of CXCL12. On the other hand, EGF increased MDA-MB-231 cell motility but had no effect on the direction of movement. Interestingly, when MDA-MB-231 cells were exposed to a gradient of CXCL12 and a uniform concentration of EGF, the two chemokines showed a significant effect on the cellular motility and migration of the tumour cells. However, when the concentration of EGF was increased, the effect of CXCL12 was nullified (Kim et al., 2013). These findings are important, as they suggest that cross signalling within the tumour niche can directly influence the migratory potential of tumour cells. Another crucial factor that contributes to tumour cell metastasis is the cells' metabolic need. Chaw

et al. showed that tumour cell migration could be simulated by serum concentration, indicating that nutrients play an important role in cellular migration (Chaw et al., 2007). These results were similar to the one Zou et al. reported in another microfluidic model. The authors revealed that when lung cancer stem cells (LCSC) and differentiated LCSC were cultured in a serum gradient, both cell types displayed an increased migration rate toward the highest serum concentration. The serum gradient was achieved by using a microfluidic channel network that can create a concentration gradient in a manner similar to CGG. Further analysis showed that the β-catenin-dependent Wnt-signalling pathway mainly regulated this migratory behaviour. Inhibition of this signalling pathway reduced the migration rates of both cell types (Zou et al., 2015).

Advanced *in vitro* models also examined the fate of chemotherapeutics inside the tumour niche. At present, understanding the effect of the TME on chemotherapeutic distribution is limited by the lack of appropriate models. For a drug to exert its effect, distribution in the tumour mass is essential. The vasculature of solid tumours presents a barrier for chemotherapeutic distribution, creating an uneven chemotherapeutic distribution within the TME. Microfluidics has made it possible for advanced *in vitro* models to study chemotherapeutics distribution inside the TME. Fig. 4 outlines the different approaches advanced *in vitro* models undertook to explore the effect of the TME on chemotherapeutic distribution and efficacy. For example, a microfluidic chip assessed the kinetic properties of doxorubicin agents in nutrient and oxygen gradients (Ayuso et al., 2016). Human colon carcinoma HCT-116 and glioblastoma U-251 MG cells were suspended in collagen I and exposed to doxorubicin. Doxorubicin was detected in all hydrogel scaffolds regions, showing a linear distribution after 2 h of exposure, suggesting that doxorubicin is evenly distributed throughout the bioengineered tumour (Ayuso et al., 2016). Another microfluidic model revealed that chemotherapeutic drug distribution and uptake might be influenced by the spatiotemporal fluctuations in oxygen levels that occur inside the TME. MCF-7 spheroids were cultured inside a microfluidic chip and were exposed to either chronic hypoxia, with a continuous oxygen concentration of 3% or cyclic hypoxia, where oxygen levels were alternated between 0% and 20%. Results showed that doxorubicin uptake was higher in cyclic hypoxia in comparison to chronic hypoxia, indicating that oxygen conditions affect doxorubicin accumulation (Grist et al., 2019). In the aforementioned TRACER device, exposure of the engineered tumour tissue to doxorubicin provided useful insights on drug distribution in an oxygen gradient. The concentration of doxorubicin decreased inside the device before plateauing toward the deeper parts of the tissue (Rodenhizer et al., 2016). These results were similar to the doxorubicin penetration observed in mouse xenografts, suggesting that an accurate understanding of chemotherapeutic distribution inside solid tumours requires precise modelling of the heterogeneous components of the TME (Primeau et al., 2005). The efficacy of doxorubicin, temozolomide and tirapazamine in an oxygen gradient were also analysed in the aforementioned microfluidic chip from Ayuso et al. (Ayuso et al., 2016) (see Fig. 4A). Doxorubicin significantly induced death in regions closer to the peripheral chambers, where oxygen tension was higher. However, no effect was detected in hypoxic regions. In the same device, the efficacy of the alkylating agent temozolomide was investigated in U-251 MG cells. Similar to doxorubicin, higher levels of cell death were detected in the most oxygenated and more proliferative regions of the microdevice. The hypoxia-activated drug tirapazamine was also studied in this microfluidic system. In contrast to doxorubicin, in hypoxic regions, tirapazamine dramatically reduced the viability of cells. Since tirapazamine needs low oxygen levels for activation, cells in well-oxygenated regions of the device remained viable (Ayuso et al., 2016). Taken together these studies demonstrate that advanced *in vitro* models provide the opportunity to analyse the effect of various microenvironmental conditions on chemotherapeutic distribution and their overall kinetic properties, as well as examining the efficacy of these agents under different microenvironmental conditions.

Wang et al. developed a multi-gradient microfluidic device, where they could create simultaneous oxygen and chemotherapeutic concentration gradients (Wang et al., 2013) (see Fig. 4B). Here, the oxygen gradient was initiated and maintained by flowing oxygen scavenger Na_2SO_3 inside the cellular chamber. On the other hand, the chemotherapeutic gradient was generated using a CGG, creating chemotherapeutic concentration variations in the cellular compartment. The chemotherapeutic gradient has made it possible for this model to assess the effect of dose-dependent exposure on their bioengineered tumour mass. The comparative analysis of the anticancer drugs tirapazamine and bleomycin was conducted in human cervical carcinoma cells (HeLa)

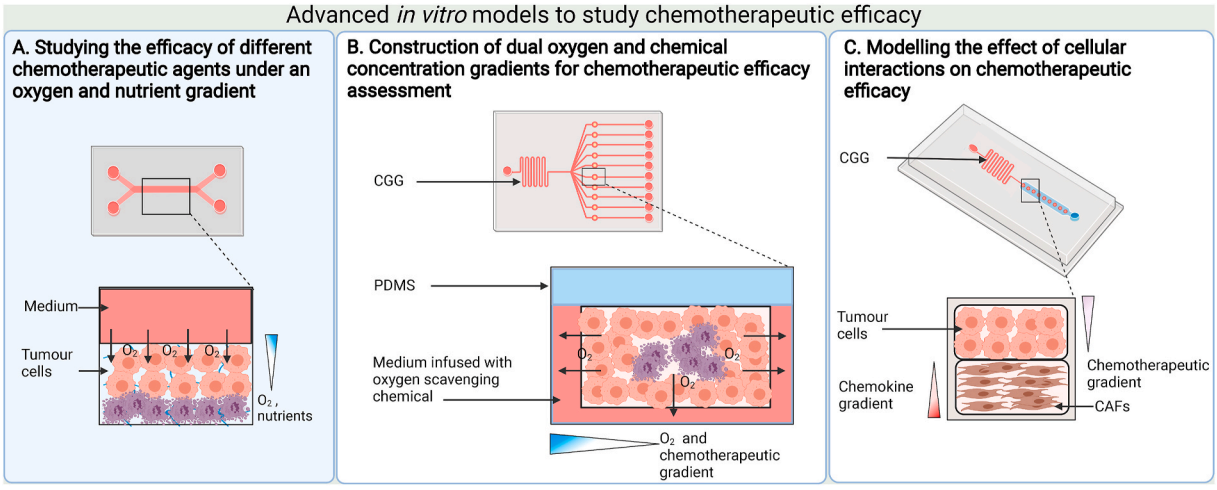

Fig. 4. Advanced *in vitro* models to study chemotherapeutic efficacy. Schematics of different microfluidic approaches to examine chemotherapeutic efficacy and distribution under different microenvironmental conditions. A) The efficacy and distribution of different chemotherapeutic agents can be studied under an oxygen gradient in a microfluidic device. The high cellular density creates uniform oxygen and nutrient gradients within the cellular compartment and also induces the formation of a pH gradient and waste products. B) Example of a microfluidic chip to establish an oxygen and chemical concentration gradient to assess the therapeutic efficacy. The oxygen gradient is created by using PDMS, gas permeable material to absorb atmospheric oxygen into the device. Within the cellular compartment, $NaSO_3$ acted as an oxygen scavenger creating an oxygen gradient. On the other hand, the chemical gradient was generated by using a CCG. C) A microfluidic chip to analyse the effect of tumour stroma and growth factors on tumour cell survival and chemotherapeutic efficacy. The chemotherapeutic concentration gradient can be created via a CGG by utilising diffusive mixing.

and lung carcinoma cells (A549). The sensitivity of both agents was assayed under an oxygen gradient between 0% and 21% O_2. The viability of cells in hypoxic regions of the device declined proportionally to the concentration of tirapazamine used, indicating a dose-dependent effect with this drug in lower oxygen areas in the chip. In contrast, bleomycin did not affect the cells in the device's hypoxic regions, whereas a reduction in cell viability was observed in highly oxygenated parts of the tumour tissue (Wang et al., 2013). In another microfluidic device, Ying et al. highlighted the effect of cellular interactions between stromal cells and tumour cells on their response to chemotherapeutics. Within their device, A549 tumour cells were co-cultured with cancer-associated fibroblasts (CAFs) and were exposed to a concentration gradient of paclitaxel that was created using a CGG (Ying et al., 2015) (see Fig. 4C). Next, the role of different chemokines and growth factors secreted by CAFs on paclitaxel efficacy was examined. Within the gradient of paclitaxel, hepatocyte growth factor (HGF), which CAFs secrete, reduced cellular apoptosis induced by paclitaxel (Ying et al., 2015). HGF is known to bind to Met and, as such, to activate the downstream phosphoinositide 3-kinase (PI3K)/AKT pathway, which regulates chemoresistance and cell apoptosis. The ability of microfluidic chips to rapidly generate chemotherapeutic concentration gradients allows them to serve as a platform for developing personalized therapeutic schemes. For instance, Xu et al. used different lung cancer cell lines and primary lung cancer cells collected from eight different patients suspended in BME (basement membrane extract) containing Laminin I, Collagen I, Collagen IV, Vitronectin, and Fibronectin (Xu et al., 2013). The bioengineered 3D cellular structure was exposed to different concentrations of chemotherapeutic agents generated via a CGG. Tumour cells were either a monoculture or co-cultured with human lung fibroblasts to test the influence of cellular interactions on chemotherapeutic efficacy. This device was used to screen for chemotherapeutic sensitivity and provided appropriate therapeutic schemes for the patients (Xu et al., 2013).

Overall, advanced *in vitro* models with their dynamic nature offer the opportunity to recreate molecular gradients found in the TME. Integration of microfluidics with tissue engineering technologies enables these models to analyse the influence of stromal cells, oxygen and chemical conditions on tumour cell behaviour with full spatiotemporal control. Another important application of these model systems is assessing the efficacy of chemotherapeutic agents as they can evaluate the contribution of these conditions on tumour cell response to chemotherapeutic agents. The presence of flow within these models allows the study of chemotherapeutic distribution inside the tumour niche in a manner that resembles the *in vivo* conditions, indicating that advanced *in vitro* models can be used in pre-clinical screening of new chemotherapeutic agents.

4. Outlook

Advanced *in vitro* models provide a transformative approach for modelling the microenvironment of solid tumours *in vitro*. They offer the chance to develop reliable, highly reproducible and potentially high throughput model systems that could represent human physiology and cancer pathology *in vitro*. These models aid in investigating the TME in a more relevant manner and may provide a robust predictive platform for clinical and therapeutic evaluation studies. Advanced *in vitro* models aim to include different mechanical, physical and chemical cues present within the TME of solid tumours. They can also integrate the cellular heterogeneity that is a characteristic feature of the TME. However, several questions remain on which factors should be incorporated *in vitro* as a minimum to produce a relevant model for cancer pathophysiology. To date, it appears that recreating the different gradients that are present in the microenvironment is essential for accurate predictions.

Another important factor when it comes to developing an *in vitro* model for cancer studies is the exact research question posed and subsequent analyses needed. For example, studying the effect of oxygen on cellular migration or chemotherapeutic response requires certain functionalities in the model. As such, one platform is unlikely to be sufficient to address all research questions that exist. More importantly, the different conditions within the TME do not exist in isolation; they dynamically interact and influence each other, potentially leading to tumour cell responses. Therefore, when modelling the TME, the interplay between the different conditions should be considered to be included in the design. Advanced *in vitro* models can create dynamic models that can be tailored to analyse oxygen gradients either alone or in combination with soluble chemokine gradients. Within these models, tumour cells can also be co-cultured with stromal cells, enabling the inclusion of cell-cell communication or tissue-tissue interfaces while investigating their effect on tumour cell behaviour and response to therapeutics. Advanced *in vitro* models also offer the chance of capturing the temporal and spatial fluctuations in oxygen levels inside the TME. Microfluidic techniques have made it possible for these models to recreate intermittent perfusion cycles that lead to the formation of cyclic or intermittent hypoxia *in vivo*, allowing advanced *in vitro* models to maintain a spatiotemporal control over oxygen. This type of model could assess cellular adaption to cyclic hypoxia and expand our knowledge about the effect of chronic and cyclic hypoxia on tumour progression. Coupling this approach with the ability of advanced *in vitro* models to integrate different aspects of the TME, such as endogenous chemical gradients, nutrient gradients and co-culture with stromal cells, would provide deeper insights into cellular and molecular mechanisms behind the important events in cancer progression and cellular behaviour.

Owing to this versatile nature, advanced *in vitro* models enable researchers to identify, analyse and validate the functionality of new biological targets for chemotherapeutic agents. As they can model key specific events during cancer progression and analyse integral cellular interactions during these processes, advanced *in vitro* models can help characterise new potential targets for therapeutic intervention. All these features offer the opportunity for advanced *in vitro* models to serve as a reliable *in vitro* tool for preclinical screening of new chemotherapeutic agents. Furthermore, the dynamic nature of advanced *in vitro* models, in addition to their ability to control the chemical parameters, have made it possible to investigate molecular diffusion and transport, enabling these models to examine chemotherapeutic delivery and distribution inside the TME. As such, advanced *in vitro* models can be used to model the pharmacokinetic properties of chemotherapeutic agents and analyse the appropriate dosages and therapeutic regimens for human cancer *in vivo*.

While advanced *in vitro* models provide a promising platform for *in vitro* studies, several challenges remain before they can be fully integrated in cancer modelling. Techniques such as microfabrication that are typically used to develop advanced *in vitro* models require specialised equipment that is not readily available in many cancer research facilities. A further challenge is that developing advanced *in vitro* models requires a highly interdisciplinary environment. The ability to use these innovative approaches to model the TME requires the combination of knowledge from biology, medicine, bioengineering and physics. Furthermore, the level of complexity of many advanced *in vitro* models causes these models to have low operational throughput. For instance, to accurately assess influence of oxygen gradients within a microfluidic chip, various factors must align to create a powerful readout including cells, ECM coating, microfluidic controls and maintaining the gradient. Nevertheless, the continued validation and optimisation of advanced *in vitro* models has started the emergence of high-throughput devices that can evaluate cancer cell behaviour.

5. Conclusion

The different components of the TME can create adverse conditions that modify tumour cell responses. Physicochemical gradients, along with the heterogenous cell composition of solid tumours, promote tumour cell migration, invasion and affect therapeutic efficacy. Accordingly, tumour cells are influenced by their microenvironment,

leading to its increasingly recognised importance. Accurate representation of the TME *in vitro* is crucial in cancer modelling not only for new therapeutic approaches but also as a tool for understanding the molecular mechanisms behind tumour development, progression and metastasis. In general, most of the work on the TME *in vitro* so far has been carried out with 2D cell culture systems where cells are maintained on flat surfaces. Within solid tumours, cells exist in a 3D environment that is both spatially and temporally heterogeneous. 2D culture models also lack the ability to recreate the widely witnessed gradients in the TME.

Advanced *in vitro* models are emerging in cancer research as promising tools for understanding the role of the TME. Using innovative engineering approaches, advanced *in vitro* models can integrate both the cellular and non-cellular components of the TME. Advanced *in vitro* models can include ECM, stromal cells and recreate the different physiochemical gradients such as oxygen and endogenous chemokines gradients, enabling the maintenance of tumour cells in an environment that aims to resemble the *in vivo* solid tumour. Microfluidic tumour-on-chip systems are one of the most promising advanced *in vitro* models in cancer research. The use of microfluidic technology allows tumour-on-chip models to create a defined environment for cellular studies. Integrating microfluidics with different fabrication techniques can customise tumour chips to explore specific phenomena in cancer research. All these features have made it possible for advanced *in vitro* model systems to study the effect of the TME either alone or in combination with different gradients, and accordingly, these models represent a new and exciting platform for cancer research. Their potential in expanding our knowledge warrants further exploration.

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