Cancer therapies have several clinical challenges associated with them, namely treatment toxicity, treatment resistance and relapse. Due to factors ranging from patient profiles to the tumour microenvironment (TME), there are several hurdles to overcome in developing effective treatments that have low toxicity that can mitigate emergence of resistance and occurrence of relapse. De novo cancer development has the highest drug attrition rates with only 1 in 10,000 preclinical candidates reaching the market. To alleviate this high attrition rate, more mimetic and sustainable preclinical models that can capture the disease biology as seen in the patient, are required. Organoids and next generation 3D tissue engineering is an emerging area that aims to address this problem.

Advancement of three-dimensional (3D) in vitro cultures into complex organoid models incorporating multiple cell types alongside acellular aspects of tissue microenvironments can provide a system for therapeutic testing. Development of microfluidic technologies have furthermore increased the biomimetic nature of these models. Additionally, 3D bio-printing facilitates generation of tractable ex vivo models in a controlled, scalable and reproducible manner.

In this review we highlight some of the traditional preclinical models used in cancer drug testing and debate how next generation organoids are being used to replace not only animal models, but also some of the more elementary in vitro approaches, such as cell lines. Examples of applications of the various models will be appraised alongside the future challenges that still need to be overcome.

1. Cancer drug development and associated clinical challenges?

Cancer is one of the leading causes of mortality throughout the globe with the World Health Organisation (WHO) estimating that cancer is the first or second leading cause of death in 91 countries and in the UK, 1 in 2 people will be diagnosed with cancer at some point in their lifetime [1]. Moreover, rise of an ageing global population, alongside increased exposures to environmental risk factors mean that the incidence and...
mortality rates are increasing year-on-year [2]. In 2020 cancer accounted for nearly 10 million deaths worldwide with the commonest cancers leading to death being lung and colorectum [2]. Each year approximately 400,000 children develop cancer. This highlights an increasing burden on medicine and oncology research to provide stronger treatments [3]. In 2020 cancer accounted for nearly 10 million deaths worldwide. Furthermore, each year nearly half a million children develop cancer, highlighting this chronic disease as an increasing burden on medicine and providing an urgent requirement for cancer research to develop new more directed and low toxic treatments [3]. The main challenges posed in the clinics are treatment toxicity, treatment resistance, the role of Cancer-associated Fibroblasts (CAFs) and relapse, all of which are discussed below.

Currently the approaches used to combat cancer in the clinic varies depending on the cancer type. Cancer surgery remains one of the key methods for solid tumours however these physical interventions can lead to elevated risk of metastatic recurrences along with elevated pro-inflammatory mediators [4]. Surgical intervention is not an option for liquid cancers such as blood malignancies and these rely on chemotherapeutic treatments. Non-surgical approaches have varying efficacy depending on the targeted disease. Many factors influence the ability of a cancer to evade a treatment, such as the severity of the disease, patient profile and immunity status. Clinical challenges also vary depending on adult or childhood cases.

In the UK 28% of patients undergo chemotherapy treatment including all stages of cancer. Current chemotherapy approaches are cytotoxic and effect multiple organ systems. In blood cancers, a study of 1872 childhood Acute Lymphoblastic Leukaemia (ALL) patients highlighted that approximately half of the patients displayed Grade 3 or 4 toxicity with features such as gastrointestinal, liver, renal and cardiac toxicity along with impaired cardiac function and in some cases crippling neurotoxicity [5]. Despite survival rates of fit older adults being increased with chemotherapy regimens, unfit older patients present higher risk of toxicity and complications [6]. Additionally, in children, chemotherapy-associated toxicity can affect the patient for the rest of their life with toxicity-associated mortality rates akin to the cancer itself. Quality of life following treatment is therefore a key clinical challenge and accelerated target discovery and drug development are both urgently needed.

Another difficulty with cancer management is treatment resistance. Cell-intrinsic mechanisms such as mutation and epigenetic factors remain a key component associated with resistance however additional factors are now understood to play an important role, with the cancer microenvironment an emerging area of investigation [7,8]. This cancer niche comprises of various cell types and extracellular matrix proteins and acellular aspects all of which provide meaning there are various signals being provided to cancer cells that mediate malignant cell survival, proliferation and treatment response.

For example, in cancers of the blood, microenvironment conferred treatment resistance is a major obstacle to overcome with therapeutics. Leukaemia cells exhibit a dynamic characteristic in which they can influence their environment which in turn can enhance malignant propagation through reprogramming of the bone marrow stroma [9,10].

Indeed, targeting such interactions between cancer and neighbouring cells is an area for scrutiny in therapeutic development, however currently treatments that directly target microenvironment dependent cancer biology are scarce and discovery of druggable targets remain incomplete. A recent study investigated a cell adhesion molecule, N-cadherin (CDH2) in the context of ALL and found it to be a potential druggable target through ADH-1 (Exherin®)™), an FDA approved orphan drug that had previously been used in Phase I/II trials for solid tumours [11-13]. It was found that CDH2 was a factor driving microenvironment dependent leukaemia proliferation and furthermore mediated reduced sensitivity to dexamethasone [11]- a glucocorticoid used in ALL treatment. However, target discovery studies identifying druggable molecules that directly drive niche dependent cancer biology remains as yet an under researched area.

This work suggests that cell–cell adhesion is an area that presents an opportunity for novel therapeutics. More targeted therapies such as ADH-1 described in the context of leukaemia can help alleviate some of the issues with treatment resistance.

Nevertheless, several concepts have recently emerged that elucidate mechanisms of cancer-niche interactions. For example, in ALL, cancer cells disrupt the bone marrow microenvironment, thereby generating a niche that is key in facilitating leukemogenesis and increasing protection from therapy. Evidence suggests that actin cytoskeleton-driven tunnelling nanotubules play an important role in communication between leukaemia cells and mesenchymal stem cells (MSCs). Leukaemia cells utilise these nanotubules to signal to surrounding MSCs promoting secretion of pro-survival cytokines also leading to drug resistance. Additionally, lipophilic molecules containing mitochondria and autophagosomes are exchanged between leukaemia cells and MSCs with autophagosomes and mitochondria described as the transferred material [14].

Another factor associated with treatment resistance is Cancer Associated Fibroblasts (CAFs) or malignant stroma signalling. CAFs are a key component of the TME where they modulate cancer metastasis through Extracellular Matrix (ECM) remodelling and synthesis, growth factor promotion, angiogenesis and modulation of the immune system [15].

The mechanism of generation of CAFs from normal fibroblasts remains under researched, nevertheless, molecular features of CAFs are of interest to help understand such cancerous transitions [16]. Moreover, origin of CAFs is not restricted to fibroblasts alone, but this includes multiple additional cell types, such as adipocytes, pericytes, epithelial and endothelial cells. Various signalling factors, epigenetic changes, oxidative stress and metabolic reprogramming all include pathways through which fibroblast reprogramming can occur. Not only is the tumour microenvironment important in CAF activation, but furthermore, the cell of origin can be culpable to the notorious CAF heterogeneity, which further complicates cancer treatment. Additional complexity arises as CAFs have diverse biological characteristics and functions and affect almost all current cancer treatments including chemotherapy, targeted therapies and immunotherapy. For example, chemoresistance is enhanced by secretion of factors to maintain the stenness of cancers. Epithelial-mesenchymal transition (EMT) and chromatin remodelling have also been described as a result of interleukins 6 and 8 released by CAFs. CAFs are closely linked to treatment outcomes in many solid-state tumours including colorectal cancer, pancreatic and breast cancers. Difficulties arise when targeting CAFs due to their high heterogeneity and hence no CAF-specific inhibitors have been approved by the FDA [17]. In colorectal cancer and pancreatic cancer, CAFs are so abundant and are the major source of ECM, providing a physical barrier that influences drug delivery. One such ECM component that CAFs produce in large quantities is collagen—particularly fibrillar collagens and beaded filament type VI collagen [18]. Collagen in large quantities leads to dysregulation of normal ECM homeostasis and therefore resistance to chemotherapy [19,20]. Despite evidence that substantiates the role of the bone marrow stroma in leukaemia treatment resistance, the role of ECM such as collagen and their effect on mediating leukaemia-niche interactions, immune modulation and consequently leukaemia treatment response remains ambiguous. Despite investigation into the role of CAFs in the TME being at an early stage, it is clear that these populations of cancer supporting cells are vitally important in promoting malignant proliferation and chemotherapy protection. They provide an opportunity for advancing therapeutics, however developing such treatments is difficult due to their variable biology and heterogeneity, which in turn complicates capturing such complex biology into tractable and clinically relevant preclinical models. Dormancy, or G0 cell cycle arrest, and its contribution to treatment resistance is also well known. In the dormant state, also known as quiescence, cancer cells accumulate additional mutations allowing for
increased cancer cell survival in new microenvironments, consequently leading to treatment resistance and evasion of the immune response [21]. Increasing evidence shows that within primary solid-state tumours, small populations of dormant cells are essential for continuous tumour progression [22]. Many current anticancer treatments target proliferative mechanisms and since dormant cells are not actively proliferating, dormant cells naturally evade these treatments [23]. Once these dormant cells have evaded treatment in the initial stage they accumulate more mutations, typically becoming resistant to the initial treatment and then enter proliferation thereby leading to a more aggressive and more difficult to treat disease. Pharmacological approaches that target dormant cell populations remains relatively unexplored. There are different approaches to combatting dormant cells and increasing the susceptibility to treatment. These strategies include maintaining cells in dormant states; reactivating dormant cells to increase susceptibility to current anti-proliferation drugs; and eliminating dormant cells. Each of these approaches have benefits but also caveats [24]. Dormant cells are not necessarily of detriment to cancer treatment, however, following their re-awakening they can become problematic and therefore inherently portend a risk of aggressive relapse. Despite the molecular mechanisms underlying dormancy being more understood, this remains an under-researched area. Various approaches to understanding the complexity of the interactions between neoplastic tumour cells and the environment.

To demonstrate the complex nature of these environments, the leukaemia niche will be discussed as a disease paradigm. The bone marrow is the primary site for haematopoiesis. This environment consists of many unique bone marrow cells, each of which contribute to haematopoietic cell regeneration and renewal in specialised niches. Each niche provides support to hematopoietic stem cells and lineage-committed cells through chemical cues, cell–cell interactions and cell-ECM interactions. The cellular composition of the bone marrow contains osteoblasts, osteoclasts, adipocytes, stroma, vascular endothelial cells, hematopoietic stem cells alongside immune cells [28,29], all of which contribute to maintenance of the bone marrow niche (Fig. 1). Following leukemogenesis, this environment becomes disrupted generating a leukaemia niche-whose ultimate characteristic is to support the proliferation and survival of the leukaemia cells conferring a positive advantage over any other cell type. Leukaemia cells can remodel the environment around them in order to promote their survival at the detriment of other cells within the bone marrow [30,31].

1.1. Modelling the cancer microenvironment in 3D

An intrinsic property of all biological systems is that these are composed of a diverse and dynamic cellular ecosystem, further marked by redundancy and degeneracy of its cellular and ECM components. Indeed, the cancer microenvironment consists of various cell intrinsic and cell extrinsic aspects, all of which contribute to tumour growth and invasion, immune response evasion and resistance. In order to recapitulate the microenvironment faithfully in preclinical models, a holistic consideration of both cell-autonomous and non-cell-autonomous aspects is required. Furthermore, generating a model that allows study of dormancy, relapse and resistance are of key importance in order to address the clinical challenges. As increased attention has been directed towards the role of the microenvironment it has become more apparent on the complexity of the interactions between neoplastic tumour cells and the environment.

Fig. 1. The bone marrow is a complex microenvironment comprising of many different cell types and acellular components. Cells found in healthy bone marrow include endothelia, adipocytes, T cells, hematopoietic stem cells (HSCs), osteoblasts, osteoclasts, mesenchymal stem cells and fibroblasts. Acellular aspects include extracellular matrix proteins (ECM) which provides support for cellular migration, differentiation and proliferation. This complexity is difficult to replicate with preclinical models but must be strived towards in order to understand mechanisms that drive cancer and treatment resistance. Created with BioRender.com.
In response to treatment, the leukaemia niche can confer treatment resistance via remodelling of the surrounding environment. This cellular dynamism is very difficult to model due to the multicellular nature of the bone marrow and the role of ECM components in the physical restructuring of the tissue.

Having the capacity to model such interactions is crucial for understanding the factors that drive treatment resistance, relapse and dormancy that generate challenges in the clinic.

1.2. Cancer stem cells

Cancer stem cells (CSCs) are subpopulations of tumours that are self-renewing. They facilitate tumour initiation, can influence therapy resistance and can promote metastasis. Following their discovery in Acute Myeloid Leukaemia (AML) they have since been found in all solid tumours. Despite these stem cells possessing typical stem cell markers, they maintain their renewal capacity via altered pathways such as STAT3, NOTCH, PI3K and WNT/β-catenin. These stem cells can also promote tumorigenesis and progression by influencing the hallmarks of cancer [32]. In addition to their altered pathways for self-renewal, they have plasticity to transition between stem and non-stem states allowing survival in response to treatment or environmental stress [33].

There are two ways in which cancer cells can be treatment resistant and these are defined as “innate chemoresistance” and “acquired chemoresistance”. Innate chemoresistance refers to cancer cells that naturally possess the mechanisms that allow them to eliminate the cytotoxic effects of chemotherapeutic agents. Acquired chemoresistance refers to resistance in cancer cells due to chronic exposure to a treatment, enriching the cancer cell population, of which CSCs are present to drive the tumorigenesis.

An additional complexity with CSCs is that they are a major source of resistance to conventional treatments due to innate chemoresistance, and their mechanisms of stemness and tumorigenicity are still not fully understood. Resistance to cisplatin, a commonly used compound to interrupt lung cancer cell transcription and DNA replication, presents significant difficulties in the clinic due to the widespread nature of its use in early stages of treatment [34]. Studies of treating lung cancer cell lines with cisplatin for 24 h demonstrated an enriched subpopulation that were CD133+. Functional analysis found these subpopulations to have higher cisplatin resistance [35].

Despite CD133 being a classically used biomarker for CSCs in a wide range of tumour tissues, it has also been reported that CD133+ cells are also able to give rise to tumours in immunocompromised mouse models [36]. This highlights the heterogenous nature of CSCs with observations in one cancer type not necessarily replicated in others. Additionally, micromilieu and environmental cues can influence key biomarker expression, such as CD133 [36]. Capturing this heterogeneity in models presents a challenge. To help capture the correct biomarker profile in models, co-culture of tumour cells alongside niche cells such as mesenchymal stem cells or CAFs have been demonstrated to maintain CD133 expression [36]. A review of numerous studies exploring the prognostic power of CD133 in cancer found in general that it can be used with prognostic value, despite some inconsistencies in some studies [36]. Furthermore, certain disease models such as ALL do not comprise defined CSC (or leukaemia initiating cells) populations. Indeed, it is well established that the ability to generate and propagate leukaemia in ALL is stochastic, not confined to a pre-defined “stem” cell population and is furthermore independent of immunophenotype. Nevertheless, treatment resistance, treatment refractoriness and relapse remain major hindrances in treating ALL, implying the urgent need to improve our understanding of in-depth mechanisms and functional characterisation of treatment resistant ALL cells- elucidating which mandate tractable research tools that faithfully replicate cancer cell function as in patients [37,38].

As a therapeutic target, CSCs seem like on paper at least an ideal, if not relatively unexplored, candidate. In the context of colon cancer, Gao et al. [39] have used CRISPR screens to identify cholesterol biosynthesis in cancer stem cells as an opportunity for therapeutic targeting. Cholesterol biosynthesis genes are overexpressed in colon cancer compared to non-cancerous cells. They identified cholesterol biosynthesis-associated genes HMGCR and FDPS and established drugability of these targets with FDA-approved drugs. Additionally, they found that there was a synergistic effect when cholesterol biosynthesis inhibitors were paired with a conventional chemotherapy drug, overcoming drug resistance [39]. This suggests that cholesterol biosynthesis is a druggable pathway in the context of colon cancer and could be applied to other cancer types.

However, the plastic nature of CSCs, allowing them to transit from differentiated cells to a CSC status can allow them to evade typical treatment regimens whilst also potentially evading CSC-targeted treatments. Given environmental factors can drive this transition, current preclinical models can have difficulty capturing this in vitro due to the difficulty of incorporating these acellular aspects.

1.3. Drug attrition in cancer drug development and reasons.

Identification of novel cancer therapeutics has been aided by the development of new technologies and increased knowledge of individual disease mechanisms. However, there are still key issues surrounding translation of novel candidates into Phase III clinical trials and drug registration. Candidate cancer therapies have the lowest success rate and are 23% less likely to pass Phase III clinical trials than any other field [40].

The typical approach for de novo drug discovery and development is a lengthy and expensive one. Per new approved drug, a financial outlay of approximately $3 million is required to take a novel candidate through all the steps required to have an approved compound. The procedure is slow too, with average time taken being between 10 and 17 years to get a new compound to market [41]. Additionally, the process is very inefficient, with 95% of drugs tested at Phase I not reaching the market and only 1 in 10,000 pre-clinical candidates ever reaching the market at all [42].

The pipeline for de novo drug development has several steps associated. The initial steps involve target identification and drug design which highlights large number of candidates. Still animal models are used at the next stage to screen these candidates in vivo, again ruling out many candidates throughout this process. An ever-growing stage of this pipeline is in vitro testing, and this includes many preclinical model types such as cell line models, spherical and organoids and organ/body on a chip. These preclinical models provide a safe platform for testing novel compounds before Phase I clinical trials and are being used more commonly.

The main area for drug attrition is the translatability from preclinical work into Phase I clinical trials. The current models used for preclinical testing, be it in vivo or in vitro are still not necessarily representative enough of the target organ or tissue. In order to alleviate the attrition rates, more robust and mimetic preclinical models must be developed that capture the complex nature of the cancer microenvironment.

There are many theories as the methods to reduce drug attrition rates in the de novo cancer therapeutic pipeline. One of the key areas that has already shown reduction in drug attrition is using predictive biomarkers to select particular patients who are most likely to benefit from a targeted therapy [42]. Phase 0 clinical trials have also been suggested and used in some cases [43] as a bridge between preclinical modelling and Phase I clinical trials. Pharmacokinetic and mechanism data can be collected from a small subset of patients exposed to a non-therapeutic drug dose, highlighting whether a candidate has the desired biological effects. There are other factors that can reduce attrition based around the efficient transition through the drug development pipeline and close collaboration between all parties involved in the development of these therapeutics.

Finally, an important method of alleviating some of the attrition
rates is through robust, more mimetic preclinical models. The focus of this review is to highlight the types of preclinical models available and how these models are contributing to the stronger understanding of cancer mechanisms and how we can use these models to develop better anti-cancer drugs.

2. Preclinical approaches to modelling cancer

2.1. Animal models and their use in cancer therapeutic studies

According to 2021 UK Home Office Statistics, 1.73 million experimental procedures were carried out on live animals in Great Britain. Of this population, 12% were used for oncology - the third largest area of focus. Despite a general trend in decreasing use of animals following the 2014 EU Directive (2010/63/EU) to ensure animals used for scientific purposes are more protected, they are still heavily used in medical research. The use of animal models such as mouse, zebrafish and Drosophila have greatly impacted our understanding of cancers and how specific mechanisms contribute to disease.

2.2. Animal replacement models and non-animal technologies as preclinical models

In vitro modelling is a well-known area for replacement of animal models, not only in cancer research but in other areas too. A movement away from animal models is promoted now in preclinical modelling as more is understood on the physiological differences between animal and man. Furthermore, use of animal products in ex vivo cultures is also now understood to result in variable results due to batch variance. Despite a drive to use synthetic materials in cultures, this is still not widespread so still in vitro culture does not mean “animal-free”.

2.2.1. Cell lines

Cell lines are considered one of the least complex and least mimetic of the available in vitro preclinical models, however they remain a popular choice to researchers due to their ease of culture and availability. They provide standard model cells to study cancer pathophysiology without the inherent variance associated with patient-derived samples. Cancer cell lines maintain the cytogenetic abnormalities of the disease whilst providing a more established and consistent method of cell culture. These benefits make them an attractive model system. Since the establishment of the HeLa cell line in 1951 there have been many cancer cell lines generated in the laboratory. During the 1980s and 1990s use of recombinant growth factors and specialised media has opened the door for many therapeutic models to be developed with the first being retinoic acid and its targeting of the PML-RAR alpha fusion gene in NB4 cell lines [44]. Another cell line of importance in leukaemia is the K562 cell line which is the model associated with the development of the first tyrosine kinase inhibitor STI-751 (Imatinib). This inhibitor can target the site of the genetic abnormality t (9; 22)-correlated BCR-ABL fusion protein that has been demonstrated to arrest leukaemia cell growth [45].

As more knowledge on specific cancer mechanisms and abnormalities is known further cell line models are necessary. Over time, as new anticancer therapeutics are developed, a safe model system is required with the correct genetic makeup suited to the targeted disease. Additionally, the emergence of -omics approaches have revealed the molecular and cellular alterations of each cell line providing further insight into their potential uses in cancer drug discovery [46]. Following important work by independent groups of Barretina et al. [47] and Garnett et al. [48] we now have a deep understanding of the genetic molecular and metabolic makeup of these cell lines allowing researchers to select the best option for their studies.

Despite cell lines still being an important model system, there is currently a drive to move away from cell lines where possible and select more complex and therefore more mimetic models. This is due to several limitations associated with using 2D cell line culture. Firstly, when cultured in vitro they do not rely on cell signalling from neighbouring other cell types, so their growth is not controlled by the environment as it is in vivo. Specifically in leukaemia cell line culture this is compounded even further as they are cultured in suspension, where the biological significance of cell–cell contact can be undermined as most cell lines do not tend to form cell aggregates or clumps in order to proliferate. The lack of cell–cell interactions would mean that tissue architecture is not accurately present therefore does not represent spatial organisation.

Comparisons of cell lines and matched tumour cells from patients has highlighted that there is considerable variation in gene expression and enrichment of microenvironment modification-related genes [49] Li et al. describes their procedure of understanding the metabolic diversity of nearly 1000 cancer cell lines from 2D cancer subtypes where they link cancer metabolome to various genetic features associated with cancer. They found that distinct metabolic phenotypes exist in cancer cell lines and these characteristics have direct implications for therapeutics and these relationships are not necessarily seen in tumour tissue [50].

2.2.2. Patient-derived models

Use of patient-derived cells in tissue culture has been a popular way of incorporating disease into cultures. There are different ways to do this with the most popular way being typical 2D culture. As with cell lines, these cells offer a relatively straightforward method for having available cells with the correct genetic abnormalities, however beyond that there is limited growth potential, lack of cell-environment interactions and difficulty in obtaining samples. Due to limited availability of these samples, it is critical that these are maximised in culture.

Co-cultures with feeder cells from the typical microenvironment can extend the lifespan of patient-derived cells in the lab. In leukaemia, culture of patient-derived blasts in a 2D-on-plastic mono-culture approach, generally leads to apoptosis of the leukaemia cells. Therefore, in order to robustly proliferate these cells in vitro, feeder layers of Mesenchymal stem cells (MSCs) are required and has in fact demonstrated that leukaemia cells proliferate in these conditions [51]. Co-culture again comes with the added issue of sourcing feeder cells which again can be difficult and may have source-to-source variance leading to varied culture success. Moreover, the human body is three dimensional where cell–cell homotypic and heterotypic interactions, as well as cell signalling is influenced by cell polarity, which in turn cannot be faithfully captured in 2D mono and co-cultures [52].

2.2.3. 3D tissue slices

Utilisation of three-dimensional models, such as tissue slices in cancer studies provides patient derived-cells in native tissue so allows consideration of more environmental and cell–cell characteristics. Features such as the tumour-stroma interactions, immune landscape and epigenetic features can therefore be assessed using these approaches. Tissue slices have further advantages as they have short generation time and high success rates [53]. However, as with other patient-derived material these samples can be difficult to source and tissue slices may not be applicable for all cancer types such as liquid cancers.

2.2.4. Spheroids

Spheroid systems add a layer of complexity to traditional 2D tissue culture in that they introduce a 3D aspect to models. These models provide a much closer physiological relevance for studying disease, especially cancer where altered cell polarity can both impact the disease as well as be influenced by it [54,55]. These models have typically been applied for therapeutic testing due to their ease of creation and low cost compared to in vivo approaches. These models tend to be on the smaller scale when compared to organoid models with sizes being 100-500 µm in diameter [56]. Having models that are 3D are beneficial when studying cancers and tumours since cancer is a complex disease
encompassing the microenvironment as well as genetic changes contributing to disease.

A recent study developed a human mesenchymal spheroid as a replacement approach to replace moderate severity leukaemia drug testing. These spheroids have successfully been used to support the ex vivo culture of patient-derived leukaemia cells that are difficult to culture in vitro without feeder support [51]. Superior proliferation of leukaemia blasts was observed in 3D spheroid culture compared to 2D co-culture methods. A marked reduction in sensitivity to Dexamethasone was also seen in 3D spheroid culture in comparison to 2D [57]. This simple and tractable approach potentially allows for a patient-specific platform for drug testing of leukaemia therapeutics that also considers some of the aspects of the microenvironment [57].

2.2.5. Organoids

Organoids are complex 3D structures derived from multi-lineage cell types and can be self-organising. The development of organoid technology has opened many opportunities for disease modelling without the need for animals. Organoids can be used for a wide range of applications in translational medical research by enabling study of disease pathobiology [58]. They offer a more complex modelling system to spheroids in that they can include multiple cell types from the organ of interest alongside ECM. The ECM serves as a scaffold surrounding cells in a tissue providing a stable microenvironment that allows for cell migration, proliferation and differentiation [59]. In vitro cultures where ECM from the native tissue is present has demonstrated increased proliferation and differentiation [60].

In cancer, organoid models for a host of organs have been generated including bone marrow [61], breast [62], lung [63] and rectal cancers [64] among others.

Sachs et al. [62] describe the protocols for long-term culture of mammary epithelial organoids and have developed over 100 organoid lines from patient tissue that encompasses the broad range of breast cancer (BC). They see that using their protocols they can generate morphologically similar models that also have matched BC biomarker HER2 (seen in approx. 20% BC cases) status to the original tumors. Using a set of drugs targeting the HER pathway they generated dose response curves. They observed that models overexpressing HER2 were sensitive to the drugs and were drug-resistant in the absence of HER2 among others.

Yao et al. [64] have used a rectal cancer organoid derived from patients with locally advanced rectal cancer. They have treated their organoid model with neoadjuvant chemoradiation which is enrolled in Phase III clinical trial and found that the chemoradiation response displayed by their organoid models are recapitulated in patients with accuracy, specificity and sensitivity implying the use of the organoid models as a companion diagnostic tool in rectal cancer treatment [64].

Whilst organoid models using cells of patient-origin are vastly important to modelling disease, they present a problem in lack of sample availability and therefore can mean a limited cell resource.

2.2.6. Induced pluripotent stem cells (iPSC) in organoid modelling

The emergence of iPSC technology has meant that samples of somatic cells can now be taken and reprogrammed to an embryonic stem cell (ESC)-like state [65]. The pluripotent nature of iPSC then allows differentiation into any cell type belonging to the three germ layers. That is, ectoderm, mesoderm and endoderm [66]. Furthermore, as these cells are derived from somatic cells and do not require destruction of embryos, they are well regarded from an ethical point of view [67].

iPSC-derived lineages provide an optimal cell source for organoid modelling- this is a powerful technology specifically in disease modelling, drug screening and precision medicine. iPSC indeed have been used to generate a number of organoids representative of several tissues. Models of the brain [68,69,70], kidneys [71] and heart [72], for example, have been generated using cells derived from iPSC lines. These models furthermore complement several applications including microfluidic systems, diagnostic in vitro preclinical models or transplantable therapies (Fig. 2).

Given the significance of the oncogenic niche, cancer preclinical models in terms precision medicine, is inherently complex and such models greatly benefit from genome editing [73]. The iPSCs generated contain specific cancer causing genetic aberrations and when differentiated produce cells of the desired tissue and with the relevant cancerous phenotype. The first case of these kind of models being used was in 2015 when iPSCs were derived from patients with Li-Fraumeni syndrome- a rare hereditary genetic disorder associated with increased risk of cancer [74].

In addition, this technology has been further used in developing kidney organoids [75] through a generated receptor tyrosine kinase (c-MET) mutated iPSC. This approach provides benefits, for example in c-MET mutated iPSC and could potentially allow for modelling of other cancers such as glioblastoma [76].

2.2.7. Bio-gels

Bio-gels or hydrogels provide a 3D culture approach in which ECM proteins can be incorporated in order to support cell proliferation. Many bio-gel formulations exist depending on the microenvironment the model is looking to recreate. Matrigel is a well-established, commercially available product that has been used in a large number of studies to develop organoids of the colon [77], stomach [78], pancreas [79] and many others. There are some downsides with using Matrigel however, it has an undefined nature and has batch-batch variations [80,81], which deter experimental reproducibility and consequently obstruct uptake of these models by a wider research community. In addition, the mechanical properties of bio-gels required to recapitulate the ECM-derived cellular support is of emerging significance. The mechanical properties exhibited by Matrigel is heterogeneous [82] resulting in potentially inconsistent models. Finally, as this product is derived from mouse it prevents any transplantation applications into human due to immunogenicity [83].

With these pitfalls in mind, defined and animal component free synthetic formulations are becoming more popular. Synthetic materials allow for stringent control of mechanical and chemical biogel properties whilst allowing for higher reproducibility. Alongside synthetic polymers, biomacromolecules from non-animal sources such as alginate, are popular options due to their low cost and ease of crosslinking through ionic gelation [84]. Use of these molecules have the caveat of not necessarily being tissue-specific for the desired model, and therefore this needs to be enhanced with chemical cues or proteins specifically relevant to the ECM of interest [85].

One consideration when developing ECM hydrogels is that the cultured cells will begin to produce their own ECM by digesting the original hydrogel and remodelling the gel around them. This remodelling can occur via several mechanisms such as adhesion, migration, contraction, degradation and ECM deposition and since innate ability of cells can be highjacked in lab-based cultures to facilitate ECM production that can generate tissue-like structures with optimal biomimicry. The mechanical properties of a hydrogel can also dictate cellular phenotype and activity. Presence of mechanical stimuli or chemical modifications can also drive the cell’s production of ECM components.

Presence of ligands or motifs within a hydrogel formulation allow for cell-gel binding through integrins or other transmembrane glycoproteins.

Abdullah et al. [61] have developed a 3D organoid model of the human bone marrow that is based on a Matrigel/collagen hydrogel using iPSCs committed to mesenchymal, endothelial and hematopoietic lineages. Using these models, they recreated an equivalent fibrosis model in order to recapitulate the hematopoietic niche remodelling seen in haematological malignancies. A gradient of TGF-β dosage yielded a dose response of hallmarks of fibrosis such as collagen 1A (COL1A1) and alpha smooth muscle actin (αSMA [ACTA2] with collagen deposition observed following TGF-β treatment. This is
also seen in patients with myelofibrosis. There was also reduced vascularisation seen suggesting multi-lineage remodelling, similar to that seen in fibrotic adult bone marrow. Additionally, introduction of TGF-β inhibitor normalised COL1A1 and ACTA2 levels. These results suggest that their organoid is an efficient method of modelling bone marrow fibrosis allowing pharmaceutical screening [61].

2.3. Financial and ethical Sustainability

In vivo experimentation is not a sustainable approach for testing novel therapeutics and leads to long model development and experimental times and projects with very high financial burdens. In comparison in vitro modelling provides a relatively more cost effective and sustainable route in target discovery, drug screening and testing of both novel single agent and combinational treatments. Finances surrounding the housing and upkeep animals is high due to specialist living conditions, food and care. In vitro modelling can provide a cheaper alternative with varying associated costs (Table 1).

3. Next generation non-animal technologies as preclinical models

3.1. Scalable 3D models

A key caveat for complex 3D models is how scalable and high throughput they are, especially in relation to ability to deliver data and information in compliance with clinically relevant timeframes. This may be associated with the ease of model creation; it may relate to the wide reach that these models have and how specialised the working setup or expertise is at end user stage. Scalability, tractability, accessibility and biomimicry are indeed key features to balance in generating meaningful preclinical models. Model selection currently relies on a trade-off between throughput production and in vivo recapitulation (Fig. 3).

Table 1

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<td>Medium-High</td>
<td>1–3 Months</td>
<td>Lowly developed</td>
<td>Low potential</td>
</tr>
</tbody>
</table>

However, emergence of new technologies has driven model development away from such compromise and towards successful generation of replicative 3D models in a high-throughput manner. Automation in tissue culture applications has opened up the possibility of high scale model development. Utilising these technologies can replace the repetitive nature of many routine tissue culture techniques which typically is carried out by human operators, and is therefore subject to human-induced errors, which can furthermore negatively impact standardisation, reproducibility and consequently endorsements of such models. However, through automation of these tasks, an increase in not only scalability but also reproducibility can be achieved with the
potential to standardise tissue culture protocols to promote wider uptake throughout academia and industry. Inclusion of bioprinting or similar technologies can provide an opportunity to fully automate entire pipelines including cell source maintenance, organoid production and downstream cultures with drug distribution via robotics.

Furthermore, advancements in artificial intelligence (AI) and machine learning (ML) can help identify complex relationships between input parameters and reduce the need for time-consuming and costly experiments. These developments have the potential to bring about rapid advances in the fields of tissue engineering and optimising tissue culture protocols. In addition, through the larger data sets generated via ML, previously unobserved results can be identified through algorithms that can learn extensively from such comprehensive data sets [86].

3.1.1. 3D bio-printing

3D Bio-printing is a fabrication technology that has been applied to preclinical models in order to increase reliability, reproducibility and scalability [87]. In bio-printing, small units of cells and polymers or ECM components are precisely dispensed to generate tissue-like structures. The methodologies for dispensing cells must therefore be compatible with deposition of live cells whilst maintaining high accuracy and high-resolution. 3D bio-printing technology utilises bio-gels as mentioned previously and have all the benefits of 3D cultures.

Within the field there are several bio-printing approaches, each with unique benefits and therefore applications in 3D modelling. The most popular techniques are inkjet, extrusion and laser-assisted printing.

Inkjet printing setups are like conventional 2D inkjet printers whereby a cartridge containing the live cells and biomaterials is connected to a printhead. During printing, the printhead is squeezed to produce a droplet of accurately controlled size. This approach is beneficial due to relatively low costs, printing speed and approximately 80–90% cell viability [88]. A limitation of this approach is that printing high viscosity liquids is difficult and could potentially block the printhead nozzles and within the cartridge a “settling effect” takes place, where initially evenly distributed cells begin to settle at the bottom of the cartridge, increasing viscosity and blocking the printhead further.

Extrusion printing is derived from modifying inkjet setups to allow printing of viscous liquids that conventional inkjets cannot print. The key difference in material produced is that extrusion printing generates continuous cylindrical lines instead of droplets due to the utilisation of a continuous printing force exerted by an air-force pump or mechanical screw. Despite extrusion printing providing an option for printing difficult materials, the cells are exposed to higher mechanical stress reducing viability.

Laser-assisted printing is a more complex setup to inkjet in that it requires a donor layer that responds to laser stimulation on top of the bio-ink [89]. Once stimulated by the laser, a portion of the donor layer is vaporised generating a bubble on the bio-ink interface, propelling a droplet of bio-ink. The droplet then falls onto a crosslinking substrate to create a cell-laden gel [90]. The benefit of this technique is that the cell and gel-forming material never touches the printing apparatus reducing mechanical stress on cells, yielding higher cell viability (95%). However, due to the more complex setup this approach has higher associated costs.

Even though these are the most established techniques for 3D bio-printing, there are novel printing technologies emerging on a regular basis. One such novel technology is Reactive Jet Impingement (ReJI) bio-printing. High cell density is a key aspect when developing tissue-
like models, however printing such densities using the previous approaches is difficult leading to blockages. The ReJI approach provides a solution to this problem by delivering the benefits of drop-on-demand printing whilst having the ability to deposit physiological cell densities. The concept of ReJI relies on 2 microvalves connected to different cartridges that are angled towards each other so simultaneous deposition of the droplets leads to them meeting in mid-air [91,92]. At this impingement point, the gel forms a creating a cell-laden gel that drops onto the printing plate. By having two separate cartridges and microvalves, amount of viscous material being passed through each valve is reduced, therefore reducing blockages. Using the ReJI technique da Conceicao Ribeiro et al. [91] were able to print high densities of mesenchymal stem cells (MSC) in a Collagen/Alginate/Fibrinogen (CAF) hydrogel. At high cell densities (40 million cells) the MSCs began to differentiate towards osteoblasts, as confirmed by qPCR and calcium deposition [91]. Despite this study not directly producing an organoid for cancers, it demonstrates an application for generating high-throughput organoid models which in turn have physiologically accurate cell densities and consequently form microtissues rapidly. This aspect of model generation is crucially important in capturing the tissues like microenvironment that is needed for organoid models used for cancer studies. It also presents a high-throughput approach that can help increase model availability and reduce time needed for model generation, leading to data yields in more clinically relevant timeframes compared to in vivo models.

An area where 3D bio-printing has been applied in cancer research is in breast cancer where Swaminathan et al. have investigated whether directly bio-printing spheroid models of breast tissue could be done, providing an immediate platform for drug response testing. Human breast epithelial cell lines were printed either as individual cells or spheroids in monocultures and in co-cultures with vascular epithelial cells. Through extrusion printing, individual breast cells were printed in Matrigel and two other alginate-based hydrogels. Despite the cell viability of these prints being between 81 and 96%, only cells in the Matrigel formed spheroids after 5–8 days of culture. When the pre-formed spheroids were printed 82–98% cell viability was observed and this viability was maintained over 7 days post-print. They also validated these printed spheroids through a drug-response assay to chemotherapeutic agent paclitaxel that is used for breast cancer treat.

3.2 Organs-on-chip

One of the limitations with organoid models is that they do not represent the tissue-tissue interfaces, organ-level structures, fluid flows and mechanical cues that are present within the body [94]. Techniques have been developed using microfluidic cell culture technology. These in vitro approaches known as organs-on-chips (or organ chips) are being utilised for modelling cancer microenvironments. This technology relies on manipulating fluids at a microscale level [95]. These systems are beneficial as many parameters can be tightly controlled and therefore more accurately assessed in relation to cancer cell biology.

Typically, these models are assembled using polydimethylsiloxane (PDMS) scaffold covered in porous membranes into either organ-specific structures or wells suitable for multiple tissue cultures [96,97]. Where these models differ from other 3D in vitro cultures is that controllable shear stress, shear flow and nutrient concentration gradients create mechanical forces on the cultures that are critical for tissue development and organ morphogenesis. These mechanical stimuli are interpreted by the cells triggering biochemical signalling directing cell adhesion and migration [98]. In disease, these mechanical stimuli can be disrupted highlighting the importance of incorporating these aspects into pre-clinical models.

An area where microfluidic systems have been used in cancer is to investigate metastatic potential of breast cancer cells. Organ-on-chip models to represent invasion/chemotaxis and extravasation were created to quantify these behaviours towards specific tissues. Lung, liver and breast microenvironments were created using Matrigel embedded tissue-specific cells. Invasive MDA-MB-231 breast cancer cells invaded both the lung and liver microenvironments whereas non-invasive breast cancer cells did not. These data were consistent to published clinical data suggesting the representative ability of these systems in mimicking metastatic phenotypes in vitro [99].

Linking multiple human organ chips, termed body on chip, has been presented as an alternative model for some animal tests associated with drug pharmacokinetics. Herland et al. [100] observed that use of fluidically coupled multi-organ-chip-based system could be used to predict pharmacokinetic parameters of nicotine that are highly comparable to human clinical studies. Inclusion of endothelium in these models allowed physiological mimicry [101] and therefore predictions of drug transport across endothelial- parenchymal barrier [100]. Utilising this approach here suggests it could be applied to other pharmaceutical fields such as cancer to predict pharmacokinetics instead of animal approaches.

There are, as with all other models, areas for further development before the technology is ideally suited as a replacement for animals. Application of the current models have highlighted their use in easily observed and measured functions. A replacement model should be able to recapitulate multiple physiological aspects and development of these microfluidic systems is required in order to capture these aspects.

Utilising these models in a high throughput manner is still not an option due to the difficulty in scaling up model production. These models are complicated to handle reducing the widespread application outside of specialised laboratories and groups.

3.3 Testing immunotherapy

Over the last decade the use of the immune system to fight cancer has revolutionized patient outcomes at least in certain cancer types. One major treatment involves anti-tumour T cells being harnessed following their activation via targeted monoclonal antibodies. However, some patients’ cancers are resistant, and not all cancer types are benefiting from this potential. There are also toxicities induced such as autoimmunity. Extending the use, or the development of improved or new drugs, requires considerable in vivo preclinical investigation in animal (mouse) models, and often these include using immunocompromised animals, that do not faithfully mirror patient or human immune systems. In vivo models are starting to be replaced by in vitro multi-cellular, multi-dimensional models, that are becoming able to reproduce in vivo mechanisms [102,103]. Modelling immunotherapy in vitro requires creating an overlap between the lymphoid tissues in which immune responses develop, the cancer tissues in which the resulting immune responses need to act against the tumour cells, and the vessels that transport the immune cells between the two [104,105]. Co-ordinating these compartments into a meaningful model system is a challenge for researchers, but one that has potential to become achievable [106].

Furthermore, the current gold standard preclinical cancer models remain in vivo patient derived xenograft (PDX) models, where cancer cells from the patient are transplanted into immunodeficient mice. For example, the NSG, NOD scid gamma mouse is used to create an orthotopic PDX leukaemia model where cancer cells from patients are transplanted into the mouse bone marrow (femur), and the animal is subsequently monitored for successful engraftment over the next few weeks to months. Of note, the NSG mouse lacks B and T cells and are furthermore deficient in functional natural killer cells [107]. Despite
being pivotal in cancer drug discovery, the necessarily altered immune system in these animals is associated with significant caveats, for example, inability to accurately model immune mediated treatment response of new anti-cancer candidates, including but not limited to immunotherapy-based combinational treatments. Such caveats impede drug development and further exacerbate drug attrition rates, an obstacle that is crippling the clinics as well as the pharmaceutical industry.

Incorporating humanised immune systems in next generation \textit{in vitro} models is important for faithfully recapitulating patient-specific immune response, the tumour microenvironment and furthermore in providing a clinically relevant platform for immunological studies. Various approaches have currently been developed in order to allow study of immunotherapy response, including reconstitution approaches such as submerged cultures or holistic approaches such as air–liquid interface models. Early promise has been seen when human immune cell have been co-cultured with patient derived cells. Patient-derived organoids with a fibroblastic and immune component have been demonstrated as having the ability to propagate primary tumour fragments whilst also displaying the T cell clonal diversity mirroring the patient’s peripheral blood [108].

3.4. Microbiome

Recently, there have been great advances in the understanding of the role of the microbiome and its implications for tumour biology. Through direct and indirect modulation of the TME, microbes can impact tumour survival. In colorectal cancer, the impact of the gut microbiome is an area of keen interest. The microbiome composition of colorectal cancer sufferers differs from healthy individuals suggesting a correlation between the two features. In mouse models, the effects of certain microbe communities on colonic epithelial cells have been investigated and found that many pathways were impacted by the gut microbiome including cell proliferation, inflammation and immune cell infiltration [109, 110].

With this in mind, development of models to incorporate the microbiome is a future step that will increase the complexity of the models providing another aspect of non-tumour cell characteristics and represent the TME more closely.

4. Precision medicine

Precision medicine refers to the process of developing therapeutics that are expected to be effective for particular groups of patients. Precision medicine considers multiple environmental and biological aspects to generate candidate therapies. The current trend for therapy development relies more on precision medicine with genomic biomarkers. Patients that are screened for biomarkers alone, however, do not necessarily benefit from genomic precision medicine strategies [111, 112]. Therefore, addition of other screening methods, such as phenotypic screening, are required alongside genetic profiles in order to build a more accurate picture.

Incorporation of patient-specific cells into organoid models provides a platform for generating patient-specific drug response data which can then be applied to patients with similar characteristics, including but not limited to immunotherapy-based combinational treatments. Such caveats impede drug development and further exacerbate drug attrition rates, an obstacle that is crippling the clinics as well as the pharmaceutical industry.

Yoshiki Sasai and his colleagues pioneered methods to mimic \textit{in vitro} some of the strong regulatory systems of neurulation, such as cell differentiation, spatial patterning, and morphogenesis to generate brain structures and the retina ‘in a dish’ – now known as brain and retinal organoids [114-116]. \textit{In vitro} modelling of brain organoids has progressed, first with the optimization of protocols for neutralization of floating embryoid bodies (aggregates of embryonic stem cells) and then with the understanding that neural cells can be maintained continuously as floating aggregates in Matrigel (extracellular matrix components). Protocols these days are either focused on guiding/directing regional identity in order to generate specific regions of interest more reliably [117], or they are unguided [68, 118]. Brain organoid technology has since progressed from unguided whole-brain organoids to the cortex, midbrain, hippocampus, cerebellum, spinal cord, and other regional brain organoids [119-121].

Brain and retinal organoids mimic a significant proportion of the cellular variety and developmental architecture of the native human brain and retina. However, the neurophysiology of neural circuits within these organoids is still not well understood. Recent studies showed oscillatory dynamics in human cortical and brain organoids [128, 129]. Earlier studies showed retinal neuronal activity and light-driven activity [130-134]. The combination of organoid techniques with cutting-edge neuroscience technologies will pave the way for new scientific discoveries and better understanding of neurological disorders. However, while organoids offer immense potential, they currently lack key essential traits such as the complexity and size of the human brain and retina.

Retinoblastoma organoids have been developed from patient-derived iPSC cell sources with germline RB1 mutations and have been used to induce retinoblastoma in immunocompromised mice. The blasts formed from the organoid system have indistinguishable molecular, cellular and genomic features from human retinoblastoma providing insight into the origins of the disease as well as the implication of RB1 mutations in the mechanisms of tumorigenesis [135].

Another area of need is to model off site metastatic infiltration of solid and systemic cancers (for example leukaemia) to the Central Nervous System (CNS). This is highly relevant in paediatric leukaemia where most children present with subclinical CNS infiltration, predominantly to the leptomeninges [136, 137].

Standard leukaemia treatments, such as chemotherapy, may not be suitable for treating CNS infiltration due to the blood–brain barrier blocking therapeutic molecules. Identifying CNS disease nevertheless remains indispensable in avoiding disease recurrence; however current CNS directed therapy is associated with significant neurocognitive toxicity. The leukaemia niche, especially fibroblast cells in BM-microenvironments mediate cancer treatment resistance, thereby affecting clinical outcome. Nevertheless, the CNS-microenvironment and its interactions with ALL remain relatively unexplored due to a lack of appropriate preclinical platforms that can model effect of the leptomeningeal niche in ALL dormancy and treatment response. This in turn hampers new drug development.
are many additional areas to explore in developing better treatments such as biomarker identification, trials of low toxicity agents and optimising drug delivery systems in CNS leukaemia [137].

Capturing these aspects in vitro is difficult due to the complex nature of the movement of the leukaemia from the bone marrow to the CNS. Organoid models of the blood brain barrier are therefore crucial for understanding leukaemia infiltration into the CNS and to study blood brain transport of drugs. Organoids that can recapture some of the signalling and transporter proteins found at the blood brain barrier have been developed by co-culturing endothelia, pericytes and astrocytes. These organoids have been used to model drug transport across the blood brain barrier and analyse drug penetration [138]. However, limitations of these organoid models is that they do not capture patient-derived leukaemia cell infiltration within the CNS, and therefore ignore the cellular components of CNS metastasis.

Microfluidic systems that replicate the bone marrow microenvironment alongside the peripheral blood and CNS could provide an opportunity to study systemic and organ-specific response to therapy, and mechanisms behind malignant infiltration of the CNS. The ability to replicate these aspects would provide a strong preclinical model. Addition of a component to replicate the blood brain barrier into this model, such as described above, could further provide a wider perspective to study CNS leukaemia and accelerate drug discovery and drug development to tackle leptomeningeal disease.

4.1.2. Muscle organoid model in cancer

Cancer is the most studied cause of sarcopenia, a condition described by the decline in muscle mass, strength and physical function. Sarcopenia is associated with a high risk of physical frailty, falls, mortality, prolonged hospitalisation, complications from cancer surgery, and chemotherapy toxicity [139]. The catabolic processes in sarcopenia are a result of not only the presence of malignancy but also the chemotherapy treatment (cisplatin, irinotecan, doxorubicin, and etoposide) and certain targeted drugs. Cancer treatment may cause direct muscle atrophy by activating the transcription factor NF-kappa B [140], which is associated with the upregulation of ERK1/2 and p38 MAPKs that disrupt the AKT/mTOR pathway [141]. Despite intense investigation and clinical trials, the major therapies prescribed to assist in the preservation of skeletal muscle mass are lifestyle measures, particularly exercise combined with nutritional supplementation. Understanding the physiological and pathological conditions associated with this condition is necessary to support the development of new therapies. In recent years several in vitro muscle models have been developed [142] to study muscle physiology and disease. The model will be used to investigate novel pathophysiological mechanisms in sarcopenia and to test new drug treatments. Animal-free models present several advantages compared to animal models. In addition to replacing animals and their products, animal-free models are more relevant to human diseases, as engineered tissues are developed using human cells, synthetic embedding scaffolds and growth conditions that can replicate the human condition.

4.1.3. Colonoid models and their applications

There has been limited success with available treatments of colorectal cancer which are mainly based on chemotherapy and/or radiation therapy, most of which have been around for decades. Therefore, there is an unmet need for representative preclinical models of colorectal cancer in which more targeted therapies using small molecular inhibitors and monoclonal antibodies can be validated. While animal models can be very helpful at the initial stages of drug testing, they often fail to mimic the conditions in human tissues. For example, underlying cellular and molecular mechanisms can be significantly different, and therefore that small molecular inhibitors and monoclonal antibodies will work differently, or not at all. Thus, many treatments fail when transferred into the clinical setting. One challenge in this investigation is the widespread negligence of the physiological oxygen gradient present in the healthy colon wall, which is not recapitulated in 2D monolayer cell cultures. Electron parametric resonance (EPR) oximetry has been used to identify a vertical oxygen gradient within the colon. In contrast to normal air, with a partial pressure (pO2) of 145 mmHg (approx. 21%), the baseline levels in epithelial cells lining the colonic mucosa exist at a much lower pO2. EPR oximetry estimates the colonic muscle wall at approx. 42–71 mmHg (7–10%), approx. 42 mmHg (6%) in the vascularised submucosa and between 3 and 11 mmHg (0.4–2%) in the colonic lumen [143]. The oxygenation of colon tissue and creation of the O2-gradient are influenced by local differences in metabolism due to a high cell turnover (colonic epithelium is renewed every 5–6 days), submucosal vasculature and the availability of ambient O2 in the colon lumen and is largely ignored in existing experimental models [144]. Although this O2-gradient can be recapitulated in spheroid models, these lack the complexity of including various cell types that are allowed to differentiate within the model as they would in vivo. Primary cell cultures are also short-lived due to most normal colon tissue cells being already terminally differentiated. Colonoids could provide a promising alternative to animal models that recapitulate the human in vivo environment for long-term studies.

Progress in stem cell research over recent years has meant that we are now able to isolate primary stem cells. The formation of a successful colonoid requires imitation of the intestinal stem cell (ISC) niche at the base of the colonic crypt, in order to recapitulate the conditions that ultimately determine ISC fate. To generate a colonoid, colonic crypts, which contain human adult stem cells, are isolated from biopsies or surgically resected tissue. Colonoids may also be formed using human embryonic stem cells, or inducible pluripotent stem cells (iPSCs) [77,145,146]. Colonoid culture medium must be able to activate the wingless-related integration site (Wnt) signalling pathway, which is essential in ISC differentiation. This is achieved by the inclusion of R-spondin and recombinant WNT protein. However, the conditioned medium of Wnt3A-expressing cells is often preferred due to the hydrophobicity and multiple post-translational modifications of WNT [147]. The culture medium must also contain a cocktail of inhibitory molecules to inhibit the anti-proliferative effect of transforming growth factor beta (TGF-β) (gastrin), inhibit bone morphogenetic protein (BMP)-induced differentiation (Noggin), block the negative feedback effect of p38MAPK on epidermal growth factor receptors (SB202190), and prevent anoikis (Rho-associated protein kinase (ROCK) inhibitors) [77,145,148-150].

The applications of colonoids within biomedical research are vast. They provide a method of assessing anticancer drug toxicity that is specific to the human condition and can be monitored in long-term experiments. In addition to the study of colon cancers, colonoids enable representative study of colonic homeostasis and nutrition. These models may also be useful for individual patient analysis with a view to developing personalised medicine specific to the patient condition, thus improving prognosis.

5. Next-generation cancer disease modelling

5.1. High throughput applications of organoids

The use of organoids has many applications within cancer research and can be used for very simple or complex techniques. Typically, organoid systems have been used to explore candidate therapies or to screen current therapies against patient-derived cells. These models provide a more cost effective alternative to in vivo PDX models whilst maintaining genetic features of the disease.

However, the use of organoid models is not restricted to drug studies. Advancements in 3D imaging techniques have allowed visualisation of these complex models, allowing studies of cell–cell and cell–ECM to take place. Time-lapse imaging techniques have been used to monitor nanotubule formation and mitochondria trafficking in organoid systems of glioblastoma [151] – a feature of cancer cells alleviating oxidative stress. High throughput and high content techniques can also be applied to
organoid models. Targeted organoid sequencing (TORNADO-seq) uses targeted RNA-seq to highlight expression of gene signatures allowing for phenotypic evaluation of cells within the organoid. In response to treatment, TORNADO-seq can elucidate information as to mode of action of drugs. A major application of this technology in colorectal cancer has included identification of 56 drugs that could induce intestinal epithelial differentiation - a key driver in cancer progression with stem cell-like cells playing a role in tumour maintenance and metastasis.

High content imaging can provide quantitative analysis on organoids to highlight variable morphologies within 3D cultures. Light-sheet microscopy has been used to capture these cellular features whilst reducing the photobleaching or phototoxic effects that confocal imaging would have. In addition, application of light-sheet microscopy in a specialised setup, provided a platform for 3D live imaging of organoid development. Combining high throughput imaging with high density organoids could potentially be of strong interest for studying cancer progression. For example, a new technology described by Beghin et al. [153] captured features such as mitosis, apoptosis, organoid shape and cellular organisation via an automated microscopy technique, which conventionally is manually assessed by trained biologists [153]. Compilation of these images into readily available banks can help standardise organoid model features whilst also contributing to the development of Artificial Intelligence (AI) quantitative analysis. Such applications in cancer is particularly exciting as it facilitates high throughput drug screening and drug testing.

5.2. Spatial transcriptomics as a research tool to characterise multicellular organoid models

Traditionally, analysis of the genes expressed in tissue or organs has been carried out via bulk RNA seq and more recently single cell, or single nuclei seq. These methods, while very informative, are limited to profiling quantitative gene expression levels present in a particular piece of tissue or cell. Moreover, the location of where these genes are specifically expressed within the tissue, or the position of the cell within the tissue they were isolated from remains unknown.

Spatial Transcriptomics is a powerful technology, capable of visualising gene expression of all mRNAs in a section of tissue, within a positional context. Each capture region contains 5000 spots, each spot consisting of a high-density group of spatially barcoded probes that contain a sequence adaptor, a spatial barcode which is unique to that spot, a 12 base UMI, and a poly-T region designed to capture polyadenylated mRNA. The workflow of this technique can be defined into 4 steps. Firstly, a tissue section (FFPE or frozen) is fixed, H&E stained and imaged for histological purposes. Following this, the tissue is gently permeabilised, allowing the mRNA to be released and held in position by the capture probes. Probes are then removed from the slide and sequencing libraries are prepared from the capture RNA and sequence, approximately 50,000 reads per capture spot. The processed Visium data is then overlaid with the original, captured image to determine which genes are expressed, and where these specific genes are expressed within the tissue section. Reports can be generated from this data, highlighting the spatial cell cluster analysis (Fig. 4). Additionally, a list of differentially expressed genes in each cluster can be generated to allow further analysis.

Spatial Transcriptomics is a particularly beneficial technique when studying human disease in both adult and developing tissues, this can be carried out in a single tissue section or consecutive sections throughout a tissue sample or organoid to gain this information in three-dimensional context. By highlighting changes in the transcriptome between normal and diseased tissue we can implicate the underlying mechanisms behind diseases, increasing the potential of new targeted treatments to be developed as a result, in addition to gaining a better understanding of

10X Visium Workflow

From organoid tissue to visualisation of spatially expressed genes

1. Organoids prepared (FFPE or OCT Sections)
2. Imaging of optimisation slide for visualisation
3. Preparation of gene expression slide
4. Barcoding and Library Construction
5. Sequencing and data processing
6. Visualisation of spatially expressed genes

Fig. 4. 10X Visium Workflow. Samples are prepared via fixation and stained for H and E and imaged. The tissue is then permeabilised releasing mRNA that is held via capture probes. Probes are removed and sequencing libraries are prepared. The processed data is then added to the original captured image to determine spatial expression in a tissue or organoid. Created with Biorender.com.
how these diseases initially develop and evolve. Human organoids are increasingly being used to understand human organ-specific physiology in both healthy and diseased tissues, and in turn, are replacing the traditional method of disease modelling in animals. As such, they have emerged as powerful tools for personalised and precision medicine. This is mainly achieved by genetic modification of organoids, usually from patient cells or fetal/embryonic material [154].

Clinical research in organoids is a new and innovative method of directly researching human disease without having to do patient clinical trials or carry out these studies in animal models. Thus, it is imperative to validate organoid tissues alongside normal and diseased human tissue to further increase the reliability and accuracy of human disease modelling in organoids and spheroids, in turn, further reducing the need for animal models of disease. Spatial Transcriptomics is an informative and reliable method of validation for this purpose.

6. Challenges and future scope

Despite the trend of extensive publications and patents emerging in the field of 3D in vitro cancer models, further model development is mandated to facilitate their clinical and commercial translatability [155]. In vitro preclinical cancer models are expected and required to be compliant for high throughput drug screening, and such, tractable models are essential to facilitate target discovery and subsequent functional validation assays, as well as have wide applications in personalised medicine. With this in mind, 2D traditional cultures still remain core in vitro preclinical models for testing cancer therapeutics, largely owing to the inherent simplicity and accessibility of such model systems. Indeed, a key barrier to the wide endorsement of complex multicellular 3D models as the go-to preclinical approach is the technical complexity associated with developing these to the extent where they are scalable, efficiently biomimetic, widely transferable and show superior clinical and commercial translatable.

The several aspects that currently limit the translation of these models from bench to bedside are as appraised below.

6.1. Cancer heterogeneity

Cancer heterogeneity refers to the clonal and molecular complexity and cell–cell heterogeneity that is seen in clinical samples obtained at diagnosis of disease. This can be difficult to model given interpatient variability as well as intrapatient tumour heterogeneity. Additional complexity arises between primary and secondary tumours. Modelling the complex genetic and protein expression profiles of these tumours is challenging and is currently not necessarily recapitulated in the current in vitro models. Techniques such as single cell sequencing are approaches that provide comprehensive information on the intratumour heterogeneity, thereby allowing some progress in decoding complex clonal relationships [156] however further development, such as the ability to dissect the cancer and its niche at single cell level and within a 3D spatial context are necessary.

6.2. Tumour microenvironment

Emerging evidence substantiating the impact of the cancer microenvironment is allowing for in vitro models to become more mimetic, however many preclinical models still do not incorporate key acellular aspects of the oncogenic microenvironment such as the ECM or fluid movement, with several models limiting focus on cellular aspects alone. Moreover, the diversity of cellular components, including aspects like lineage plasticity is a tissue microenvironment is often neither deciphered nor modelled. As mentioned previously, there are several aspects, some of which remain under researched, of cancer-niche interplay that contribute to disease progression and treatment resistance. Currently, addition of some of these aspects into preclinical models hinders their scalability, consequently mandating specialist experimental setups and development of next generation research tools that facilitate all of model development, model characterisation, cell fate tracking and necessary genetic manipulations.

6.3. Scaling up

Scaling up model production is necessary for translation to the clinic. Equipment allowing for high throughput model generation is usually very specialised to the model specifically or the laboratory setting. Approaches such as 3D bioprinting are excellent proven methods for increasing throughput model production, whilst retaining accuracy, but they require specific bioprinting materials and setups. Scaling up models also requires vast quantities of cell source in order to produce these models within clinically relevant time frames. Incorporation of primary or patient-derived cells is desirable for these models however, these can be difficult to source and such state-of-the-art research projects are heavily interdependent on inter-disciplinary collaborations, including but not limited to, hospitals, academia and industry. iPSCs, especially patient-derived iPSC lines, does alleviate some of the tension here by providing an infinite cell source but iPSCs have their own caveats such as controlling directed differentiation into the desired phenotypic lineage, and retaining the desired phenotype of differentiated cells within the disease models [157].

7. Conclusion

The urgent need for improved preclinical models to successfully alleviate drug attrition in cancer drug development has revolutionised the field of in vitro cultures, specifically with the emergence of next generation ex vivo 3D preclinical models. Organoid models in the correct culture settings can aim to faithfully recapitulate the cancer as it would arise, propagate and respond to treatment in the patient. Indeed the first step to developing such models is to define the molecular complexity of the cancer itself and furthermore decode the mechanisms driving the complex interplay between cancer cells and their surrounding niche. Furthermore, the cellular diversity of the tumour microenvironment coupled with its dynamism is a key contributor to both cancer progressions, cancer dormancy, re-awakening of dormant cells and consequently treatment response. Besides capturing these key aspects, a clinically translatable model should provide sufficient tractability to facilitate scrutiny of complex biology such as cancer cell fate tracking within the context of its niche. Moreover, addition of acellular components to these models such as synthetic and tissue-minetic ECM are needed to enhance their capacity for in vivo (animal) predictability. Ultimately, optimal next generation 3D preclinical platforms will aim to successfully meet 4 key objectives: optimal clinical translatable, transferability of experimental set up between different laboratories, reproducibility of outputs and scalability to ensure endorsement by both academic and industrial sectors. Innovation of such cutting edge 3D models will enable extensive implementation in preclinical studies to meaningfully impact future cancer research to a point where it can effectively replace animals in cancer drug testing.

CRediT authorship contribution statement

Sean Hockney: Visualization, Writing – original draft, Writing – review & editing. Jessica Parker: Visualization, Writing – original draft, Writing – review & editing. Jasmin E. Turner: Visualization, Writing – original draft, Writing – review & editing. Xanthea Todd: Writing – original draft, Writing – review & editing. Stephen Todryk: Writing – original draft, Writing – review & editing. Roben Ger Gieling: Writing – original draft, Writing – review & editing. Gerrit Hilgen: Writing – original draft, Writing – review & editing. Deepali Pal: Conceptualization, Supervision, Funding acquisition, Writing – original draft, Writing – review & editing.
Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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