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Next-generation cancer organoids

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Organotypic models of patient-specific tumours are revolutionizing our understanding of cancer heterogeneity and its implications for personalized medicine. These advancements are, in part, attributed to the ability of organoid models to stably preserve genetic, proteomic, morphological and pharmacotypic features of the parent tumour in vitro, while also offering unprecedented genomic and environmental manipulation. Despite recent innovations in organoid protocols, current techniques for cancer organoid culture are inherently uncontrolled and irreproducible, owing to several non-standardized facets including cancer tissue sources and subsequent processing, medium formulations, and animal-derived three-dimensional matrices. Given the potential for cancer organoids to accurately recapitulate the intra- and intertumoral biological heterogeneity associated with patient-specific cancers, eliminating the undesirable technical variability accompanying cancer organoid culture is necessary to establish reproducible platforms that accelerate translatable insights into patient care. Here we describe the current challenges and recent multidisciplinary advancements and opportunities for standardizing next-generation cancer organoid systems.

Tumour heterogeneity. Heterogeneity in cancer is often regarded as the most substantial roadblock in the development of effective, patient-specific therapies[1,2]. Specifically, patients often present diverse tumour phenotypes that dynamically evolve throughout disease progression and clinical treatment. This extreme heterogeneity within and across patient cancers often explains widespread patient therapeutic responses, including intrinsic and acquired therapeutic resistance[2]. Additionally, it exacerbates the difficulty of designing successful clinical trials that target broad patient populations, as specific therapeutic mechanisms may not be effective against tumours with varying origins and phenotypes[1,3]. Therefore, successful advancement of personalized cancer therapies will depend on our ability to systematically define and model cancer heterogeneity.

Cancer heterogeneity can be subdivided into two overarching categories: intra- and intertumoral. While intratumoral heterogeneity is defined by spatiotemporal cellular and extracellular diversity within a given patient's cancer, including both primary tumour and metastatic populations, intertumoral heterogeneity describes population-level variations between patients with the same cancer subtypes[2]. Due to advancements over the last decades, the application of several omics methods—primarily bulk and single-cell DNA/RNA sequencing—to patient tissue samples has revealed that genomic and transcriptomic instability in tumours can support cancer's ability to dynamically select, harbour and amplify subpopulations of phenotypically distinct neoplastic clones, leading to overall cancer cell heterogeneity[4,5]. Recently, the cooperative use of several multidisciplinary techniques has highlighted that genetically divergent intratumoral subpopulations can exhibit distinct cellular phenotypes, transcriptomic profiles, epigenetic signatures, metabolic rates and morphological features that directly influence cancer progression, metastasis and therapeutic response[6].

While this progress has expanded our understanding of neoplastic cell heterogeneity, tumoral diversity has also been attributed to several other less explored microenvironmental sources, including the presence of non-neoplastic cells, niche-relevant soluble factors and the altered extracellular matrix (ECM)[7,8]. In particular, tumour microenvironment (TME) cells—including fibroblasts, immune cells, endothelial cells and so on—exhibit extreme biological diversity, which directly and indirectly influences neoplastic cell phenotype through complex reciprocal signalling mechanisms mediated through soluble cues, cell–cell contact and ECM remodelling[8]. Intertumoral heterogeneity can also arise from altered tumour cell origin, stage of diagnosis, treatment status, tumour recurrence, patient systemic health and unique environmental risk factors[2,6].

Overall, the ability to accurately recapitulate the vast inter- and intratumoral biological heterogeneity in cancer models is a fundamental and unresolved goal in cancer biology. Understanding the sources and implications of spatiotemporal tumour heterogeneity will undoubtedly improve our evolving definition of cancer and aid in the design of effective patient-specific treatment strategies. On the other hand, a notable field-wide effort for improving cancer culture standardization and reproducibility has also come to the forefront of cancer research. As a one-size-fits-all approach to modelling human tumours probably does not exist, realizing both of these seemingly paradoxical goals is an extremely challenging moving target, albeit central to clinical translation of precision oncology.

Organoids for modelling interpatient tumour heterogeneity

More recently, three-dimensional (3D) organoid culture of human tumour tissue has emerged as a relatively low-cost and representative platform to model cancer heterogeneity and interactions with the TME in vitro[9] (Fig. 1), while simultaneously addressing several limitations of traditional cancer models (Box 1). Here, we define cancer organoids as 3D self-organized assemblies of neoplastic cells derived from patient-specific tissue samples that mimic key histopathological, genetic and phenotypic features of the parent tumour. This Review largely focuses on cancer organoids comprising neoplastic cells—and occasionally TME cells—from human, patient-derived, epithelial-based tumours, but acknowledges the importance of expanding these tools to broad tumour subtypes.

In one of the first demonstrations of cancer organoid culture, Sato et al. successfully generated organoid models from patient-derived

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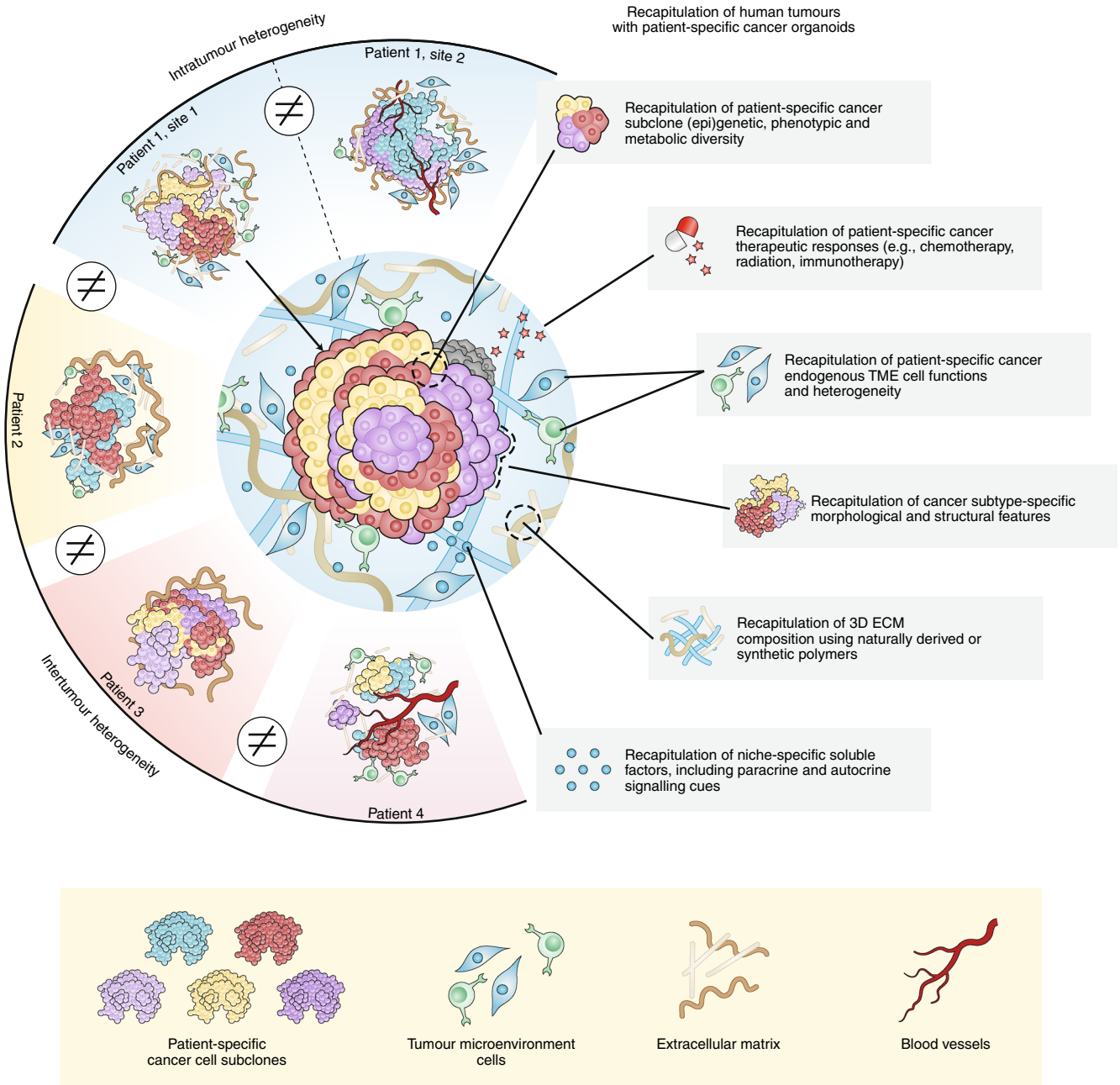

Fig. 1 | Cancer organoids recapitulate defining characteristics of patient-specific tumour heterogeneity. Each patient's cancer presents a host of unique cellular and environmental characteristics that contribute to the vast biological heterogeneity within and across tumours. Organoid models derived directly from human tumour tissue have been shown to accurately recapitulate this inherent intra- and intertumoral biological heterogeneity. Specifically, cancer organoids can recapitulate the extreme (epi)genetic and phenotypic diversity of distinct neoplastic cell subclones, as well as their collective tumour-specific morphological features. Cancer organoids also enable modelling of TME heterogeneity, including the presence and functions of non-neoplastic, TME cells, the signalling of niche-specific soluble factors and the altered composition of the ECM. As a result, cancer organoids are a promising tool to model patient-specific responses to anticancer therapies in the clinic.

diseased tissues including colorectal cancer (CRC), expanding on previous protocols for generating healthy gastrointestinal organoids[10,11]. From this seminal work, organotypic cultures have been successfully generated from several primary tumour types and have been demonstrated to more faithfully recapitulate characteristics of the original tumour compared with traditional cell lines[9]. Cancer organoids have been exploited as a high-throughput platform for patient-specific testing of clinical and emerging anticancer treatments including chemotherapies[12–15], immunotherapies[16] and radiation therapies[17] as well as numerous studies of disease progression and tumour niche factor requirements[18–20]. The success rate of organoid generation from select cancer subtypes is often reported to be >70% (refs. [13,15,16,21])—notably higher than that for traditional cancer cell lines (~20–30%)[22]. Successful cancer organoid and cell line derivation is often defined by the ability to expand, passage and cryopreserve cells that retain genetic and histological characteristics of the original tumour, although no standardized definition has yet been established. Finally, in vitro cancer organoid platforms are amenable to modelling TME cell heterogeneity and heterotypic cell interactions through the coculture of non-neoplastic

Box 1 | Limitations of traditional cancer models

The advancement of personalized cancer treatment will require models that can reliably recapitulate both intra- and intertumour heterogeneity. Despite facilitating important insights into disease pathology, traditional methods lack the ability to sustain genetic and phenotypic heterogeneity of human patient-derived tumour samples while simultaneously facilitating comprehensive cellular and environmental manipulation. For example, two-dimensional in vitro culture of immortalized cell lines has developed into arguably the most popular cancer modelling approach, owing to its high throughput, the availability of large collections of comprehensively characterized lines such as the Cancer Cell Line Encyclopedia[131], and the overall cost-effectiveness. Despite these advantages, immortalized cells are extensively passaged, often genetically modified, and adapted to grow in hyperoxic conditions (~20% oxygen) compared with average tumour oxygen levels (~1–5%), and commonly undergo clonal selection of fast-growing neoplastic populations. As a result, these models are not able to recapitulate the neoplastic heterogeneity and drug response of the parental tumour[132]. The common use of tissue-culture plastic and glass as two-dimensional culture substrates additionally limits the ability to model intrinsic 3D tissue anatomy and complex cellular interactions with the ECM. Moreover, these models often ignore the tumour's stromal, vascular and immune microenvironment, a key element for modelling next-generation cancer therapies that target desmoplasia, angiogenesis and immune responses. Finally, there are a lack of (patient-matched) two-dimensional cell lines that are able to model normal tissue in comparison with immortalized cancer cell lines, thus limiting insights into studies of cancer disease progression and cross-talk between tissue-specific neoplastic and normal cell types.

Alternatively, patient-specific in vivo animal models, such as patient-derived xenografts and genetically engineered models, serve as more complex cancer models that intrinsically incorporate 3D tissue organization and offer system-level analysis of tumour progression and treatment efficacy[133]. Furthermore, animal models, primarily patient-derived xenografts, are increasingly being used in tandem with in vitro human culture models to compare in vivo and in vitro results. However, in vivo animal models are costly, relatively low throughput and subject to increasing pressure for replacement solutions on the basis of ethical concerns. Patient-derived xenograft models offer limited genetic and environmental manipulation, rely on immunocompromised mice that lack the adaptive immune system, and suffer from clonal selection pressure upon engraftment and propagation of human tumour tissue, leading to genetic and phenotypic divergence from the parent tumour[134,135]. On the other hand, genetically engineered models enable unique studies of tumour onset and progression, while also providing native interactions between neoplastic and TME cells. However, their overall genetic manipulation is still relatively limited, and iteratively introducing novel mutations is a slow process. As a result of these limitations, traditional preclinical cancer models are not able to accurately predict clinical success of anticancer therapies, leading to expensive and time-consuming human clinical trials with staggeringly low success rates (~3%)[132,136].

cell types, including cancer-associated fibroblasts (CAFs)[20,23] and immune-cell types[16].

To date, several studies have modelled interpatient heterogeneity by creating 'living biobanks' of patient-specific organoids derived from cancer tissues including colorectal[10,18,24], pancreatic[13,20,21], prostate[25], ovarian[14,26], bladder[27], liver[28], breast[15], lung[29,30], oesophagus[31], gastric[19], endometrium[32] and brain[33]. These reports highlight cancer organoid maintenance of parent tumour biology, including (epi)genetic, proteomic, morphological and pharmacotypic features. Notably, Tiriac et al. established pancreatic cancer organoids from a genetically and phenotypically comprehensive cohort of 138 patient tumour samples[13]. Detailed pharmacotyping of these organoid lines revealed population-level genetic and transcriptomic signatures associated with anticancer drug response that mirrored patient clinical outcomes. Interestingly, the pharmacological signatures garnered from their in vitro organoid studies enabled ex post facto prediction of improved treatment responses for several patients in clinical trials. Similarly, Fujii, Shimokawa et al. created a living biobank of 55 CRC organoid lines derived from a wide spectrum of tumour phenotypes including both primary and metastatic lesions[18]. Unique patient-specific organoid samples maintained distinct histopathological features and genetic signatures of their in vivo counterparts. Through careful genetic characterization and manipulation of in vitro culture conditions, the authors underscore cancer organoids' ability to model genomic heterogeneity across patients and characterize its effects on altered niche factor requirements and metastasis.

Addressing limitations of current cancer organoid culture

Despite their potential as valuable models of patient-specific cancer biology, organoids exhibit several limitations that preclude their (pre-)clinical implementation. In particular, for many cancer subtypes, the efficiency of organoid derivation and reliable in vitro expansion is extremely low and unpredictable (<30%)[16,25,34] and few studies have focused on non-epithelial cancers (for example, glioblastoma (GBM)[33] and rhabdoid[35] tumours). Furthermore, established organoid cultures often only include neoplastic cancer cells and do not support long-term coculture of other TME cell types. Finally, the mechanistic roles of the ECM in driving cancer organoid phenotype and drug sensitivity are largely unknown due to the lack of appropriate 3D culture platforms to model these interactions.

In part, these limitations can be attributed to the current use of non-standardized and ill-defined culture protocols across cancer organoid studies, which introduces technical variability into in vitro organoid cultures and reduces their accurate representation of the cancer's intrinsic biological heterogeneity. Specifically, the origins of technical variability include non-standardized cancer tissue sources and subsequent processing, ill-defined and non-specific medium formulations and the use of heterogeneous, animal-derived 3D matrices that lack the tunability to mimic properties of the native tumour ECM (Fig. 2).

In this Review, we present our outlook for the development of next-generation cancer organoids and the advancements in organoid culture needed to accelerate their clinical translation for personalized medicine. For each of the three aforementioned sources of technical variability, we discuss current protocol limitations and recent innovations in addressing these limitations, and propose future opportunities for culture standardization. Overall, we have designed the flow of this Review to be relevant to researchers across several disciplines. As a result, we begin with an introductory discussion on limitations and advancements in organoid generation and medium formulations and build to engineered ECMs, particularly relevant to the *Nature Materials* readership.

Addressing outstanding sources of technical variability in organoid protocols is critical to comprehend cancer's inherent biological heterogeneity and advance the predictive power of these models. We acknowledge that, once technical variability is reduced, the next step will be experimentally validating that intrinsic biological heterogeneity is preserved within reproducible organoid cultures and exploring how heterogeneity is influenced by environmental stimuli. The ideal methods and protocols for quantification of biological

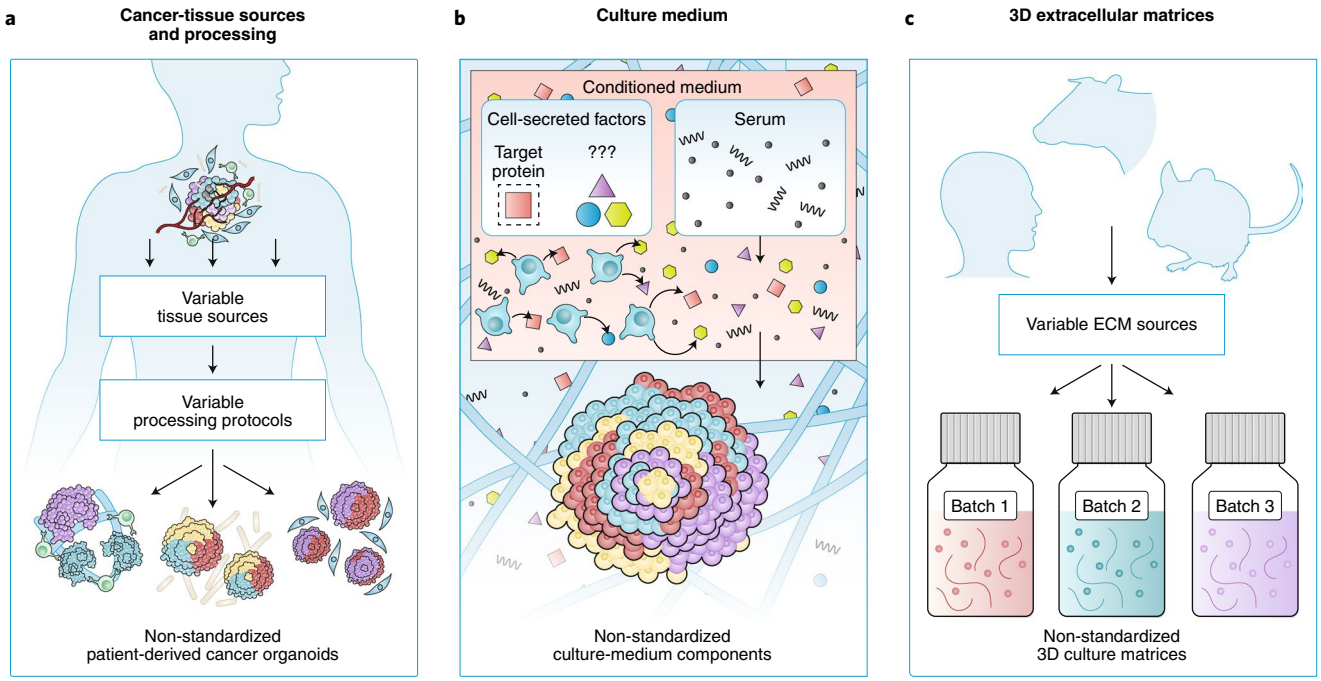

Fig. 2 | Current techniques for cancer organoid culture introduce technical variability into biologically heterogeneous cultures. Protocols used throughout cancer organoid derivation and culture are non-standardized, which is detrimental to reproducibility and limits the ability to reliably represent the tumour's inherent biological heterogeneity. **a**, Variable sources and methods of collecting human tumour tissue, as well as the protocols used for its downstream processing into 3D organoid cultures, lead to the formation of non-standardized organoid models that may only represent a subset of the patient's cancer. **b**, The use of ill-defined and heterogeneous medium components for organoid cultures, including conditioned medium and animal-derived serum, directly inhibits the controlled presence of soluble cues and unpredictably alters organoid phenotype. **c**, 3D culture of cancer organoid samples within animal-derived ECMs is limited by substantial batch-to-batch variability and xenogenic contamination. These matrices also have a complex and ill-defined composition as well as overall poor tunability, which limits mechanistic studies of matrix influence on cancer biology.

heterogeneity are a topic of much discussion and debate within the cancer community, and we point the interested reader towards these excellent articles: refs. [36,37]. Overall, without the application of standardized tools and strategies, future studies will continue to be afflicted by issues of reproducibility and poor patient tissue recapitulation. Importantly, the advancements discussed here are a critical framework for reducing technical variability across all next-generation cancer models.

Organoid generation from tumour tissue

Over the last decade, the methods of organoid derivation—including selection of the source of tumour tissue and its downstream processing—have varied widely (Fig. 3). For example, cancer organoids have been derived from primary tumours[24], metastatic lesions[12], circulating tumour cells[25] and tumour cells from liquid effusions[14], which are collected using several techniques, including solid and liquid biopsies, surgical resections and rapid autopsies[13]. Following tumour tissue collection, patient samples must be processed for downstream culture, often culminating with encapsulation in a 3D matrix. While there exist multiple approaches to initial tissue processing, two prevailing strategies have emerged: (1) complete tissue dissociation and encapsulation of single cells and (2) enzymatic and mechanical tissue mincing and 3D encapsulation of millimetre-scale tumour fragments. Alternatively, cancer organoids can also be produced directly from healthy organoid models through the introduction of driver mutations that replicate cancer onset and progression (Box 2). While each organoid derivation strategy enables the testing of unique hypotheses surrounding cancer biology and treatment, current methods for their implementation are not standardized, which jeopardizes their meaningful contribution to clinical research. Here, we describe current limitations associated with initial tumour tissue sourcing, subsequent tissue processing and overall organoid culture platforms, while also highlighting innovative and reproducible advancements that focus on eliminating technical variability.

Limitations of current techniques. *Sources of tumour tissue.* Capturing the extensive patient-specific biological heterogeneity of cancer using organoids requires sourcing tissue samples that reflect the tumour's spatiotemporal diversity. However, current cancer organoid models are predominantly derived from single biopsies or small fragments of surgically resected tissue, and therefore do not accurately encompass the parent tumour's cellular and morphological diversity or its in vivo temporal evolution. Despite the high efficiency of cancer organoid derivation from some cancer subtypes, several clinical characteristics—including cancer subtype, histopathological grade, intratumoral cell-type heterogeneity, patient treatment status and tumour recurrence—are known to alter the ease of organoid generation from patient tissue[14,18,21,28]. Unfortunately, for many of these cases, the specific mechanisms mediating the successful establishment of organoids from select tissue samples over others is not well understood. Furthermore, this misrepresentation of select patient populations may lead to misguided drug discovery and biomarker development if populations missing from organoid biobanks respond differently to anticancer treatments. Finally, the simultaneous generation of healthy tissue-matched organoid cultures has provided important insight into disease evolution[13,20,21,24]. However, fast-growing cells can contaminate tumour tissue samples, limiting studies that aim to solely recapitulate neoplastic cell biology. Strikingly, analysis of tumour

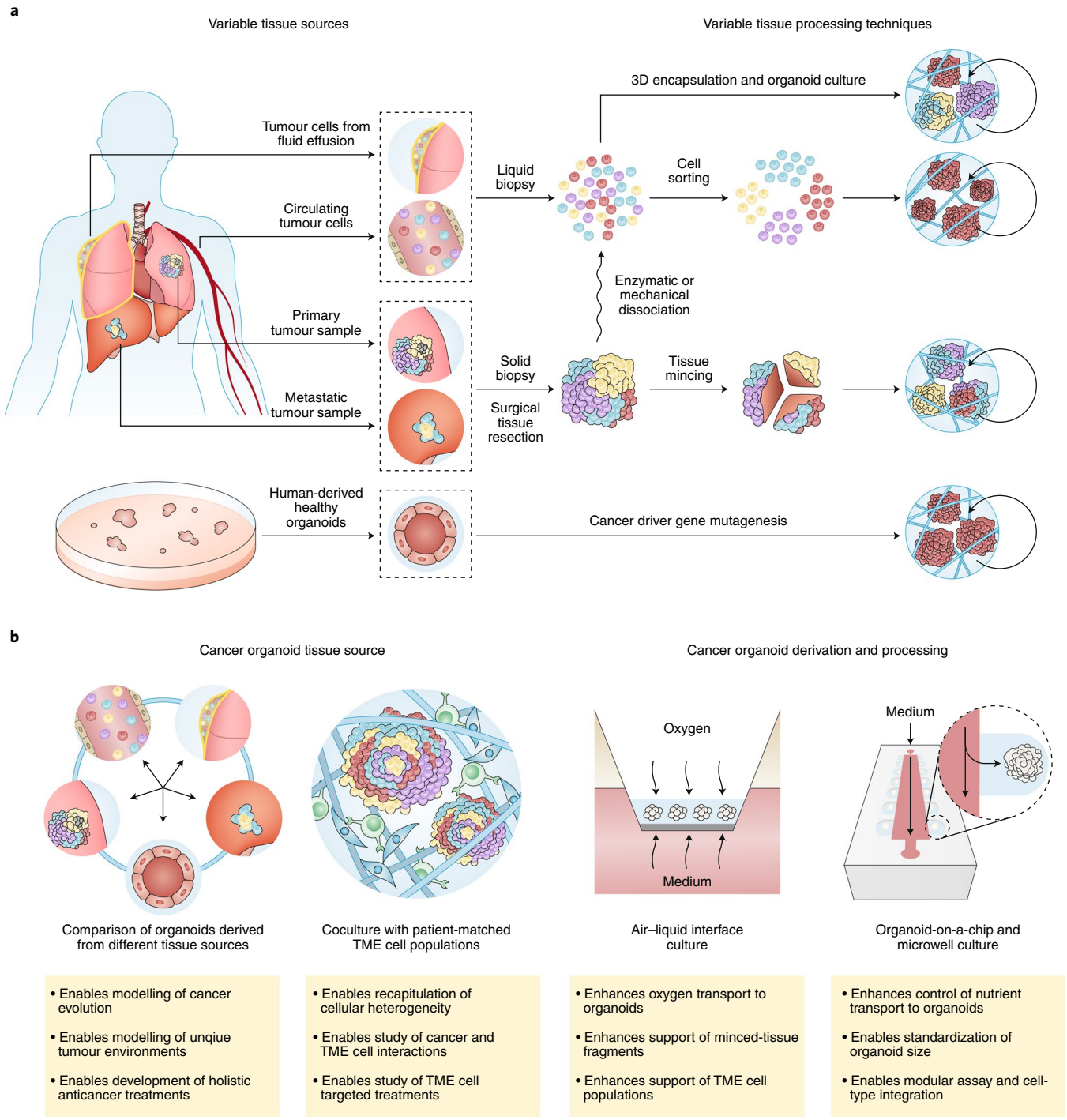

Fig. 3 | Patient-specific cancer organoid derivation is limited by non-standardized methods of tissue procurement and processing. a, Current methods of cancer organoid derivation. Several sources of tumour tissue from distinct stages of cancer progression, including primary tumours, circulating tumour cells and secondary metastatic lesions, have been collected to generate patient-specific cancer organoid models. Similarly, several subsequent tissue processing techniques, including tissue mincing, complete tissue dissociation and/or cell sorting, have also facilitated cancer organoid generation upon encapsulation in a 3D matrix. However, the variable implementation of these methods—each introducing its own sources of technical variability—has led to the formation of non-standardized cancer organoid cultures. **b**, Recent advancements in cancer organoid derivation. The comparison of cancer organoids derived from multiple tissue samples per patient and the introduction of cancer-associated TME cell types (left) has enabled an enhanced understanding of tumour cellular heterogeneity and the impact of heterotypic cell interactions on cancer cell biology. Additionally, advancements in cancer organoid culture platforms (right) have enabled environmental control of nutrient mass transport and overall standardization of organoid size and spatial organization.

cell purity in one study of lung cancer organoids from 59 patients revealed that 58% of organoids were overgrown by contaminating healthy airway cells[34].

Several of these limitations in sourcing tumour tissue for organoid generation are a result of clinical necessity and are beyond the experimentalist's control. For example, tumour tissue availability

Box 2 | Oncogenic mutagenesis of healthy organoid cultures

The derivation of patient-specific cancer organoid models from primary patient tumour tissue presents inherent limitations. First, for rare cancer subtypes, access to a sufficient number of patient tissue samples may not be possible. Moreover, while some cancers have high organoid derivation rates, organoid cultures of many other cancer subtypes have thus far been difficult to establish from patient-derived tissue for unknown reasons. Second, the complex genetic landscape within and across patient cancers hinders researchers' ability to define causal mechanisms of cancer phenotype and evolution. To address these limitations, methods have been developed to derive cancer organoids from healthy human organoid cultures via directed mutagenesis of oncogenes[137]. Organoid models of specific cancer subtypes can also be derived directly from human embryonic stem cell or patient-specific induced pluripotent stem cell cultures, which may further reduce the need for invasive tissue extraction[96]. These models provide a unique opportunity for determining mechanisms of cancer progression and how distinct mutation patterns may drive cellular response to anticancer therapies.

Despite these advantages, cancer organoids derived from genetically modified healthy cells suffer from technical limitations that reduce their biological and clinical impact. For example, current methods are limited to the sequential introduction of a relatively small number of mutations per sample, and therefore do not reproduce the full spectrum of genetic heterogeneity within a patient's unique tumour. Additionally, such cultures require a priori knowledge of distinct mutational drivers of the specific cancer subtype of interest, which may not be available. Recent advancements have aimed to mitigate or address these limitations. For example, novel gene editing tools based on clustered regularly interspaced short palindromic repeats (CRISPR)–Cas9 have been developed to standardize and improve human organoid genetic manipulation. These include CRISPR-associated base editors for selective introduction of single-nucleotide base edits without the need for double-stranded breaks[138] as well as CRISPR-mediated homology-independent organoid transgenesis (CRISPR-HOT) for efficient knock-in of exogenous DNA based on non-homologous end-joining-mediated sequence introduction[139]. In addition to better tools for genetic manipulation of organoids, increased cellular-level genetic characterization of patient cancers via single-cell DNA and RNA sequencing will catalyse the generation of more sophisticated and comprehensive cancer organoid models derived from healthy human cultures. The combination of this comprehensive, patient-specific mutational information with concomitant in vitro organoid perturbation studies will drive unique insights into carcinogenesis. Overall, the ability to reliably generate cancer organoid models from patient-specific healthy tissue samples will provide a fundamentally distinctive outlook on disease progression and offer a platform for the critical study of exceptionally rare human cancer subtypes.

and collection method are factors that can substantially alter organoid generation yet are not controlled by researchers. Furthermore, patients may or may not be treatment naïve before tissue collection, which may influence organoid phenotype and drug response in vitro. The time between patient tissue collection and subsequent 3D encapsulation is also variable across protocols and could greatly alter organoid generation efficiency.

Tissue processing for organoid generation. Non-reproducible tumour tissue processing similarly contributes to overall non-standardized cancer organoid cultures. In one commonly used approach, patient-derived tissue is dissociated into single cells—often through enzymatic and/or mechanical treatment—and encapsulated within a 3D matrix submerged in medium[38]. Full dissociation of tissue samples enables expansion of clonal organoids, which may be contextually advantageous or disadvantageous. However, enzymatic dissociation can result in off-target cleavage of cell-surface proteins and requires distinct, tissue-specific dissociation conditions[38]. Tissue dissociation techniques furthermore result in non-reproducible cell cluster sizes, ranging from single cells to clusters ~100 μm in diameter. Finally, the removal of native neoplastic cell interactions with the TME through complete tissue dissociation can often lead to disruption of complex cell–ECM interactions and negative selection against non-neoplastic or non-epithelial cell types.

Alternatively, patient tissues can undergo mincing and subsequent 3D encapsulation of intact millimetre-scale tumour fragments. In contrast to full tissue dissociation, this method promotes maintenance of native tissue architecture and TME cell components that could regulate organoid formation and phenotype. However, similar to the above, manual tissue mincing results in non-reproducible fragment sizes that will directly foster non-uniform environments for encapsulated cells such as the development of oxygen and nutrient gradients throughout large tissue clusters. Blunt mechanical tissue mincing may also damage samples, further reducing viable cells for organoid generation. Finally, despite the advantages associated with the maintenance of native tumour architecture, the use of intact tumour fragments provides limited control over the encapsulation of defined cell populations with reproducible interactions with their 3D environment.

Advancements in standardization. *Patient sample collection and characterization.* Advancements in standardizing cancer organoid generation will begin with organoid-based studies that aim to define intra- and intertumoral heterogeneity. For example, in a study by Roerink, Sasaki, Lee-Six et al., four to six spatially distinct tumour tissue sections across three patients with untreated CRC were used to generate clonal organoids from single cells[39]. Whole-genome and targeted oncogenic sequencing enabled the generation of phylogenic trees of somatic mutations at single-cell resolution, revealing genetically diverse subpopulations of cancer cells within and across patient tumour samples. Further characterization of each organoid sample showcased clone-specific epigenetic and transcriptomic signatures, as well as heterogeneous drug response profiles. These results demonstrate that tissue samples collected from a single tumour region do not accurately reflect the vast spatial heterogeneity of neoplastic clones, and that multiregion tissue sampling will permit the formation of more precise cancer organoid models.

Through careful study design and patient selection, researchers have generated multiregion cancer organoid models using primary and metastatic neoplastic tissue from single patients to model intratumour heterogeneity. For instance, Kopper et al. recapitulated intrapatient genetic, transcriptomic, morphological and pharmacotypic heterogeneity in organoids derived from primary ovarian tumours and multiple metastatic sites within the same patient[14]. Vlachogiannis et al. used a similar approach to highlight the strength of colorectal and gastroesophageal cancer organoids to model both intra- and intertumoral pharmacotypic heterogeneity in response to anticancer agents[12]. Excitingly, the therapeutic responses measured across in vitro organoid samples recapitulated tissue-matched primary tumour or metastatic lesion responses within a given patient receiving similar clinical treatment.

Despite the inability for clinical tissue collection to be fully standardized, it is imperative that advancements in organoid culture protocols focus on alleviating limitations that may be outside the researcher's control. In one example, Walsh et al. describe a method for generating viable cancer organoids from frozen primary human

breast cancer tissue, circumventing issues associated with timing of tissue extraction and immediate sample availability[40]. Specifically, flash-frozen tissue samples could be thawed following 6–12 months in storage and retained a drug response profile similar to that of fresh organoid cultures derived at the time of biopsy from the same tissue sample.

Organoid derivation and culture environment. Beyond efforts to standardize collection of representative tumour tissues, advancements in microfabrication and microfluidic technologies for standardizing downstream tissue processing, organoid derivation and pharmacological testing are also being implemented. For example, Brandenberg, Hoehnel, Kuttler et al. engineered U-shaped microwell arrays that enable reproducible formation of healthy gastrointestinal and CRC organoids with user-defined initial cell numbers[41]. Scalable and automated imaging of organoids cultured in microwell arrays revealed increased homogeneity of organoid size and morphology. They further demonstrated the platform's utility via high-throughput drug screening of 80 clinically relevant drugs on CRC organoids and identified pharmacotypic hits using automated image analysis.

To improve standardization of organoid derivation from intact tumour tissue fragments, Horowitz, Rodriguez et al. developed a microdissection protocol for the generation of submillimetre, cuboidal-shaped sections of human glioma xenograft tumours, termed 'cuboids', improving tissue fragment uniformity compared with traditional tissue mincing techniques[42]. Specifically, 88% of the glioma cuboids were within the desired size range (300–600 μm), and further analysis validated their ability to retain the native TME. Similarly, Li et al. demonstrated that 3D air–liquid interface (ALI) culture can support primary organoid generation, oncogenic transformation and long-term in vitro culture of minced murine gastrointestinal tissues[43]. In ALI culture, tissue is embedded within a 3D matrix atop the permeable membrane of an inner transwell dish. Medium is then added to an outer dish, enabling diffusion of nutrients through the bottom membrane and leaving the top of the matrix exposed to air, resulting in enhanced oxygen transport compared with submerged culture[44]. Notably, ALI culture of millimetre-scale tissue fragments enabled formation of cancer organoids containing both epithelial and mesenchymal cells[43].

Modelling TME cell heterogeneity. Recent work has also focused on developing culture platforms that can accurately model TME cell heterogeneity and heterotypic cell interactions. Utilizing the benefits of ALI culture, Neal, Li et al. showcased the ability to derive cancer organoids from >100 tumour samples that retain native CAFs and immune cell types to test personalized immunotherapies[16]. The authors demonstrated the ability to recapitulate the in vivo tumour-infiltrating T-cell repertoire and model patient-specific PD1/PDL1-dependent mechanisms of immune suppression. In another report, Schnalzger et al. developed a platform to test cancer immunotherapies using human chimaeric antigen receptor-engineered natural killer cells targeting patient-derived CRC organoids[45]. Live cell imaging enabled tracking of natural killer cell recruitment and antigen-specific cytotoxicity against individual organoids expressing a range of cancer-relevant targets.

Other groups have explored the specific effect of CAFs on cancer organoid populations. For example, Öhlund, Handly-Santana, Biffi, Elyada et al. described the coculture of pancreatic ductal adenocarcinoma organoids and CAFs[23]. Interestingly, RNA-seq analysis highlighted CAF heterogeneity through identification of two distinct CAF subpopulations (that is, myofibroblastic or immunoinflammatory phenotype) that each exhibited unique interactions with cocultured pancreatic ductal adenocarcinoma organoids. Similarly, Ebbing, van der Zalm et al. cocultured oesophageal adenocarcinoma organoids with patient-derived CAFs and found that stromal-derived interleukin-6 drove epithelial-to-mesenchymal transition and therapeutic resistance, leading to the proposition of novel biomarkers and therapeutic strategies for patient stratification and personalized treatment[46]. Interestingly, mouse CAFs from these patient-derived xenografts did not produce the same phenotype, highlighting limitations in murine models and their potential for reconstructing the human TME.

Future opportunities for organoid generation. As patient-derived cancer organoids become increasingly utilized, the implementation of reproducible techniques for organoid derivation that embrace inherent tumour heterogeneity will be pivotal to their clinical success. For example, studies that establish cancer organoid biobanks generated from several tumour tissue types offer unique insights into broad cancer phenotypes. However, while these studies have begun to explore intratumour spatial heterogeneity, modelling of temporal evolution of patient-specific cancers using organoid cultures remains relatively underexplored, potentially due to limited sample availability. Increased implementation of liquid biopsies and genetically engineered healthy organoids may provide more accessible methods of modelling cancer progression. Advancements in microfabrication techniques also have potential for standardizing cancer organoid derivation. For example, these technologies will enable the precise study of how initial cell cluster size alters organoid formation efficiency and selection of cancer subpopulations. Increased availability of techniques to monitor and quantify organoid expansion at the cellular level, such as cellular barcoding[47] and image analysis based on machine learning[48], will directly complement these advancements in cancer modelling.

Another major goal of cancer modelling is the ability to build platforms with native anatomy and complexity, including the addition of relevant TME cells. However, the diverse roles that TME cells play in cancer organoid progression and treatment have yet to be systematically studied, most probably due to the lack of reproducible protocols that support simultaneous long-term expansion of multiple cell types. Microphysiological systems offer a promising approach to create organoid/tumour-on-a-chip models with increased tissue complexity, including for the integration of mature vasculature. To date, several microfluidic devices have been designed to model cancer's interactions with vascular networks, providing a unique assessment of cancer extravasation, drug delivery and tumour growth[49,50]. Similarly, Nikolaev et al. developed a custom microfluidic platform to generate 3D tubular 'mini-intestines' with user-defined crypt and villus-like domains, which maintained a higher degree of intestinal cell-type diversity than did traditional organoid culture and supported several non-epithelial cell types within the surrounding ECM[51]. Further development of these microfluidic devices specifically designed for cancer modelling will be critical to accurately recapitulate cellular and anatomical variability across patient-specific tumours.

Culture medium

The establishment of organoid models of healthy and diseased human tissue would not have been possible without a substantial understanding of the in vivo stem cell niche and specific regulatory factors that permit the propagation of adult stem cells in vitro (for example, Wnt/R-spondin, Noggin and epidermal growth factor). Beyond the direct inclusion of these stem cell factors as purified proteins, additional factors promoting cell proliferation and differentiation tailored to the in vivo niche have also been introduced into tissue-specific medium formulations. Building from the initial medium formulations used for healthy intestinal organoids, Sato et al. highlighted genotype-dependent soluble-factor requirements for colon adenocarcinoma organoid cultures[10]. Specifically, medium components required for healthy human colon organoids, including R-spondin, Noggin and epidermal growth factor, were

Table 1 | ECMs for normal and cancer organoid models

Material	Biomimicry	Gelation trigger	Microstructure	Biodegradable	Typical stiffness	3D cell encapsulation	Commercially available	Tissue	References
Mammalian biopolymers									
Murine EHS sarcoma matrix (basement membrane extract) (laminin/entactin, collagen type IV) (for example, Corning Matrigel, Trevigen Cultrex, Gibco Geltrex)	Epithelial ECM	Temperature	Amorphous to nanofibrillar	Yes (MMP)	0.01–0.3 kPa	Yes	Yes	Bladder, brain, breast, endometrium, oesophagus, intestine, kidney, liver, lung, ovarian, pancreas, prostate, stomach	10,13–15,18–21,24–33,96,97
Collagen type I	Stromal/ connective ECM	pH	Nano- to microfibrillar	Yes (MMP)	0.1–10 kPa	Yes	Yes	Brain, breast, oesophagus, intestine, kidney, liver, lung, pancreas, stomach, thyroid	16,98–103
Fibrin	Blood clot/ regenerative ECM	Enzymatic	Microfibrillar	Yes (MMP, plasmin)	0.01–1.5 kPa	Yes	Yes	Intestine, liver, pancreas, vasculature	88,104
HA	Neural/stromal/ connective ECM	Various (physical, chemical, enzymatic)	Amorphous	Yes (hyaluronidase)	0.01–2.5 kPa	Yes	Yes	Brain, breast, intestine, kidney	80,90,100,101,105–107
Gelatin methacrylate (GelMA)	Stromal/ connective ECM	Various (free radical polymerization, temperature)	Amorphous	Yes (MMP)	0.1–100 kPa	Yes	Yes	Heart, intestine, liver, ovarian	108–110
Decellularized matrices	Specific to native tissue	Various (native structure, chemical, enzymatic, temperature)	Various (amorphous, nano- to microfibrillar)	Yes (MMP, tissue specific)	Seldom homogeneous or documented, typically of the order of 0.1–10 kPa	Depends on decellularization method	Generally not	Intestine, liver, pancreas, stomach, vasculature	111–113
Other biopolymers									
Alginate	Bioinert, can be functionalized	Divalent cations	Amorphous	Physical dissociation only	0.01–10 kPa	Yes	Yes	Brain, intestine, kidney, lung, vasculature	87,102,114,115
Silk	Non-specific adhesion, can be functionalized	Various (physical, chemical)	Various (beta-sheet crystals, nanofibres, sponges, microspheres)	Yes (MMP)	1–200 kPa	No (in most common protocols)	Precursor available, but typically requires custom modification/ biofabrication	Intestine, kidney	86,116
Nanocellulose	Bioinert, can be functionalized following surface oxidation	Various (physical, chemical)	Various (nano- to microfibrillar, colloidal suspension)	Physical dissociation only	0.01–1 kPa	Yes	Yes	Intestine, liver	117,118

Continued

Table 1 | ECMs for normal and cancer organoid models (continued)

Material	Biomimicry	Gelation trigger	Microstructure	Biodegradable	Typical stiffness	3D cell encapsulation	Commercially available	Tissue	References
Engineered materials									
PEG	Bioinert, can be functionalized	Various (chemical, enzymatic)	Amorphous	Dependent on user functionalization	0.01–10 kPa	Yes	Yes, custom modification sometimes required	Brain, breast, endometrium, heart, intestine, liver, pancreas	80,82–85,103,119–122
Self-assembling peptides (for example, RADA16/PuraMatrix, bQ13)	Bioinert, can be functionalized	Various (ionic strength, pH)	Nanofibrillar	Physical gel dissociation, building blocks degradable by peptidases	0.1–6 kPa	Yes (transient exposure to low pH)	Yes	Brain, intestine, prostate	123–125
Poly(lactic/(-co-) glycolic) acid, polycaprolactone	Bioinert, can be blended or coated	Physical (dried from organic solvent)	Various (amorphous, fibrillar by electrospinning, sponges)	Hydrolysis	1–50 MPa	No	Yes	Brain, breast, lung	126–128
Polyacrylamide	Bioinert, can be functionalized if copolymerized with acrylate	Free-radical polymerization	Amorphous	No	0.1–1,000 kPa	No (in most common protocols)	Yes	Kidney	129
Oligo(ethylene glycol)-substituted polyisocyanopeptides	Bioinert, can be functionalized	Temperature	Nanofibrillar	Physical dissociation mostly	0.01–1 kPa	Yes	Yes	Liver	130
ELP	Elastin mimetic, tunable bioactivity through protein design	Various (chemical, temperature)	Various (amorphous, nano- to microspheres)	Yes (elastase, dependent on protein sequence)	0.01–10 kPa	Yes	No	Intestine	89,90
Hybrid materials									
Combinations of the above polymers	Various, depending on the polymer blend	—	—	—	—	—	—	Brain, breast, intestine, liver, lung, vasculature	80,90,100–103

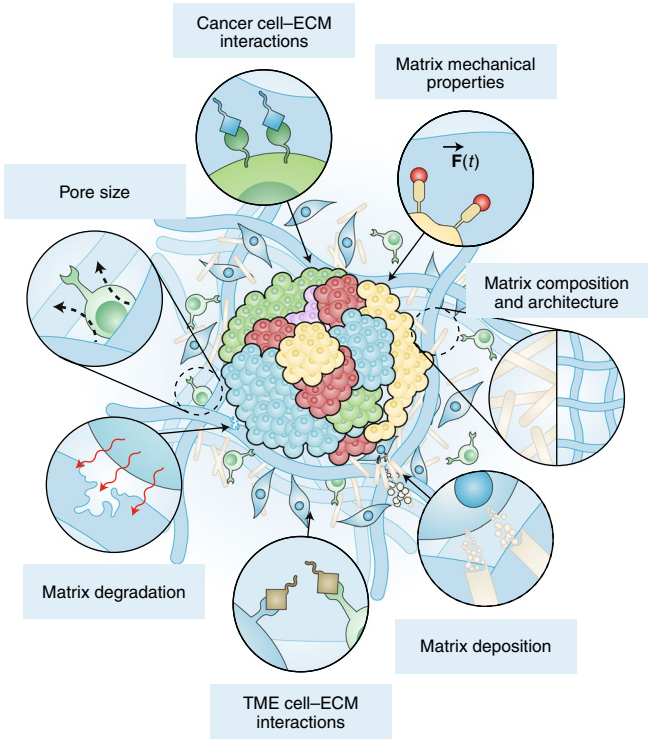

Fig. 4 | The ECM influences cancer organoid phenotype through several biochemical and mechanical interactions. The ECM plays critical roles in driving cancer phenotype, disease progression and therapeutic response in vivo. 3D engineered matrices with tunable biochemical (for example, ligand presentation, soluble-factor sequestration) and mechanical (for example, matrix viscoelasticity, degradation, architecture, pore size) properties are poised to answer previously untestable hypotheses surrounding mechanisms of these important cancer–matrix interactions. Additionally, reciprocal interactions between the ECM and TME cells, such as fibroblasts and immune cells, can also be modelled using in vitro engineered matrices.

often dispensable for adenocarcinoma organoid growth. Numerous cancer organoid studies have since highlighted how genetic and phenotypic neoplastic cell heterogeneity can necessitate the use of medium formulations catering for unique patient samples[18,20].

As researchers continue to identify the drivers of tumour biological heterogeneity, the development of patient-relevant medium formulations for cancer organoid culture will undoubtedly become increasingly complex, yet equally important to model. However, current components used for cancer organoid medium (for example, purified growth factors, conditioned medium and animal-derived serum) are prohibitively expensive, non-reproducible and/or highly heterogeneous, precluding precise patient-specific modelling of the tumour niche. Here, we discuss these limitations in detail and highlight recent advancements for standardized and scalable cancer organoid medium formulations.

Limitations of current techniques. *Expensive growth factors and conditioned medium.* Cancer heterogeneity is driven, in part, by the complex and reciprocal soluble-factor signalling between several TME cell types. Given that most cancer organoid models consist of pure populations of neoplastic cells, signalling factors that promote cancer cell growth—which are often secreted from TME cells in vivo—must be externally supplemented via medium formulations. However, medium cocktails that include several growth factors and nutrients can quickly become prohibitively expensive and non-scalable for high-throughput applications. The inclusion of some components as purified recombinant proteins can also be limited by poor solubility and insufficient stability for long-term storage[52], resulting in diminished protein activity[53].

As a result of innovations across several laboratories, the use of conditioned medium from mammalian cells engineered to produce Wnt3a, Noggin and/or R-spondin has greatly reduced the cost and improved overall accessibility of implementing cancer organoid models across a variety of tissue types[52]. Despite this advancement, the direct dilution of conditioned medium into complete organoid medium formulations poses several limitations for culture standardization and reproducibility. For example, conditioned medium can suffer from substantial batch-to-batch variability, which manifests as variable target-protein activity levels. Conditioned medium also contains diverse factors beyond the target protein(s), which have unpredictable effects on encapsulated cancer organoid phenotype and drug response. This phenomenon is compounded by the inclusion of residual serum, often fetal bovine serum (FBS), in conditioned medium, which can be required for the culture of mammalian expression systems.

Animal-derived serum. The direct and indirect inclusion of animal-derived serum within cancer organoid medium formulations results in a non-standardized culture platform with limitations similar to those described above. Specifically, FBS—originating from the liquid fraction of fetal calf blood purified of cellular debris and coagulation factors—contains a rich cocktail of soluble and matrix signalling factors, including full-length proteins, peptides, carbohydrates, lipids, hormones and several small-molecule nutrients known to support in vitro cell culture[54]. Despite their widespread use, animal-derived sera remain ill-defined and contain components that unpredictably influence cancer organoid growth. Proteomic and metabolomic studies underscore sera's complexity and have led to the discovery of 1,800 unique gene products within human serum[55]. This complexity is compounded by variability in soluble-component concentrations across batches and suppliers, owing to its animal origin as well as geographic and seasonal differences in serum collection. Finally, the use of animal-derived serum also introduces xenogenic components, which could have undesired effects on encapsulated organoids and restrict human-specific immunological studies, while also posing a risk of zoonotic, bacterial or viral-based infections[56].

FBS is often indirectly introduced into complete cancer organoid medium formulations via conditioned medium, as serum is frequently required for in vitro mammalian cell expansion and optimal protein production. Direct use of FBS is also typical for organoid cryopreservation and intermediate steps throughout organoid derivation and passaging protocols. Importantly, FBS can have substantial and unpredictable effects on organoid derivation and phenotypes. For example, Seino et al. reported that the use of conditioned medium containing FBS reduced the long-term culture of healthy pancreatic organoids patient-matched to pancreatic ductal adenocarcinoma organoid samples[20]. Overall, the reliance on conditioned medium and animal-derived serum throughout cancer organoid culture hampers efforts to develop standardized models and limits comparisons of data across experiments and laboratories.

Advancements for standardization. *Production and purification of recombinant proteins.* While the dilution of conditioned medium into complete medium formulations limits medium standardization, a number of advancements have reduced its dependence on animal-derived serum. For example, given that Wnt proteins become lipidated and hydrophobic throughout intracellular processing, effective solubilization of Wnts expressed and secreted in vitro requires the presence of stabilizing factors often supplied through serum-containing medium. Specifically, Mihara et al. identified the glycoprotein afamin, found in bovine serum, as a key stabilizer of 12

distinct Wnt proteins during in vitro protein expression[57]. Wnt3a–afamin complexes showed enhanced biological activity and better promoted human intestinal organoid expansion than did Wnt3a purified using detergent-based methods[52]. From this observation, they developed a coexpression system consisting of mammalian cells transfected with both Wnt- and afamin-encoding vectors, which has now been implemented across several cancer organoid studies to generate Wnt conditioned medium in the absence of serum. Despite this advancement, FBS is often still included during initial mammalian cell expansion before collection of conditioned medium, which increases the potential for contamination of serum-derived components and influences protein production on a batch-to-batch basis.

To meet the reproducibility needed to translate cancer organoid technologies to the clinic, researchers must transition to standardized methods of recombinant protein expression and purification. With this motivation, Tüysüz et al. developed phospholipid- and cholesterol-based liposomes that enhance the stability and maintain the activity of recombinantly produced Wnt3a[53]. Compared with detergent-based solubilization, liposome-stabilized Wnt3a showed enhanced expansion of healthy human duodenal organoids and supported serum-free derivation of healthy human intestinal and liver organoids with an efficacy similar to that of serum-containing Wnt3a conditioned medium. The authors also showed that their liposome formulation could support the purification of up to 80% of Wnt3a from contaminants in conditioned medium.

For medium components that have less restrictive requirements for protein purification, other methods of recombinant protein expression (for example, bacterial and insect cell based) may be more controlled, high throughput and cost effective. However, bacterial platforms often have limited mechanisms for directing protein folding and post-translational modifications, which may be required for maintaining proper target-protein biological activity. Purified proteins can also be plagued by species-specific contaminants such as endotoxins. To address these concerns, Urbischek et al. developed a unique R-spondin 1 and Gremlin 1 expression and purification workflow in *Escherichia coli*[58]. As both proteins require specific conditions for proper folding and configuration of disulfide linkages, the disulfide-bond C isomerase was coexpressed with the target protein in *E. coli*, followed by in vitro disulfide shuffling. Cell-based activity assays, including the support of both healthy human colon epithelium and colon adenocarcinoma organoids, indicated that the purified proteins could match the activity of commercially sourced proteins with negligible endotoxin contamination. Compared with commercial sources, the authors estimate a reduction in protein cost per litre of organoid medium by 500- and 350-fold for R-spondin 1 and Gremlin 1, respectively.

Engineered signalling molecules. In addition to the inclusion of full recombinant proteins in medium formulations, several engineered agonists of cancer-associated signalling pathways have been developed as cost-effective alternatives with similar biological activities. In one example, Janda, Dang et al. used de novo design and protein engineering strategies to develop water-soluble, surrogate Wnt agonists that induce Frizzled–LRP5/6 heterodimerization and phenocopy downstream beta-catenin signalling[59]. In a follow-up study, they expanded this toolkit by developing 'next-generation surrogate' Wnts that induce similar levels of downstream Wnt signalling at 50 times lower concentration than the previous generation[60]. Agonists also increased formation efficiency of several healthy human organoids in vitro compared with Wnt3a conditioned medium. In addition to Wnt agonists, a similar approach was used to develop R-spondin surrogates that bind and restrict degradation of Frizzled and associated LRP5/6 receptors independent of their natural interaction with LGR proteins[61]. By linking these domains with a protein that targets cell-specific surface receptors, R-spondin surrogates can selectively potentiate downstream Wnt signalling within a particular cell type. Future use of these selective agonists within cancer organoid cultures may enable novel insight into how specific clonal cancer cell subpopulations respond to targeted Wnt/R-spondin pathway activation.

Future opportunities for medium. The development of next-generation cancer organoid medium will require a meticulous, patient-specific understanding of the in vivo tumour niche and standardized approaches to model these insights in vitro. While recent studies have successfully identified required or dispensable medium components for cancer organoid culture, they largely focus on only a handful of interconnected pathways (for example, Wnt/R-spondin, epidermal growth factor, transforming growth factor beta, bone morphogenetic protein). Moreover, current stratification of patient organoid samples into subcategories with specific medium formulation requirements is often driven solely by mutational status; however, studies show that shifted expression of select signalling factors, such as Wnt1, in cancers can drive tumorigenesis even in the absence of genetic alteration or total gain/loss of function[62]. Therefore, future efforts to characterize patient-specific soluble-factor signalling within the in vivo TME across a variety of pathways and cell types should be prioritized and is currently being driven by efforts in single-cell RNA sequencing and proteomic profiling[63]. Furthermore, the collection and characterization of patient-matched human serum in cancer organoid studies has begun to offer insights into soluble factors that may directly influence organoid phenotype[28,46], while also serving as a potential replacement for animal-derived serum for applications in precision oncology. Specific attention to differences between healthy and cancer stem cell niches will be critical to understanding cancer initiation and how current medium formulations may bias clonal selection and expansion of cancer organoid populations. By standardizing tumour characterization processes before and during organoid generation, tumour-specific in vitro medium formulations can be developed that best model the in vivo signalling environment of an individual patient, resulting in more representative studies of their cancer progression and treatment.

Beyond soluble-factor presence and concentration, several other important physiochemical properties of medium formulations have been underexplored in cancer organoid culture. For example, growth-factor signalling is known to be highly context dependent, and thus will be influenced by several factors including the presence of binding partners/inhibitors, the surrounding pH and the composition/architecture of the ECM. Additionally, the effects of varying oxygenation levels in the medium of in vitro cancer organoid cultures have not been widely explored, despite hypoxic conditions being critically important in regulating cancer cell phenotype in vivo. Soluble factor concentrations, oxygen concentration profile and pH within the in vivo TME are also spatially heterogeneous, owing to the non-uniform distribution of several cell types and altered vasculature. However, traditional cancer organoid culture methods (that is, submerged in a bath of medium) are not able to accurately recapitulate this heterogeneity to study their effects on cancer phenotype. Technologies and platforms to spatiotemporally control cancer organoid medium should therefore be a focus of future research. In addition to microfluidic-based approaches, emerging microfabrication techniques such as two-photon patterning have demonstrated four-dimensional presentation of active growth factors[64]. As cancer organoid cultures become more complex with the addition of multiple TME cell types, future effort will also be required to identify the necessary medium components to maintain non-neoplastic cells in culture and support heterotypic cell interactions.

Extracellular matrix

The in vivo ECM is a dynamic polymer network comprising proteins, polysaccharides and proteoglycans that serve structural and biochemical functions. In contrast to healthy tissues, where ECM turnover is highly regulated, tumours typically exert marked and disorganized remodelling of the composition, architecture and mechanics of their surrounding ECM, which can account for ~60–90% of the total tumour mass[65,66]. This altered ECM has been widely shown to influence neoplastic and TME cell biology through a host of biochemical and biophysical interactions. For example, biophysical cues such as matrix (visco)elasticity, pore size, degradation and architecture as well as biochemical cues such as ligand presentation and growth factor sequestration have been linked to cancer cell phenotype, disease progression, metastasis and drug response[66]. As a result, the ECM and its related downstream cellular signalling pathways have recently become targets for several anticancer therapies[67]. Overall, our comprehensive understanding of these cancer–ECM interactions and their exploitation as drug targets will require model systems with reproducible and tunable control of matrix properties.

The leap in in vitro tissue culture dimensionality has reframed our perspective on how 3D tissue organization and cell–ECM interactions can markedly alter neoplastic cell phenotype. In particular, these insights were driven by seminal work from the Bissell laboratory exploring the use of naturally derived ECMs for modelling normal and neoplastic mammary epithelium. In one example, Weaver et al. described the use of hydrogels derived from the decellularized basement membrane of murine Engelbreth–Holm–Swarm (EHS) sarcoma, or 'EHS matrix', to instruct the formation of 3D polarized structures from mammary epithelial cells[68]. Notably, they identified that tissue polarity and β4 integrin-mediated engagement with laminin conferred apoptosis resistance following cytotoxic drug treatment, highlighting how 3D cellular organization and cell–ECM interactions can regulate cancer progression and treatment. Similarly, Kenny, Lee et al. highlight the considerable effect that culture dimensionality and 3D cell morphology have on the transcriptional profile of several human breast cancer cell lines[69]. Overall, these studies emphasize dynamic reciprocity between the cell and ECM, which continues to drive the development of biomaterials to direct cellular behaviours.

Building from these seminal works, cancer organoid models offer the potential to recapitulate 3D tumour ECM composition, architecture, mechanics and cell–matrix interactions though their culture within hydrogel matrices in vitro (Fig. 4). Despite this potential, there have yet to be comprehensive studies using cancer organoids to model intra- or intertumoral ECM heterogeneity, and only a limited number of studies have explored how specific ECM properties influence patient-derived cancer organoid pathogenesis and anticancer treatment responses in vitro. One key reason is that most 3D in vitro cancer organoid experiments rely on the use of ill-defined and poorly tunable animal-derived scaffolds, which preclude standardization and limit mechanistic understanding of organoid–ECM interactions. Here, we discuss limitations of the most commonly used matrices for cancer organoid cultures (that is, EHS matrix and collagen), and highlight advancements in engineered matrices with reproducible control over biochemical and biophysical ECM properties for organoid culture.

Limitations of current techniques. *Murine EHS matrix.* Over the last decade, the most common matrix for 3D culture of both healthy and cancer organoids has been EHS matrix, sold under the trade names Corning Matrigel, Trevigen Cultrex and Gibco Geltrex. Following murine tumour extraction, several ECM proteins—predominantly laminin (~60%) and collagen IV (~30%)—are retained in reconstituted EHS matrix[70], and ultimately provide both structural and biological support to encapsulated cells. EHS matrix has been widely adopted across cancer organoid studies due to its ability to provide a rich milieu of tumour-derived ECM components, growth factors and cytokines that enable proliferation and maintenance of a variety of neoplastic and TME cell types. Additionally, EHS-matrix gelation is a simple process that is primarily triggered by laminin self-assembly as well as crosslinking of laminin and collagen by endogenous nidogen 1 following an increase in temperature above 10 °C (ref. [70]). Overall, EHS matrix has provided a tumour-relevant environment for the establishment of many cancer organoid subtypes, enabling countless in vitro studies for modelling patient-specific cancer biology.

Despite its ability to support 3D human cancer organoid culture, EHS matrix is animal derived, and therefore exhibits substantial batch-to-batch variability and contains ill-defined and xenogenic impurities that can unpredictably influence organoid phenotype[71]. For example, Matrigel contains over 14,000 unique peptides and nearly 2,000 different proteins[72], many of which are known to alter cancer cell phenotype. Even specially processed, growth-factor-reduced formulations of EHS matrix still suffer from only ~53% batch-to-batch similarity in protein content[72]. Additionally, EHS matrix lacks tunability of its biochemical and mechanical properties, rendering it unable to recapitulate patient-specific characteristics of the tumour ECM. For example, the tumour ECM is often stiffer than healthy matrix (for example, human healthy breast tissue ~400 Pa; human invasive breast cancer tissue ~5 kPa)[73], yet EHS matrix is much weaker (~100 Pa)[74]. EHS matrix's viscous nature may also limit its use in scaled pharmaceutical applications due to the difficulty of automated liquid handling. Overall, these collective limitations make it impossible to determine causative mechanisms of matrix-induced cancer cell behaviour and, along with its relatively high cost, prohibit its use in high-throughput drug screens and clinical use. Even if these limitations in material composition, reproducibility and automation could be mitigated, scaling EHS-matrix production to levels necessary for widespread use in pharmaceutical screenings would require a substantial animal burden, which may be considered ethically problematic.

Collagen matrix. The extreme desmoplastic response in many solid tumours is often associated with increased collagen (commonly I–IV) deposition and remodelling. This increased presence of collagen regulates several aspects of cancer through a complex assortment of biochemical and biophysical signalling cues[75]. As a result, collagen type I matrices have become an increasingly common biomimetic and less expensive alternative to EHS matrix for in vitro cancer organoid models. However, as collagen is often derived from animal sources, the resultant matrices suffer from similar limitations to EHS matrix, namely batch-to-batch variability, limited mechanical and biochemical tunability, and contamination with ill-defined and xenogenic components. Additionally, the collagen hydrogel microstructure (for example, fibril diameter, alignment) is highly dependent on the rate of pH and temperature change during gelation[76]. As a result, collagen gelation performed under variable environmental conditions can lead to architectural heterogeneity and undefined collagen fibril size across samples, which can critically influence cell interactions with the matrix[77]. While several studies have identified methods of controlling collagen matrix mechanical properties and architecture[76], they often require the addition of potentially toxic agents or require specialized chemical modification to the collagen protein, which can disrupt native crosslinking and ligand availability.

Advancements for standardization. Several biomaterial platforms have been engineered for 3D in vitro culture of cancer cell lines, spheroids and non-human primary cancer tissues, leading to important insights in our understanding and treatment of cancer (see reviews [78,79]). However, engineered matrices have yet to be routinely applied to human cancer organoid cultures, and thus

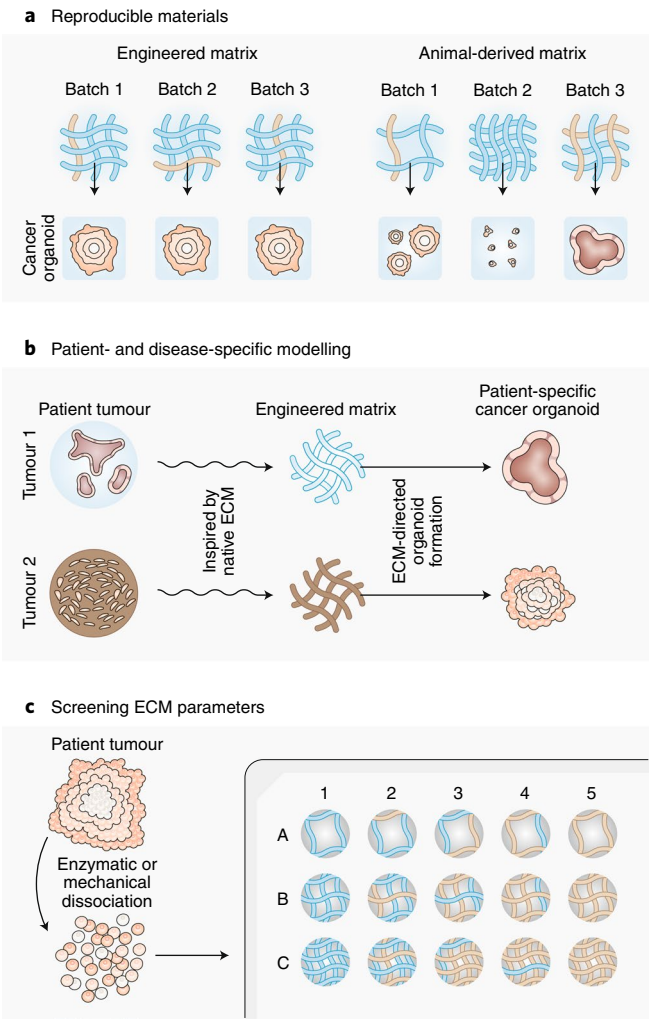

Fig. 5 | Engineered matrices for standardizing cancer organoid models. **a**, Compared with animal-derived matrices, engineered matrices offer high batch-to-batch reproducibility and enable standardization of cancer organoid formation and culture. **b**, Designer engineered matrices can be formulated to mimic patient- and disease-specific composition and structure of the native tumour ECM. **c**, High-throughput screening of tunable engineered matrices can provide insight into the roles of biochemical and mechanical matrix properties in cancer organoid biology.

their implementation will serve as a future opportunity for understanding roles of the ECM in regulating patient-specific cancers. Therefore, in this section, we focus broadly on recent advancements in the development of engineered matrices tailored for human and/or tissue-derived organoid culture, which we summarize in Table 1.

In one example of engineered materials designed for primary human cancer organoid culture, Xiao et al. encapsulated patient-derived GBM within a hybrid material consisting of synthetic polyethylene glycol (PEG) decorated with the RGD integrin-binding peptide and crosslinked with recombinant hyaluronic acid (HA), an extracellular polysaccharide commonly upregulated in GBM[80]. GBM organoids grown in high-HA-content hydrogels showed upregulation of CD44, an HA-binding cell-surface receptor and cancer stem cell marker, compared with traditional gliomasphere suspension cultures. Interestingly, GBM organoids cultured in engineered hydrogels with reduced HA content showed a roughly threefold increase in drug sensitivity compared with stiffness-matched matrices with high HA content, while *CD44* knockdown abrogated this drug-resistant phenotype. These results demonstrate the ability for tunable matrix platforms to provide unique perspectives into matrix-mediated mechanisms of cancer organoid phenotype and correlated drug response.

PEG-based synthetic matrices. Beyond cancer models, several examples of designer matrices have been developed for healthy organoids derived from pluripotent stem cell and adult tissue, primarily for intestinal tissue[81]. In a pioneering study, Gjorevski et al. introduced 3D synthetic PEG-based matrices for the culture of primary adult mouse intestinal organoids derived from purified Lgr5$^+$ intestinal stem cell colonies[82,83]. Interestingly, they found that unique matrix properties were required to support initial intestinal stem cell colony formation and subsequent differentiation into organoids. PEG matrices of intermediate (~1.3 kPa) stiffness decorated with the integrin-binding RGD peptide best supported intestinal stem cell colony formation through a Hippo pathway/Yes-associated protein-dependent mechanism. Conversely, subsequent intestinal organoid differentiation required a PEG matrix with softer (~190 Pa) stiffness and the incorporation of full-length laminin 111. Using a similar PEG-based platform with tunable polymer density and integrin-binding peptide presentation, Cruz-Acuña, Quirós et al. demonstrated the ability to culture intestinal organoids derived from human pluripotent stem cells in a fully synthetic matrix[84]. Excitingly, organoids grown in PEG matrices differentiated into mature intestinal tissue upon injection in vivo. Finally, Hernandez-Gordillo et al. developed a similar PEG-based platform with tunable ligand concentration and biophysical properties that supported the culture of primary human-derived intestinal and endometrial organoids from several patients[85]. They found that low-stiffness (~100 Pa) PEG hydrogels crosslinked with enzyme-degradable peptides and decorated with the integrin-engaging, collagen-mimetic peptide GFOGER best supported organoid formation efficiency and proliferation similar to EHS-matrix controls.

Biopolymer-based engineered matrices. While purely synthetic PEG-based material systems offer several benefits for tunable hydrogel design, they often suffer from high swelling and lack the cellular-scale architectural features found in the native matrix. To address some of these limitations, researchers have developed biopolymer-based engineered matrices for organoid cultures. For example, engineered matrices for intestinal organoids based on purified silk protein[86] and alginate polysaccharide[87] provide systems with alternative biochemical, mechanical and architectural properties, while retaining superior homogeneity and reproducibility compared with animal-derived matrices. Fibrin gels formed from purified human plasma fibrinogen have also been proposed as semidefined, animal-free matrices with tunable stiffness, pore size and chemical functionalization. Specifically, Broguiere et al. showed that fibrin gels supported the growth of murine and human epithelial organoids from healthy and cancer tissue when supplemented with purified laminin[88].

Recombinantly engineered proteins are another promising biopolymer scaffold for 3D organoid culture that is tunable and chemically well-defined, and enables selective integration of native, bioactive protein domains. For example, DiMarco et al. developed an engineered recombinant elastin-like protein (ELP) matrix for the culture of primary murine intestinal organoids[89]. ELPs are genetically engineered, modular proteins that allow for the independent integration of cell-instructive amino-acid sequences and site-specific crosslinking domains, enabling the decoupling of biochemical and mechanical matrix cues. The authors show that intestinal organoids grown in soft (~200 Pa) ELP matrices with high RGD ligand concentration supported the highest organoid formation efficiency, matching that of collagen matrix controls. In another study, Hunt et al. utilized an engineered hybrid matrix comprised of HA and

ELP—termed HELP hydrogels—for 3D encapsulation, proliferation and differentiation of adult human, tissue-derived intestinal organoids[90]. The authors show that an interplay of matrix signalling cues (that is, RGD ligand concentration, HA presence, matrix stiffness and matrix stress relaxation) regulated intestinal organoid growth rate and formation efficiency from single cells. Notably, encapsulated organoids could be serially cultured within HELP hydrogels for at least 12 passages, at which time organoids showed growth rates statistically similar to those of EHS-matrix controls.

Future opportunities for ECM. Overall, these pioneering studies tailored to healthy organoids will undoubtedly inform the development of novel materials specifically engineered to model the in vivo TME, paving the way for reproducible, disease-specific and customizable models of 3D cancer–ECM interactions in vitro (Fig. 5). However, several current limitations preclude their full adoption for cancer organoid models. In particular, a central goal within the biomaterials community is the development of carefully engineered material platforms to dissect the minimal parameters necessary to match the biological output and efficiency of EHS matrix. In tandem, researchers aim to use these platforms to precisely model mechanisms of matrix-mediated organoid biology. Despite substantial advancement towards these goals, the culture efficiency of organoids is often lower in engineered matrices than in EHS matrix, and synthetic platforms that enable organoid culture of one tissue or species are often not directly applicable to others, further limiting their widespread use. These limitations of engineered matrices could potentially be attributed to their limited biodegradability/remodellability as well as their relatively minimal incorporation of ECM components and cell-interactive ligands compared with more complex naturally derived matrices. Future advancements in polymer science and materials engineering must address these critical limitations of current synthetic platforms, without foregoing general ease of use and availability to a broad range of researchers.

Another limitation of current engineered matrices is that they often lack sufficient spatiotemporal control over biochemical and mechanical properties to appropriately model the dynamic TME. To address this limitation, several groups have developed platforms to reversibly and irreversibly modify both biochemical and mechanical matrix cues in both space and time[64,91,92]. While these studies have incorporated several different cell types, few studies to date have incorporated this technology into culture matrices for organoids. In one example, Hushka et al. demonstrated a photodegradable synthetic 3D platform for light-induced, on-demand matrix softening, which initiated epithelial crypt formation in intestinal organoid cultures[93]. Unique control of spatial organization can also be achieved through 3D bioprinting of organoids and organoid-forming stem cells embedded within injectable matrices[94]. In particular, these models will rely on the careful design of cell-compatible and printable bioinks in combination with post-printing crosslinking methods. In tandem with these material innovations, advancements in methods to measure cancer cell interactions with the dynamic ECM will be necessary to fully understand its biological impact. In one example, Krajina et al. developed a real-time, non-invasive dynamic light scattering microrheology technique to simultaneously measure cell-mediated matrix fluidization and stiffening in an in vitro 3D breast cancer model[95].

Many current studies that showcase engineered matrices for organoid culture are reliant on initial organoid derivation in animal-derived materials, which will bias cell selection and phenotype. To comprehensively understand how matrix properties influence cancer organoid behaviour, patient samples must be encapsulated directly into synthetic matrices for organoid derivation. These organoid samples must also be efficiently passaged, expanded and characterized, which requires scalable and on-demand methods of synthetic matrix dissociation and cell purification. Finally, as synthetic matrices for 3D cancer organoid culture become increasingly applied, their use in high-throughput analyses, such as chemotherapeutic screens, will drive demand for cost-effective and bioactive materials with scalable matrix crosslinking strategies.

Conclusion

Breakthroughs in cancer biology are consistently driven by novel approaches to studying and modelling cancer: from the establishment of the first human cancer cell culture from the cervical tumour of Henrietta Lacks that redefined our ability to study patient tumour samples in vitro, to the recent accessibility of single-cell sequencing technologies that are providing unprecedented resolution and unbiased insight into the cellular makeup and phenotypes of malignancy. With innovations in modelling patient-specific tumours using organoid models, we are yet again redefining our understanding of biological heterogeneity across cancer subtypes. While these advancements have accelerated our ability to ask fundamentally new questions about cancer heterogeneity, successfully answering these questions and translating our findings into curative clinical treatments hinges on our ability to refine and reproducibly apply these technologies across cancer patient populations.

It should be noted that every cancer model exhibits intrinsic limitations for recapitulating patient-specific tumours, and selection of the appropriate model is fundamental to each study's success. Importantly, as we continue to develop a more complete understanding of the fundamental drivers and treatments of cancer, our ability to improve upon and standardize these models is of increasing importance. In this Review, we have highlighted novel approaches for standardization of next-generation cancer organoid culture and offered our outlook on the next steps for reproducibly and accurately modelling tumour heterogeneity using organoids. Future implementation of these techniques to improve cancer organoid culture will require interdisciplinary effort from clinicians, biologists and engineers. Overall, our collective success in engineering robust cancer models will reshape the way we approach personalized care and accelerate the translation of clinically effective treatments that greatly improve patient outcomes.

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Author contributions

B.L.L. and S.C.H. conceived the Review. B.L.L. wrote the Review. R.A.S., B.L.L. and N.B. conceived and illustrated the figures and table. B.L.L., R.A.S., N.B., M.P.L. and S.C.H. edited the Review. All authors read and approved the final manuscript contents.

Competing interests

The authors declare no competing interests.

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