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Mini-review

Tumor organoids: From inception to future in cancer research

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ABSTRACT

Tumor models have created new avenues for personalized medicine and drug development. A new culture model derived from a three-dimensional system, the tumor organoid, is gradually being used in many fields. An organoid can simulate the physiological structure and function of tissue in situ and maintain the characteristics of tumor cells *in vivo*, overcoming the disadvantages of traditional experimental tumor models. Organoids can mimic pathological features of tumors and maintain genetic stability, making them suitable for both molecular mechanism studies and pharmacological experiments of clinical transformation. In addition, the application of tumor organoids combined with other technologies, such as liquid biopsy technology, microraft array (MRA), and high-content screening (HCS), for the development of personalized diagnosis and cancer treatment has a promising future. In this review, we introduce the evolution of organoids and discuss their specific application and advantages. We also summarize the characteristics of several tumor organoids culture systems.

1. Introduction

Our understanding of the origin of cancer has increased greatly over recent decades. Although progress has been made in diagnosis and treatment, cancer remains a problem worldwide. A major challenge is the development of effective therapies, from basic research to clinical application. Many cancer models only poorly recapitulate a patient's tumor [1]. Consequently, many drugs that perform well in cancer models fail in clinical trials [2]. Recently, an *in vitro* model was developed that can be exploited in basic and clinical cancer research, the organoid.

The main tumor research model remains patient-derived cancer cells (PDCs), which, together with patient-derived xenografts (PDXs), have made tremendous contributions to cancer research. However, they have limitations [3] that are increasingly exposed by our deepening understanding of the complexity of tumors. Monolayer cell culture lacks the diversity of cell types, spatial organization, and overall *in vivo* microenvironment of tumors, adversely affecting stem cell culture [4]. Animal models can simulate the *in vivo* situation and reflect the interaction between the various systems, but their clinical use is difficult because of their low success rate, long culture cycle, and high cost [5]. Recently developed three-dimensional (3D) culture technologies have led to the development of more relevant to pathophysiology of human healthy tissue and cancer models. Organoids derived from 3D technology have emerged as an independent research tool. By simulating the growth environment *in vivo*, cultured 3D cell clusters have a structure and function highly similar to the tissues and organs. The organoid culture can be started with small tissue samples obtained from biopsy or surgery. For seeding, small enzymatic tissue fragments from epithelial fragments can grow rapidly, are similar to tissue, and stable when passaged. Patient-derived organoids (PDOs) can better maintain the primary and tumor cell characteristics in long-term culture than PDCs and PDXs [6,7]. Thus, organoids have advantages over the traditional model and *in vitro* tumor model. PDOs can act as a platform for drug discovery and precision medicine and be used to study regenerative medicine and immunotherapy. For example, adaptation of PDO models for high-throughput screening would carry the additional benefit of allowing investigators to test the organoid response to a wide range of compounds agnostically, without the need for in-depth a priori understanding of the underlying molecular mechanisms at the time of chemosensitivity testing, which could reduce the time to treatment. In addition, many combinations and dosages of a drug can be tested simultaneously, an approach that is less feasible with PDX models. Thus, the successful establishment of a tumor organoid has broad application prospects in basic and clinical research (Fig. 1). In this review, we summarize the history of organoids and compare them to different tumor research models. We discuss the specific application of tumor organoids, the role of different tumor organoid culture systems, and their future prospects.

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Abbreviations

PDC	patient-derived cancer cell
PDX	patient-derived xenograft
3D	three-dimensional
PDO	patient-derived organoid
LGR5	leucine-rich repeat containing G protein-coupled receptor 5
EGF	epidermal growth factor
iPSC	induced pluripotent stem cell
CRC	colorectal cancer
CNV	copy number variant
SCLC	small cell lung cancer
MSI	microsatellite instability
MSS	microsatellite stable
HCC	hepatocellular carcinoma
HRAS	HRas proto-oncogene
TP53	tumor protein p53 gene
5-FU	5-fluorouracil
HDACi	histone deacetylase inhibitor
FAP	familial adenomatous coli
TCR	T cell receptor
IL	interleukin
IEL	intraepithelial lymphocyte
ECM	extracellular matrix
BMP	bone morphogenetic protein
FGF	fibroblast growth factor
APC	adenomatous polyposis coli
DE	definitive endoderm
HIO	human intestine organoid
HCO	human colonic organoid
ALK	activin receptor-like kinase
EpCAM	epithelial cell adhesion molecule
YAP	yes-associated protein
TAZ	tafazzin
FZD	frizzled
ROR	RAR-related orphan receptor
Lats	linker for activation of T cells
ESC	embryonic stem cell
ISC	intestinal stem cell

2. Origins of tumor organoids

2.1. Evolution of 3D models

Since the German scholar Willhelm Roux successfully isolated cells from chicken embryos in 1885, cell culture technology has germinated [8]. In 1907, Granville Harrison succeeded in culturing frog embryonic nerves and established the suspension drop culture method, which was the earliest animal cell culture method [9]. From 1910 to 1912, Carrel improved the suspension drop culture method with culture medium renewal [10]. Modern cell culture techniques were pioneered by both of them. In 1948, Earle designed a medium containing sodium bicarbonate. In 1951, Dulbecco began to use artificial synthetic medium on the basis of the work of predecessors. This is the commonly used Dulbecco's modified Eagle medium (DMEM) [11]. In 1951, Gey established the Hela cell line for the first time [12]. Two-dimensional (2D) cell culture laid the foundation for the development of cell and molecular biology [13]. Conventional adherent tissue culture involves growing cells on solid flat surfaces as 2D monolayers. The cells adhere to an artificial plastic or glass substrate and contact other cells only at their periphery. Oxygen, nutrients, and waste gradients are absent; therefore, the environment is non-physiologically uniform. The cells are forced to adopt a monolayer morphology, which is not natural for all cell types, and the 2D surface inhibits the cells from forming a multi-dimensional structure. Therefore, cells grown in monolayers on plastic surfaces do not accurately model *in vivo* cells [14,15]. Monolayer cells cannot mimic important characteristics of tumors *in vivo*, such as the physical properties and clinical relevance, or model oncogene addiction. Molecular profiling studies indicate that many cell lines do not accurately represent the tumors for which they are used as models [16]. For example, most human tumors divide slowly, and is rare or absent, resulting in poor drug response predictions. In addition, the bioactivity of

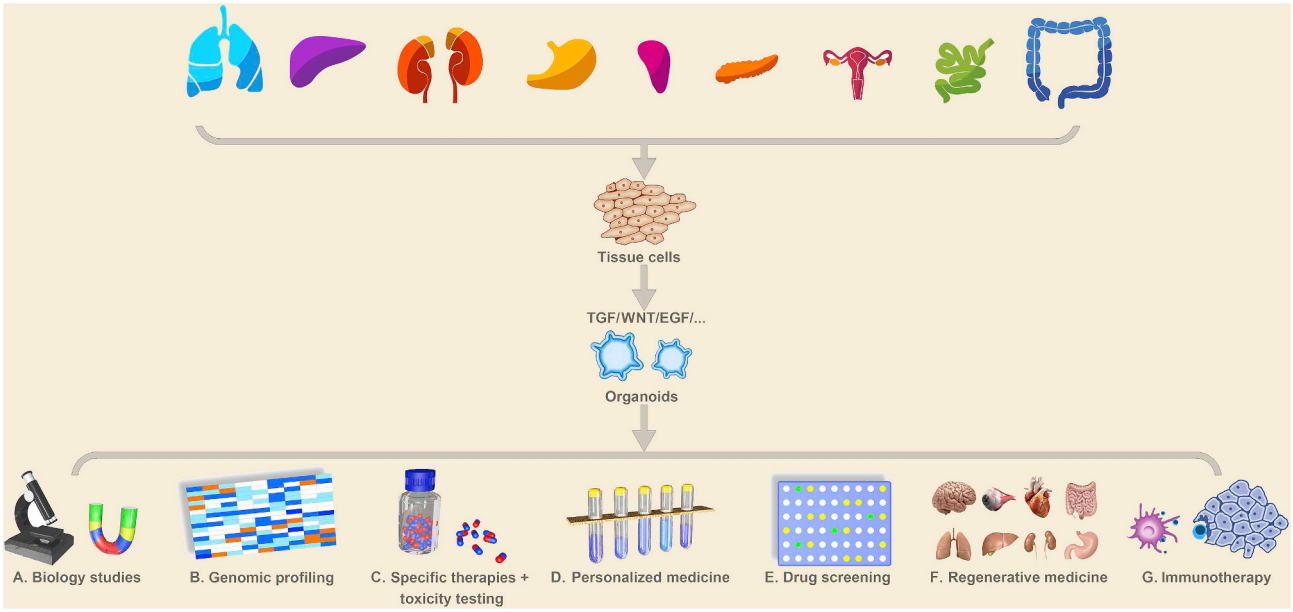

Fig. 1. Potential applications of tumor organoids. Organoids can be established from patient-derived samples of healthy or tumor tissue. Organoid technology can be exploited in basic research and genomic-profiling analyses. Organoids can also promote the development of anti-tumor drugs, including efficacy testing and toxicity testing. In addition, organoids can be potential candidates in personalized medicine and drug screening, or used to understand the principles of regenerative medicine and immunotherapy.

traditional 2D culture substrates cannot be tailored and limited in complexity, and controlled cell co-culture is impossible [17,18]. The unnatural state of 2D culture could affect cell growth, gene expression, signal transduction, and morphology, which may lead to uncertainty in *in vitro* experiments. Creating a 3D cell culture is a more relevant and practical alternative [19].

First proposed in 1968 [20], 3D culture can provide a skeleton structure for cell growth, shorten the distance between cell culture and physiology, and simulate the microenvironment of tumors and the extracellular matrix (ECM). In 1975, Rheinwald and Green successfully cultured the formation of keratinizing colonies from single cells [21]. Typical 3D culture models currently include organotypic multicellular spheroids, multicellular tumor spheroids, tumor-derived spheroids, and tumor-derived organoids [22]. Primary cancer cells with stem cell-like features are expanded *in vitro* as floating spheres. In 3D spheroids, all cells grow in close contact, reproducing the physical communications and signaling pathways observed in solid tumors [23]. As in solid tumors, the internal structures of spheroids comprise different cell layers. 3D culture not only retains the cell morphology *in vivo*, but also allows intuitive and controllable cell culture [24,25]. Culturing cells in 3D versus 2D environments provides another dimension for external mechanical inputs and cell adhesion, which dramatically affects integrin ligation, cell contraction, and associated intracellular signaling [26]. It's been reported that 3D culture system has a slower proliferation rate than 2D culture and better mimicked *in vivo* tumor growth, which can improve microenvironment by bringing it closer to *in vivo* conditions [27,28]. 3D culture can also improve our understanding of tumor biology and facilitate the study of a drug's long-term effects [28,29]. Animal models can capture some, but not all, of the important facets of human responses [30]. For example, animal and *in vitro* models cannot accurately recapitulate human physiology and disease [31,32]. 3D cancer models aim to narrow the gap between 2D cell lines, animal models, and clinical studies. Novel 3D cell culture platforms are being created that better mimic *in vivo* conditions; these platforms are sometimes called organoids.

2.2. The history of organoids

In 1975, James Rheinwald and Howard Green were the first to reconstruct a 3D tissue structure from cultured human stem cells [21]. Later, Lindberg [33] and Pellegrini [34] cultured limbal stem cells on 3T3 trophoblast cells and transplanted them into injured eyes, starting a new chapter in 3D organoid culture. In 1998, Korinek et al. found that the WNT signaling pathway is essential for maintaining stem cell viability in mice [35]. In 2007, Clevers' team proved that leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5) is a stem cell marker [36]. In addition, LGR5+ crypt cells isolated from mouse intestinal segments were cultured in a matrix gel containing epidermal growth factor (EGF), Noggin, and R-Spondin to form a crypt-villus complex similar to the intestine [36,37]. Since then, 3D intestinal tissue organoid models were developed successfully. An organoid is defined as a 3D structure grown from stem cells and consisting of organ-specific cell types that self-organize through cell sorting and spatially restricted lineage commitment [6,37]. In 2010, Barker and others successfully used LGR5+ stem cells to produce gastric functional units *in vitro* [38]. In 2011, Sato et al. [39] cultured intestinal adenomas, metaplastic Barret epithelium, and colon cancer tissue *in vitro* to establish an intestinal tumor organoid from patients for the first time. In the same year, airway stem cells were observed to form alveolar-like structures *in vitro,* mimicking the airway's response to human H1N1 virus [40]. In 2012, Mariani successfully constructed normal brain tissue organoids using induced pluripotent stem cells (iPSCs) [41]. Normal pancreas organoids were first reported in 2013 [42], human prostate cancer organoids in 2014 [43], and pancreatic cancerous organoids in 2015 [44]. Clevers' team successfully used CRISPR/Cas9 technology to modify colorectal cancer (CRC) organoids [45]. Subsequently, in 2017, liver cancer organoids were reported [46] and a breast cancer organoid biobank successfully constructed [47]. In 2018, organoids for bladder tumors [48], esophageal cancer [49], and primary breast cancer metastasis [50] were also constructed successfully. PDOs of metastatic colorectal cancer were reported to be highly similar to primary tumors in phenotypic and genotypic analyses [51]. Collectively, these studies marked the arrival of the organoid era and witnessed the emergence of a new *in vitro* model (Fig. 2).

3. Different cancer research models

PDCs are easily grown, relatively inexpensive, and amenable to high-throughput testing of therapeutic agents [52]. Currently, they are widely used in cancer research. However, in 2D culture, integrin expression and function is abnormal because of a lack of cell adhesion [53]. The lack of stromal cells and interactions between the cells and the ECM leads to significant apoptosis [54], but the balance of growth/death probably results in stable cell numbers [55]. PDCs lack important signal factors promoting cell proliferation, differentiation, and survival [56,57]. They also lack immune cell interactions, resulting in a failure in modeling the tumor microenvironment when evaluating new targets and chemotherapies [58]. Thus, PDCs cannot accurately predict the treatment response *in vivo* [56]. Furthermore, there are unexplained differences in drug sensitivity [59,60]. The genetic composition and cell behavior of PDCs *in vitro* can change over time [61,62]; therefore, they cannot cover the full genetic spectrum of tumor types [63], resulting in misrepresentation of the original tumor and non-physiological responses.

PDX implantation of patient-derived tumors, subcutaneously and/or orthotopically, in immune-deficient mice is similar to the original donor tumor in regards to histological expression and biological behavior, such as protein expression, tumor biomarker status, and genetic status [64,65], and is a widely utilized model for drug discovery, biomarker detection, preclinical drug evaluation [66] in cancer research, and drug

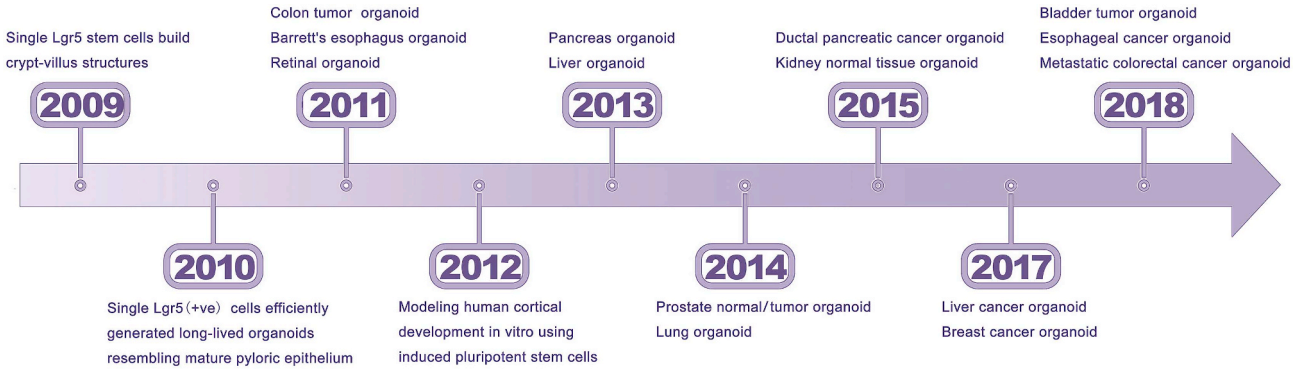

Fig. 2. Milestones of tumor organoids in recent years.

development [67]. However, the success rate of transplantation is relatively low [68], the culture time is relatively long (4–8 months) [66], and resource requirements are high, incurring higher relative costs. Although PDX can efficiently investigate resistance mechanisms and determine novel treatment approaches, studies have reported changes in specific genes and drug targets [69], and several copy number variants (CNVs) in primary tumors that gradually disappeared [70]. In a secondary xenograft study [71] of non-small cell lung cancer (SCLC), a group of tumor-specific genes lost their expression during the transition to tissue culture. Another limitation of PDX models is the generation of specific subtypes of tumors. Most PDX models have been generated from invasive and metastatic tumors, whereas non-metastatic tumors have demonstrated engraftment failure in mice [72,73]. Though some studies have shown that PDX can be developed into valuable models to study tumor-stromal interactions, their usefulness is currently limited by a loss of the human stromal compartment [69]. As the tumor microenvironment plays an important role in tumor growth, transplantation of human tumor cells into the PDX model may affect the predicted drug response, drug toxicity, and patient response [74,75]. Microenvironmental factors, such as the physical properties of the ECM and interactions with stromal cells, can influence the metabolic phenotype of cancer cells, contributing to the response to metabolically targeted therapies [76].

PDOs are a 3D culture of cancerous cells derived from tumor tissues, similar to the original specimen in terms of genome and function [77,78]. Despite the lack of stromal cells, PDOs can imitate the pathological characteristics of primary tissue at the organ level and better simulate the tumor *in vivo* [79,80], making it useful for cancer research and therapy in clinical studies (Table 1). PDOs can be cultured from cells derived from needle biopsy, the culture time is relatively short, and the culture medium can be selected according to the source tissue [39]. Inheritance and morphology remain stable after many generations [63,81]. Gene manipulation [82] and high-throughput drug screening are preponderant compared to PDX models [83]. Compared to the high failure rate of PDCs in anticancer drug research, PDOs are more realistic in recapitulating the *in vivo* environment, which can faithfully reproduce the drug response and serve as a good model for evaluating specific responses in patients with cancer [49,84]. Organoids from different areas of a patient's tumor can better mimic tumor heterogeneity. PDOs also demonstrate potential to model specific cancer subtypes that have unique genomic mutations [85].

4. Characterization and application of tumor organoids in cancer research

4.1. Simulation of the tumor

Cancer gene modeling is a key application of tumor organoids, as simple 2D culture contains very little material for analysis. Tumor heterogeneity is insufficiently stable in the PDX model [86]. Tumor organoids can be used to simulate and study the occurrence and development of specific tumors. In 2014, Boj et al. [44] used mouse endothelial neoplasia pancreatic tissue for *in vitro* culture and transplanted the organoids back into mice, which could completely mimic the progress of pancreatic cancer invasion. Clevers et al. [87] constructed tumor organoids from 20 resected colorectal tumors, obtaining consistent results from exome sequencing, RNA expression, and histological analysis. Lee et al. [48] reported 22 cases of bladder tumor organoids that maintained tumor heterogeneity during culture and xenotransplantation, demonstrating changes in their mutation spectrum consistent with clonal evolution. PDOs have been constructed for colorectal cancer [87], pancreatic cancer [44], liver cancer [88], breast cancer [47], and bladder cancer [48] that mimic the main features of each primary tumor.

4.2. Pathological features

Tumor organoids preserve the pathological features of the original tumors and can be used for basic research, such as the correlation between infectious agents and cancer development. Nuciforo et al. [88] showed that liver cancer organoids have similar histological properties, such as trabecular and/or solid growth with cytoplasmic eosinophilia and highly atypical cells, as the poorly differentiated adenocarcinomas from which they were derived. A year-long culture of tumor organoids did not alter the histological nature of hepatocellular carcinoma. For this pathology, organoids could be used to explore the link between infectious agents and cancer development. Some infectious pathogens are considered significant risk factors for cancer [89]. Organoids can clarify the relationship between pathogens and cancer by replicating some of the most common pathological features. Most pathogens colonize via the lumen of organs, and then contact the apical surface of the epithelium containing the differentiated cells. A major advantage of using organoids to study host-pathogen interactions is that they are

Table 1
Comparison of the cancer research models.

Feature	Patient-derived cell lines	Patient-derived xenografs	Patient-derived organoids	Reference
Lower resource consumption	+ + +	+	+ +	[60]
Shorter culture term	+ + +	+	+ +	[58]
Higher initiation success rate	±	+ +	+ + +	[64,65]
Lower cost	+ + +	+	+ +	[44]
3D model growth	-	+ + +	+ +	[45]
Incorporation of Signal factor	-	+ +	+ +	[48,49]
Tumor-stroma interactions	-	+ +	-	[46]
Mimic tumor microenvironment	-	+ +	+ +	[50,70,71]
Genetic stability	±	+ +	+ +	[53,56,57]
Express complete cancer spectrum	+	+ +	+ +	[55]
Genetic manipulation	+ + +	-	+ + +	[73]
Predict drug response accurately	±	+	+ +	[48,58]
Predict drug sensitivity accurately	±	+	+ +	[51,52]
High-throughput screening	+ + +	-	+ + +	[39,44]

Matching degree of the items on the left side is unsuitable (−), may (±), possible (+), suitable (++), best (+++). Patient-derived organoids (PDO) represent a more realistic response than Patient-derived cell lines (PDC) in terms of recapitulating the *in vivo* environment, and also allow for easier genetic manipulation and high throughput screening than Patient-derived xenografs (PDX).

either composed of, or can be differentiated into, virtually all of the different cell types of a particular organ [90]. Differentiated cells in the organoid model of *Helicobacter pylori* infection induced and promoted inflammatory responses mediated by the NF-kB pathway, which may play a role in the development of gastric adenocarcinoma induced by *H. pylori* [91]. Interestingly, *H. pylori*-infected mice do not develop ulcerative and cancerous tumors like humans [92]. The organoid virus infection model is also used for cancer research [93], as it allows direct investigation of the effects of *ZIKV* infection on cortical layer thickness to address questions related to cell type-specificity in a complex tissue and to investigate human-specific and developmental stage-specific features [94]. In culture, organoid lines exhibit morphologies ranging from spheroidal to asymmetric, and occasionally comprise relatively loose aggregates of cells with similar histopathological features [48]. Thus, organoids could help reveal the pathogenic mechanism and identify potential anti-tumor targets.

4.3. Genetic stability

Identification of the accumulation of mutations in cancer-driving genes provides important insights into the active mutational processes in tissue homeostasis and tumorigenesis. Organoid culture can maintain genome stability, whereas PDX models are often subject to physiological and genetic constraints. In contrast to 3D-cutured cells, chromosomes cannot correctly separate during cell division in 2D-cultured cells after multiple passages [95]. The pattern by which cells adhere to the ECM, transduced intracellularly via integrins, influences the orientation of the mitotic spindle and cell division axis in cultured cells and tissues [96]. Compared to traditional 2D culture, cultured organoids exhibit morphological and genetic stability after multiple passages [97]. Hill et al. [98] successfully constructed ovarian cancer organoids with mutations in *BRCA1* and *BRCA2*, with a genotype consistent with the primary tumor. Nanki et al. [99] successfully constructed gastric cancer organoids and found that the original features and appearance can be maintained after cell passage. Fujii et al. [100] amplified three tumor organoid lines from a single colorectal tumor organoid: two microsatellite instability (MSI) CRC organoids and one microsatellite stable (MSS) CRC organoid. They sequenced the exons before and after long-term culture ($>$ 6 months). The comparison of gene mutations showed that the MSS CRC type organoid was genetically stable. Nuciforo et al. [88] maintained the heterogeneity of primary tumors via long-term culture of tumor organoid biopsies of liver cancer patients with different etiologies and tumor stages. They observed that a substantial proportion of the hepatocellular carcinoma (HCC) organoid-specific mutations were likely present in the originating tumors at low frequencies, rather than generated *de novo*. Tumor organoids play an irreplaceable role in investigating the extent of intra-tumor diversification to characterize genetic stability.

4.4. Advantages in drug research

The precision treatment of cancer is inseparable from two key breakthroughs: Drug targets that are sensitive to drug treatment can be screened by analyzing a large number of abnormal gene data from cancer patients and validating drug sensitivity targets by analyzing a large number of *in vitro* models that can maintain the characteristics of cancer cells *in vivo*. Currently, many drugs with promising results in animal experiments are ineffective for patients due to xenogeneity between mice and humans [101]. To solve this problem, scientists have tried to transplant and study human tumors in mice. However, this approach has its limitations, such as difficulty obtaining enough tumor tissue for transplantation and the gradual adaptation of human tumors to the internal environment of mice, losing their human characteristics [102]. Moreover, 2D cell models are often unsatisfactory in drug screening [103]. The development of tumor organoids is expected to solve these problems [104]. Tumor organoids are close to the natural physiological structure, retaining the specific function of the tumor and reproducing a real drug response. Numerous studies have shown that organoids are excellent models for assessing the specific response of patients with cancer [104–106]. Ogawa et al. [107] successfully produced glioma in the laboratory by editing two genes in the cell, HRas proto-oncogene (*HRAS)* and tumor protein p53 (*TP53*). The experiment fits the reality of human tumors by manipulating only a few cells, rather than turning thousands of cells into cancer cells at once, which is similar to the process of carcinogenesis. For drug screening, 5-fluorouracil (5-FU), oxaliplatin, irinotecan, doxorubicin, and docetaxel have been compared successfully in organoid models of gastric cancer [108]. Cells in the PDC model are highly susceptible to death at higher concentrations and could not be tested for drug toxicity. Little et al. [109] successfully assessed the toxicity of cisplatin to the kidneys using human renal carcinoma organoids. The tolerance of tumor organoids to drugs is stronger than that of other models [110]. The establishment of a PDO model could improve drug safety and efficiency, and such as model could be used to study the relationship between epigenetic and genotypic mutations and drug resistance, allowing stratification of patients according to a specific treatment program [111].

Existing technology cannot accurately predict the sensitivity of patients to chemotherapy. The application of tumor organoids is expected to solve this problem. Li [112] and others successfully established esophageal cancer organoids and performed drug sensitivity experiments, showing that different tumor organoids have great differences in drug susceptibility. These data reflect the complexity of chemotherapy regimens in patients with different tumors. In addition, organoids in subculture are not invariable, as some subclones gradually become dominant clones because they are more adaptable to the environment, leading to genomic changes in the tumor organoid, which is of great value to understanding tumor evolution and drug resistance. Ye et al. [113] engineered adaptable 3D tumor organoids that presented adhesive peptides with distinct integrin specificities for B and T cell lymphoma cells, resulting in enhanced proliferation, clustering, and drug resistance to chemotherapeutics and panobinostat, a new class of histone deacetylase inhibitor (HDACi). Chen et al. [114] used colonic organoids from patients with familial adenomatous coli (FAP) as a platform for drug testing and found two compounds that decrease proliferation in FAP tumor organoids by limiting hyperactive WNT signaling. Thus, tumor organoids could be useful for drug testing before clinical therapeutic application.

4.5. Tumor organoids for immunotherapy

A proper immune response is dependent on immunogenicity of tumor cells. For tumor cells, the sufficient immunogenicity is partially determined by antigens that result from mutations (called neo-antigens). In many cases, the potency of the immune response elicited by the neoantigens of tumor is inadequate, inducing that immune cells cannot eliminate tumor cells [115]. Immunotherapy is expected to fundamentally change the way patients with cancer are treated. However, immunological studies have successfully established stable tumor cell lines that are limited to a small number of tumor tissues, reducing the utility of the method for personalized applications.

Recently, Dijkstra et al. [116] demonstrated that co-cultures of autologous tumor organoids and peripheral blood lymphocytes can be used to enrich tumor-reactive T cells from the peripheral blood of patients with mismatch repair-deficient colorectal cancer and non-small-cell lung cancer. They found that these T cells can be used to assess the efficiency of killing matched tumor organoids. In addition, the short-term maintenance of CD45-positive lymphocytes can be achieved through co-culture with patient-derived organoids of air-liquid interface tumors [117]. The application of tumor organoids in immunization may utilize autologous tumor organoids and T-cell cultures as a patient-specific model system for studying tumor immune recognition and detecting immune tumor treatment protocols. Other potential applications

include the use of tumor organoids in co-culture with T cells to expand tumor-reactive T cells to generate a large number of cells for adoptive T cell therapy and to identify tumor-reactive T cell receptors (TCRs). The addition of interleukin (IL)2, IL7, and IL15 to the culture medium allowed intraepithelial lymphocytes (IELs) to coexist with the intestinal organoids of mice for several weeks, which is expected to help these immune cells fight cancer [118]. Zumwalde [119] found that the lymphocyte compartment associated with human breast ductal epithelial organoids contains several conserved T cell populations that can be targeted, or in some cases possibly inhibited, to promote immune-mediated clearance of nascent neoplastic cells. Such an approach may provide a novel non-invasive means to treat ductal carcinoma in situ. The recent development of thymus organoids could possibly provide a more physiological *in vitro* environment to efficiently maintain and expand tumor-specific T lymphocytes [120]. However, immune incompatibility remains a major obstacle to rendering the artificial organoids as an integral part of the host. Thymus bioengineering is a promising approach to modulating the adaptive immunity of the patient and achieving immunosuppression-free tissue/organ replacement. On the other hand, Neal et al. [121] utilized an air-liquid interface method to propagate PDOs from > 100 human biopsies or mouse tumors in syngeneic immunocompetent hosts as tumor epithelia with native embedded immune cells. Robust droplet-based, single-cell determination of gene expression and the immune repertoire indicates that PDO TILs accurately preserve the original tumor TCR spectrum. Crucially, human and murine PDOs successfully modeled immune checkpoint blockade (ICB) with anti-PD-1 and/or anti-PD-L1 expanding and activating tumor antigen-specific TILs and eliciting tumor cytotoxicity. These results suggest great application prospects of organoids in tumor immunotherapy.

4.6. Application in regenerative medicine

The development of thymic organoids may provide a more appropriate physiological environment to effectively maintain and amplify tumor-specific T lymphocytes [122]. Takahashi and Sekine jointly used organoid technology to produce insulin-secreting islets that could grow blood vessels, representing a breakthrough in type 1 diabetes research [123]. In 2013, Clevers' team indicated that liver organoids have broad potential as a safe clinical source of liver cells for transplantation [106]. A transplant organization for PSC-derived organoids currently exists. To treat pediatric metabolic liver disease, researchers established the vascularized liver bud transplant program at the senior medical research center of Yokohama City University (YCU), which will solve various safety problems in pre-clinical animal experiments, including the carcinogenic effects after transplantation [101]. Another study provides "proof of concept" for retinal sheet transplantation therapy for advanced retinal degenerative diseases [124]. Thus, organoids not only offer new prospects for regenerative medicine, but also provide a new platform for disease modeling and drug discovery by reconstructing more complicated and heterogeneous structures.

4.7. Tumor organoid biobanks contribute to cancer research

In 2015, the Sanger Institute and the Habrecht Institute jointly established the world's first biobank of tumor organoids [125]. Initially established as a library of colorectal cancer organoids [100] that can be used to identify and test new drugs, it has a certain predictive potential [49]. Gao et al. [43] attempted to establish a library of prostate organoids that could be cultured *in vitro* for a long time to perform drug screening and determine the individual drug resistance mechanisms of prostate cancer [49]. To explore how individual genetic events translate into cancer phenotypes, Nanki et al. [99] established a biological library consisting of genetically engineered gastric organoids carrying various gastric cancer mutations and 37 patient-derived organoid lines, including rare genomically stable gastric cancers. In addition, Sachs et al. [47] established a living biobank of breast cancer organoids to capture disease heterogeneity, describing a representative collection of well-characterized breast cancer organoids that are available for cancer research and drug development, with a strategy to assess the *in vitro* drug response in a personalized fashion. Other tumor organoid collections have been generated for CRC metastases, liver cancer, and various cancer types (Table 2).

4.8. Tumor organoids as models of personalized medicine

Organoids obtained from patient samples will guide personalized medical treatment Nuciforo et al. [88] performed long-term culture of organoids from patients with liver cancer of different etiologies and tumor stages. Hubrecht Organoid Technology (HUB), founded by the Harrecht Institute in the Netherlands, KNAW, and Utrecht University Medical Center, has developed a powerful diagnostic tool that links patient-specific genetic and phenotypic information to preclinical and clinical drug responses [101]. Pauli et al. [126] described the development of a robust precision cancer care platform that integrates whole-exome sequencing with a living biobank, enabling high-throughput drug screening on patient-derived tumor organoids. Integration of genomic data with drug screening from personalized *in vitro* and *in vivo* cancer models could guide precision cancer care and fuel next-generation research. Vlachogiannis et al. [49] generated metastatic gastrointestinal cancer (CRC and gastroesophageal cancer) from patient-derived organoid models, demonstrating a positive predictive value (that a particular drug works) of 88% and negative predictive value (that a particular drug does not work) of 100%, suggesting that tumor organoids recapitulate patient responses in clinical trials and could be used in personalized medicine. Personalized anticancer therapies have great potential for the future of oncology [127,128]. Using a personalized tumor model similar to the original tumor, a patient's drug response can be predicted more accurately; thus, tumor organoids have advantages, challenges, and potential in individualized treatment.

5. Culture systems

Since the emergence of intestinal organoids, culture systems have included various growth factors, such as artificially added specific growth factor systems and non-artificially added systems (e.g., co-culture with mesenchymal cells) [129]. In this review, we discuss the effect and mechanism of various artificially added growth factors in partial organoid culture media (Table 3).

5.1. Extracellular matrix

The ECM comprises a large collection of biochemically distinct components, including glycoproteins, proteoglycans, and polysaccharides, with different physical and biochemical properties [130,131]. The ECM is a charged protein network rich in polysaccharide modifications and can bind to many growth factors, including bone morphogenetic proteins (BMPs), fibroblast growth factors (FGFs), and WNTs [132]. ECM with low growth factor levels is usually used in organoids and various growth factors added artificially to control growth or differentiation. The ECM acts as an intermediate in the transmission of signals between different cells. Cells can modulate both the composition and structure of the ECM [133–136] via multiple mechanisms, including the synthesis, degradation, alignment, and cross-linking of the matrix [137,138]. These changes affect signaling from cell–matrix adhesion receptors, such as integrins and discoidin domain receptor tyrosine kinases [139,140]. Moreover, the ECM limits the diffusive range, accessibility, and signaling direction of ligands to their cognate receptors [141]. These changes affect the tissue polarity, structure, angiogenesis, lymph angiogenesis, inflammatory responses, and cancer cell invasiveness [138]. The 3D version of the ECM retains

Table 2
Overview of the tumor organoid collections.

Tumor organoid type	Date	Source	number	Establish efficience	Specific experiment	Research type	Country and institute	Reference
Prostate Cancer	2014.9	Metastases and circulating tumor cells	7	~15–20%	Xenograft studies, genetically manipulation, drug response	Pharmacologic studies.	USA (Memorial Sloan Kettering Cancer Center)	[35]
Colorectal cancer	2015.5	Primary tumor	22	~90%	Drug screen	Gene-drug association	Netherland (Hubrecht Institute)	[55]
Colorectal cancer	2016.6	Primary tumor	55	~100%	Xenotransplantation, genotype-phenotype analyses	Tumorigenesis progress	Japan (Keio University School of Medicine)	[91]
Colorectal cancer	2015.1	Metastases	8	~70%	None	Tumor genetic diversity	Netherlands Cancer Institute	[68]
Various cancer types	2017.3	Primary tumor	56	~37%	Xenograft studies, high-throughput drug screen	Discover novel therapeutic for clinical trials	USA (Weill Cornell Medicine-New York)	[69]
Breast cancer	2017.1	Primary tumor	> 100	~80%	Xeno-transplantations, gene editing, drug screen	Drug development	Netherland (Hubrecht Institute)	[39]
Liver cancer	2017.11	Primary tumor	7	~100%	Xenograft studies, drug screening, karyotyping, the cancer genome atlas (TCGA)analysis, organoid-formation assay	Drug development	UK (Wellcome Trust/CRUK Gurdon Institute)	[38]
Gastrointestinal cancer	2018.2	Metastases	23	~70%	Xenotransplantation, drug screen	Drug testing for clinic	UK (The Institute of Cancer Research)	[41]

*TCGA = the cancer genome atlas.

these advantages, especially for cancer organoids. Matrix components can regulate various cellular processes, such as gene expression [142], differentiation [143], and proliferation [144]. Tight attachment between the epithelium and basement membrane is necessary for epithelial cell survival via integrin signaling, and loss of this signal likely leads to apoptosis [145]. Apoptosis occurring when epithelial cells lose contact with the ECM [146] is known as anoikis [147]. *In vitro,* intestinal tissue undergoes anoikis during the cultivation process, and the ROCK pathway inhibitor Y-27632 can prevent this phenomenon [148]. Importantly, the ECM in organoid culture can affect stem cells. Mounting evidence suggests that the ECM is an essential non-cellular component of the adult stem cell niche [138]. For example, various ECM receptors have been used as markers to enrich adult stem cells in *in vitro* and *in vivo* systems [149,150].

5.2. WNT ligands/R-spondins

Intestinal crypts have fascinated biologists because of their ability to "strongly and continuously proliferate" [151]. In 1998, Korinek et al. demonstrated the importance of the WNT pathway in intestinal stem cells, demonstrating that the WNT pathway is necessary for the formation of intestinal crypts [35,152,153]. As a potent activator of tyrosine kinase receptor signaling (such as EGF), WNT inhibits BMP/TGF-β signals and maintains stem cell characteristics [154]. WNT pathway activation by Paneth cell-produced ligands is essential for stem cells of the small intestine [155]. LGR5+ intestinal stem cells have infinite proliferation capacity, whereas LGR5+ cells can be used as markers of intestinal stem cells and can produce intestinal tissues, such as crypt and villi [156]. LGR5+ cells can also be used as markers of gastric stem cells, which have the ability to self-renew and reconstruct gastric functional units *in vitro* [157]. This may be related to the endoderm source of the gastrointestinal tract that is formed during gastrulation. LGR5 is associated with steady-state stem cells in a variety of tissues, including hair follicles, breast, and ovaries [158–160]. LGR5 is a stem cell marker and a ligand of Rspo-1 [36]. The WNT proteins provide a prototype for ligand-mediated activation of this signaling pathway, and their activities are central to the maintenance of the undifferentiated state of intestinal crypt progenitor cells [161]. Rspo-1 is a WNT pathway activator that acts through LGR5, which could significantly induce crypt hyperplasia in mice [162]. Stimulation with R-spondin/WNT is not essential for continued growth of the organoids, but strongly induces luminal cells, leading to a prostate-like pseudostratified structure of the organoids [163]. Rspo-1 plays an important role in the growth of liver ductal cancer [164]. However, LGR5 is not significantly expressed in the livers of normal adult mice. Interestingly, LGR5+ cells appear around the bile duct damaged by external factors, suggesting the activation of WNT signaling. LGR5+ cells can differentiate into liver cells and ductal cells [163]. Such protective mechanisms also exist in the pancreas and stomach [165–167] and can affect tumor stem cells to promote tumor growth [168,169]. In an *in vitro* model study, single LGR + cells could produce liver organoids and express liver cells and early bile duct cell markers in modified small intestine medium [163]. Activation of the WNT pathway is required to maintain stem cell viability in most cells, including non-parenchymal stem cells, such as hematopoietic stem cells [170]. However, in colon cancer, adenomatous polyposis coli (APC) mutations frequently occur, reaching up to 80% in human colon cancer and resulting in abnormal activation of the WNT pathway [171]. Therefore, for samples with abnormal activation of the WNT pathway caused by individual APC mutations, it is not usually necessary to add WNT pathway activation factors during the organoid culture process, though this depends on the actual situation. Thus, the cultivation system is individualized and diversified.

Table 3
Growth factors and small molecule inhibitors applied in tumor organoid cultures.

	DMEM/F12	penicillin/ streptomycin	Primocin	GlutaMAX	B27	N2	HEPES	N-acetylcysteine	gastrin
Mouse colon adenoma organoid(31)	T	T	_	T	T	T	T	T	_
Human colon cancer organoid (31)	T	T	_	T	T	T	T	T	T/F
Huamn barrett's epithelium(31)	T	T	_	T	T	T	T	T	T
Human hepatocellular carcinoma organoid(38)	T	T	_	T	T	T	T	T	T
Human cholangiocarcinoma (38)	T	T	_	T	T	T	T	T	T
Human pancreatic ductal adenocarcinoma organoid(36)	T	T	T	T	T	_	T	T	T
Mouse pancreatic ductal adenocarcinoma organoid(34)	T	_	_	_	T	_	_	T	T
Human prostate cancer organoid(35)	T	_	T	T	T	_	T	T	T
Human gastric cancer organoid(90)	T	_	_	T	T	T	_	T	T
Human esophageal adenocarcinoma organoid(103)	T	T	T	T	T	_	T	T	_
Human ovarian tumor organoids (89)	T	T	_	T	T	_	T	T	_
Human breast cancer organoid(39)	T	T	T	T	T	_	T	T	_

	Nicotinamide	EGF	FGF	HGF	Noggin	RSPO-1	WNT-3a	Y-27632	A83-01	Forskolin
Mouse colon adenoma organoid(31)	_	T	_	_	_	_	_	_	_	_
Human colon cancer organoid (31)	_	T/F	_	_	_	_	_	_	T/F	_
Huamn barrett's epithelium(31)	T/F	T	T	_	T	T	T	_	T	_
Human hepatocellular carcinoma organoid(38)	T	T	T	T	T/F	T/F	T/F	T/F	T	T
Human cholangiocarcinoma (38)	T	T	T	T	T/F	T/F	T/F	T/F	T	T
Human pancreatic ductal adenocarcinoma organoid(36)	T	T	T	_	T	T	T	_	T	_
Mouse pancreatic ductal adenocarcinoma organoid(34)	T	T	T	_	T	T	_	T/F	_	_
Human prostate cancer organoid(35)	T	T	T	_	T	T	_	T	T	_
Human gastric cancer organoid(90)	T	T	T	_	T	T	T	T/F	_	_
Human esophageal adenocarcinoma organoid(103)	T	T	T	_	T	T	T	_	T	_
Human ovarian tumor organoids (89)	T	T	T	_	T	T	_	T	T	_
Human breast cancer organoid(39)	T	T	T	_	T	T	_	T	T	_

	BMP7	γ secretase inhibitor	SB202190	Dexametha-sone	Notch inhibitor	PGE2	Dihydrotest-osterone	Exendin	Neuregul-in 1
Mouse colon adenoma organoid(31)	_	_	_	_	_	_	_	_	_
Human colon cancer organoid (31)	_	_	T/F	_	_	_	_	_	_
Huamn barrett's epithelium(31)	_	_	T	_	T	_	_	_	_
Human hepatocellular carcinoma organoid(38)	_	_	_	F/T	_	_	_	_	_
Human cholangiocarcinoma (38)	_	_	_	F/T	_	_	_	_	_
Human pancreatic ductal adenocarcinoma organoid(36)	_	_	_	_	_	_	_	_	_
Mouse pancreatic ductal adenocarcinoma organoid(34)	_	_	_	_	_	_	_	_	_
Human prostate cancer organoid(35)	_	_	T	_	_	_	T/F	T	_
Human gastric cancer organoid(90)	_	_	_	_	_	_	_	_	_
Human esophageal adenocarcinoma organoid(103)	_	_	T	_	_	_	_	_	_
Human ovarian tumor organoids (89)	_	_	T	_	_	T	_	_	_
Human breast cancer organoid(39)	_	_	T	_	_	_	_	_	T

* "T" is added throughout the process, and "-" is not added. "T/F" is first added and then removed, and "F/T" is first removed and then added.

5.3. BMP/Noggin

In the intestinal tract, regulation of the proliferation of intestinal epithelial cells by BMP is vital [172–177]. Human Noggin comprises 205 amino acids (residues 28–232) after removal of its signal peptide (residues 1–27) and is secreted as a glycosylated, covalently linked homodimer [178]. Antagonistic BMP proteins are essential for long-term maintenance of small intestinal organoids. After removal of Noggin, LGR5 expression is gradually lost, and proliferation stops within 1–2 weeks [179]. Embryonic development has guided successful efforts to direct the differentiation of human embryonic and iPSCs into specific organ cell types *in vitro*. This involves activin-induced definitive endoderm (DE) formation, FGF/WNT induced posterior endoderm patterning, hindgut specification and morphogenesis, and a pro-intestinal culture system, which eventually produces intestinal organoids [180]. When activated by BMP, human intestine organoids (HIOs) and human colonic organoids (HCOs) experience substantial maturation and express regional markers consistent with identity as small and large intestine, respectively [181]. During pancreatic organoid culture, Noggin is a necessary growth factor [182,183]. In addition, Noggin increases the number of crypts in the long-term culture of intestinal organoids [175], and goblet cells increase after BMP treatment [181]. Cell differentiation may be prevented by inhibiting BMP signaling activity and synergistic activation of the WNT pathway [39,43,184].

5.4. A83-01

A83-01 is a small molecule inhibitor of TGF-β receptor family members activin receptor-like kinase (ALK)4/5/7, which can improve the *in vitro* amplification time and clone formation ability of hepatocytes [185]. The differentiation of epithelial cell adhesion molecule (EpCAM) + ductal cells is promoted by knocking down TGF-β signaling during long-term amplification *in vitro* [185], which is necessary for human liver tissue [182]. During the cultivation of intestinal organoids, unique serum-free and mesenchymal-free growth environments are comprised of the defined niche factors Matrigel, EGF, Noggin, and R-spondin; human intestinal and colonic organoids further require p38 and TGF-β inhibitors [39,145]. The addition of recombinant BMP or TGF-β strongly inhibits organoid growth, whereas targeted deletion of SMAD family member 4 (SMAD4) mitigates their toxicity [186]. Mouse APC-deficient adenoma organoids can grow in the absence of Noggin, which supports reciprocal inhibition of WNT and BMP/TGF-β signaling. In contrast, human APC-deficient adenoma organoids remain dependent on both Noggin and A83-01, indicating that the human intestinal epithelium is more sensitive to BMP/TGF-β pathways than that of the mouse [39].

5.5. Rho/ROCK

The Rho/ROCK signaling pathway induces cell migration, cytoskeletal recombination, and stress fiber formation, which are related to physiological functions, such as tissue contraction, endothelial permeability, and growth [187]. Focal adhesion is induced by Rho, a multiprotein complex of integrin and associated proteins that is clustered by the force of actomyosin bundles ligated to this complex and promotes adhesion to the ECM. The Hippo signaling effectors yes-associated protein (YAP) and tafazzin (TAZ) are involved in the control of stem cell renewal, the size of the organoids, regeneration, and cancer [188]. Rho signaling is also required for activation of YAP/TAZ by WNT signaling, in which WNT activates Rho through the frizzled (FZD)- RAR-related orphan receptor (ROR)-G protein subunit alpha 12/13 pathway and inhibits linker for activation of T cells (Lats)1/2 [189]. Rho-mediated activation of YAP/TAZ is required for long-term survival and expansion of human embryonic stem cell (ESC) cultures [190]. Paradoxically, dissociated human ESCs exhibit Rho-ROCK-mediated hyperactivation of myosin, and the resulting contraction induces their death, which can be rescued by Y-27632 [191–193]. Activation of the Rho/ROCK pathway can enhance the connection between cells and the matrix, and promote cell adhesion [187]. In addition, Y-27632 could inhibit anoikis in various culture systems [157,194]. Y-27632 positively regulates the formation of organoids, but it is not necessary for organ survival and is not added in many cultures [182]. Moreover, Y-27632 inhibits apoptosis of prostatic stem/progenitor cells induced by dissociation in mice, improves their cloning efficiency [194], and can promote the rapid proliferation and aggregation of feeder cells [195]. Y-27632 is usually added after passage or recovery, and generally lasts no more than 3 days [47,164,196,197].

5.6. Notch

Inhibiting the Notch signaling pathway can effectively block the differentiation of hepatic organs into ductal cells [198]. Removing mitotic stimulation and administering Notch inhibitors leads to hepatocyte differentiation. After transplantation, these cells mature into functional hepatocytes [166]. In the early/mid stage of retinal differentiation, Notch inhibitors can be used to increase the proportion of photoreceptors within the laminated organoids [199]. Notch inhibition upregulates Notch ligands (e.g., Dll1 and Dll4) on secretory-lineage

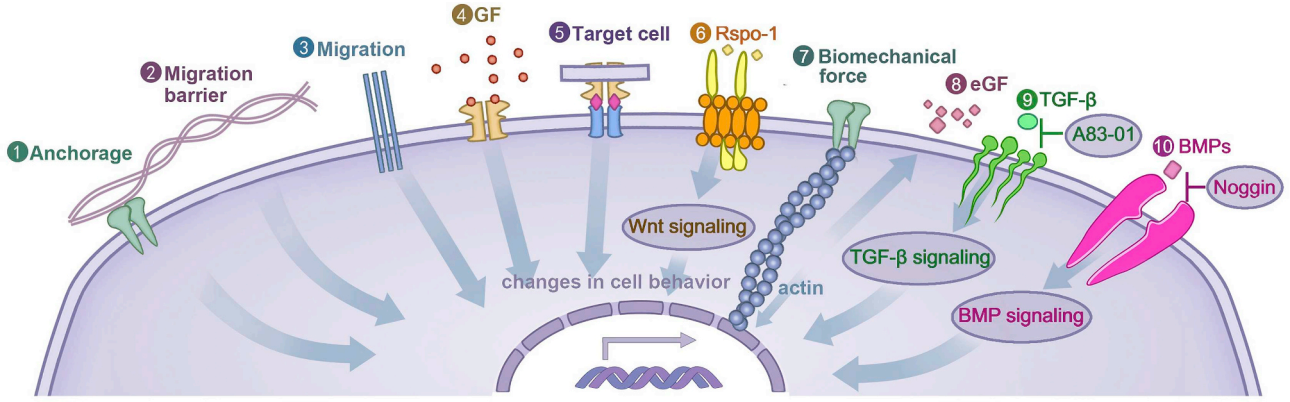

Fig. 3. Mechanisms of extracellular matrix (ECM) function. (1) Anchorage to the basement membrane is essential for various biological processes, including asymmetric cell division of stem cells and maintenance of tissue polarity. (2) The migration barrier and (3) ECM may serve to block or facilitate cell migration. (4) Growth factor(GF) and (5) the ECM can selectively bind to different growth factors and function as a signal coreceptor or presenter. (6) Rspo-1 helps maintain the stemness of organoids by activating WNT signals. (7) Cells directly sense the biomechanical properties of the ECM, including its stiffness, and change a wide variety of behaviors accordingly. (8)Endogenous factors regulate the growth of peripheral cells. (9) A83-01 can specifically block the signal of TGF-beta and contribute to the growth of organoids. (10)Noggin can block BMP signal to regulate cell differentiation. Abbreviation: eGF, endogenous growth factor.

cells, activating neighboring Notch + cells. Therefore, intestinal stem cells (ISCs) constantly require contact with Notch ligand + cells, such as Paneth cells, to form small intestinal organoids as a heterotypic checkerboard pattern of ISCs and Paneth cells [155]. Atoh1-deficient organoids lose secretory differentiation and grow in the absence of Notch + niche cells [200]. Although the detailed molecular mechanism remains unclear, histone deacetylase (HDAC) inhibitor treatment suppresses secretory-lineage differentiation through Notch activation and promotes homogeneous ISC expansion in the organoid [201].

In terms of physical properties, by functioning as a barrier, anchorage site, or movement track, the ECM provides a fulcrum and migration path or barrier for cells. In terms of the biochemical characteristics of the ECM, it is indirectly and directly related to the cell's signal transmission ability. At the same time, with the participation of signals, such as WNT and BMP, they jointly promote the formation and growth of organoids (Fig. 3).

6. Conclusions and future perspectives

Progress in generating tumor organoids that faithfully recapitulate human *in vivo* tissue composition has extended their application range. However, the complexity of organoid cultures may hinder the efficiency of further analysis and treatment [202]. Organoids may not fully duplicate the complexity and diversity of their primary cells (e.g., brain organoids) and lack immune system elements, key stromal cells, and vascular factors. In addition, the relatively rigid ECM could limit drug penetration, hampering the use of organoids in drug screening. Another limitation is that organoids have not yet been cultured from non-epithelial tumors. There are also ethical issues, including the use of embryos [203]. To overcome these challenges, Czerniecki et al. produced a liquid-handling robot protocol to produce kidney organoids for screening [204]. Microfluidics technology could facilitate the co-culture of organoids to mimic a complex microenvironment [205]. Recently developed synthetic matrices that support the growth of mouse and human intestinal organoids could possibly replace mouse-derived ECM substitutes [206]. Advances in the growth of organoids from primary glioblastoma tissue may promote the culture of other non-epithelial tumors [207]. Biobanks of patient-derived organoids should increase our understanding of inter-patient and intra-patient heterogeneity, hopefully leading to personalized medicine for various diseases. Organoids have emerged as a physiologically relevant *in vitro* model for cancer research. They can be established efficiently from patient-derived tumor tissue, making organoids highly relevant for translational applications and the future development of cancer treatments.

Conflicts of interest

The authors declare that they have no competing interests.

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