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Leveraging and manufacturing in vitro multicellular spheroid-based tumor cell model as a preclinical tool for translating dysregulated tumor metabolism into clinical targets and biomarkers

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Abstract

The grand challenge now and in the near future for the pharmaceutical industry is how to efficiently improve R&D productivity. Currently, the approval rate of the entire clinical drug development process is extremely low, and the high attrition in the phase I clinical trial is up to 95%; 67% and 33% of all drugs that enter Phase II and Phase III clinical trials fail to transit into the next stage, respectively. To achieve a higher success rate in clinical trials, developing efficient drug screening method based on more in vivo like tumor tissue is an urgent need to predict the toxicity and efficacy of candidate drugs. In comparison to 2D planar tumor model, the 3D multicellular tumor spheroid (MTS) can better simulate the spatial structure, hypoxia and nutrient gradient, extracellular matrix (ECM) deposition and drug resistance mechanism of tumor in vivo. Thus, such model can be applied for high-throughput drug screening and evaluation, and also can be utilized to initiate a series of fundamental research areas regarding oncogenesis, tumor progression and invasion, pharmacokinetics, drug metabolism, gene therapy and immune mechanism. This review article discusses the abnormal metabolism of cancer cells and highlights the potential role of MTSs as being used as efficient preclinical models. Also, the key features and preparation protocols of MTSs as well as the tools and techniques used for their analysis were summarized and the application of 3D tumor spheroid in specific drug screening and in the elucidation of drug resistance mechanism was also provided. Despite the great knowledge gap within biological sciences and bioengineering, the grand blueprint for adaptable stirred-tank culture strategies for large-scale production of MTSs is envisioned.

Keywords: Multicellular tumor spheroids, In vitro models, Tumor metabolism, Preclinical evaluation, Cancer therapy, Precision medicine

Introduction

Cancer has long been one of the leading causes of morbidity and mortality worldwide (Rodrigues et al. 2018), and is a group of diseases characterized by uncontrolled growth and spread of abnormal cells. As both direct and indirect consequence of oncogenic mutations, tumorigenesis largely depends on the reprogramming of cellular metabolism.

Increasing evidence suggested that tumors are heterogeneous not only because of 3D solid structure with different cell types and their interaction with ECM but also because of metabolic zonation caused by varied metabolic activity among different layered tumor cells

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and physical barriers in terms of substance permeability (Zhu and Thompson 2019). The individual heterogeneity of patients with different types of tumors and the tissue heterogeneity of the same patient are huge obstacles to effective treatment of tumors. Therefore, precision medicine that takes into account individual differences in each person's genes, environment and lifestyle is the future direction of cancer treatment (Wishart 2016), and from the tumor metabolic point of view, cancer treatment requires targeting specific metabolic pathways in a case-dependent manner (Fig. 1). For example, Daemen et al. successfully identified three different metabolic subtypes in pancreatic ductal adenocarcinoma (PDAC) through a broad metabolite profile, facilitating accurate diagnosis of tumor subtypes with specific metabolic requirements (Daemen et al. 2015). Oncometabolites, often referred to as biomarkers, are targets for biological drug development, and the more specific they are, the better their accuracy in targeting specific tumors (Nielsen 2017). Although a plethora of anticancer drugs have been developed in an attempt to target and inhibit tumor-specific metabolic pathways (Table 1), seeking for efficacious cancer treatment remains a worldwide challenge.

At present, chemotherapy, radiotherapy and surgery are the most commonly used clinical methods for the treatment of cancer-related diseases (Siegel et al. 2016). Advances in cancer treatment desperately need extensive in vitro models to test. 2D cell culture, first developed by Harrison in the early 19th century (Breslin and O'Driscoll 2013), is a common screening method for in vitro therapy due to its advantages of simplicity, repeatability and low cost (Chatzinikolaidou 2016). However, in traditional 2D culture, cells grow in a lack of tumor microenvironments (TME) such as layered cylindrical structure, interaction of various cytokines, gradients of nutrient and waste, which is extremely different from the state of cells in vivo (Doke and Dhawale 2015). For example, Apicella et al. found that EBC1 cell lines (an MET-addicted NSCLC cell line) derived from JNJ-605-resistant tumors (RES-J EBC1) lost drug resistance as in vitro 2D culture. While cells were re-transplanted into mice, they regained drug resistance. Also, non-resistant tumor cells were co-cultured with mouse fibroblasts isolated from resistant tumors, and the non-resistant tumor cells obtained the ability of resistance (Apicella et al. 2018). Beyond that, Wilson et al. found that cancer cells typically express multiple receptor tyrosine kinases (RTK), and increased RTK ligand levels in the tumor environment lead to oncogenic kinase inhibitor resistance. Therefore, cancer cell lines with high kinase activity acquire drug resistance when exposed to RTK ligands (Wilson et al. 2012). These results demonstrate that TME is an important factor that affects drug resistance. In addition, cell signal transduction network is changed or corrupted, which is also an important factor affecting the results of drug screening (Wang et al. 1998; Weaver et al. 2002). Furthermore, commonly used transgenic animal models, such as Patient-Derived tumor Xenograft (PDX) models, can stabilize tumor formation in vivo and have the advantage of genetic diversity, but its application is rather limited, because of costly material resources, time-consuming and lot-to-lot uncertainty factors in terms of tested mice. In addition to this, challenges associated with both dynamic observation and massive production of 3D tumor model still remain (Jo et al. 2019). To address this, designing more cost-effective but representative models that can recapitulate solid tumors in vivo is an obvious method. The in vitro 3D culture models of tumor cells have gradually developed (Chatzinikolaidou 2016). In the early 1950s, 3D multicellular tissue spheroids have been reported, and the term "3D culture model" was first put forward by Barcellos-Hoff et al. (1989). Since the 1970s, organ and 3D cell culture models have been developed as an alternative to traditional 2D cell culture and animal models for drug testing and evaluation (Costa et al. 2014). In the 1990s, the morphology of 3D culture models was found to depend on the interactions of growth factors, morphogens and matrix proteases (Simian et al. 2002).

In recent years, 3D culture of tumor cells in vitro has developed rapidly. MTS is a 3D culture model, which can represent the main characteristics observed in solid tumors in vivo and behave closer to the actual tumor pathological and physiological environment than 2D culture cells. For example, it can better imitate the tumor microenvironment, including cell proliferation and differentiation, cell-to-cell and cell-to-extracellular matrix interaction (Fennema et al. 2013). The current clinical market requires a large number of tumor models, but high-throughput and large-scale MTSs culture in vitro has remained suspended. Hence, the batch and standardized production of 3D models are garnering more attention over the past decade (Katt et al. 2016). The number of journal articles associated with MTSs and their clinical use listed from the Web of Science database has also tremendously increased over the past decade (Fig. 2).

In the following sections, we will summarize (a) metabolic specificity of tumor cells; (b) the key features and preparation protocols of MTSs as well as the tools and techniques used for their analysis; (c) the application of 3D tumor spheroid in specific drug screening and drug resistance mechanism. In addition to this, the grand blueprint for adaptable stirred-tank culture strategies for large-scale production of MTSs is prospected.

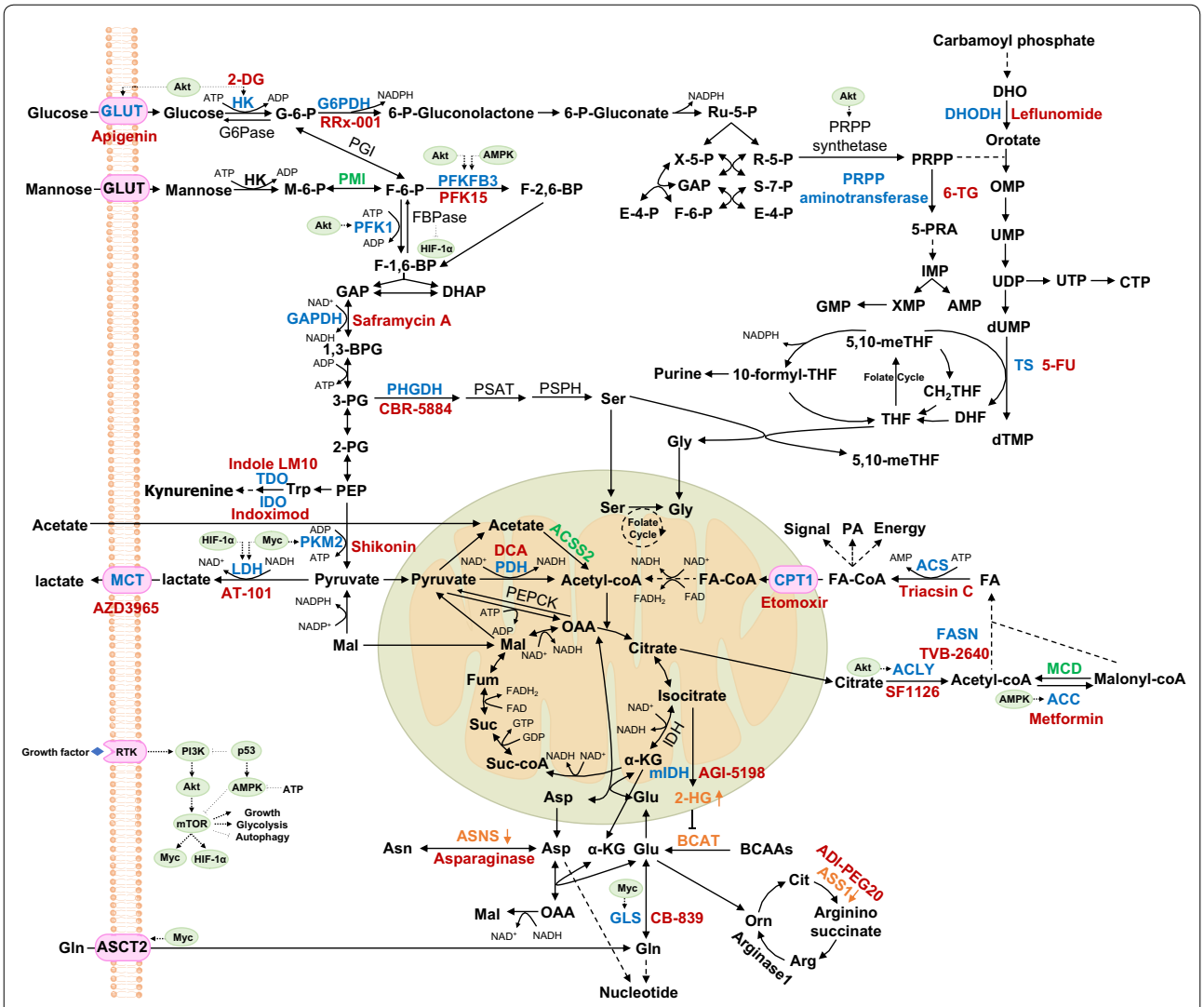

Fig. 1 Schematic diagram of targeted metabolic pathways in cancer therapy. Through the regulation of various signaling pathways, the metabolism of cancer cells changes compared with normal cells. Therefore, targeting tumor metabolic pathways is an important direction for anticancer treatment. The accumulation of mannose-6-phosphate (M-6-P) impairs further glucose metabolism. Tumor cells with low level of mannose phosphate isomerase (PMI) are sensitive to mannose, which can also become sensitive to mannose by siRNA-targeted PMI (Gonzalez et al. 2018). Glycolysis breaks down glucose into pyruvate and produces a variety of intermediates into the pentose phosphate pathway, TCA cycle, lipid metabolism, and amino acid metabolism. The pentose phosphate pathway provides reducing equivalents and precursors for nucleotide synthesis. In addition to energy metabolism, the TCA cycle also provides precursors for lipid and amino acid synthesis. Acetyl-CoA is an important intermediate of energy metabolism. In human brain tumors, the contribution of acetate oxidation to the acetyl-coA pool is enhanced, and acetyl-coA synthetase 2 (ACSS2) is highly expressed, which can be a potential target for tumor detection and treatment (Mashimo et al. 2014). Acetyl-CoA carboxylase (ACC) and malonyl-CoA decarboxylase (MCD) are key enzymes in the fatty acid synthetic pathway, catalyzing the interconversion of acetyl-CoA and malonyl-CoA. At present, anticancer drugs targeting ACC have been applied in clinics, and siRNA inhibition of MCD is adverse to cancer cells, which makes MCD a potential therapeutic target (Currie et al. 2013). 2-HG, a metabolite of mIDH (mutated isocitrate dehydrogenase) tumors, has become a useful prognostic cancer marker (Salamanca-Cardona et al. 2017). 2-HG inhibits branched chain amino acid transaminases (BCATs) activity, which increases glutamate from glutamine catalyzed by glutaminase (GLS). Therefore, anticancer drugs targeting mIDH and targeting GLS can be used in combination therapy (McBrayer et al. 2018). Arginine succinic acid synthase (ASS1) and asparagine synthase (ASNS) cannot be expressed in some tumor tissues, which can also become potential targets for cancer detection and treatment (Ananieva 2015). Target metabolic enzymes (Blue); Anticancer drugs (Red); Potential targets (Green); Tumor biomarkers (Orange). Up arrow and down arrow denote the increased and decreased level of metabolites or enzymes, respectively

Table 1 Anticancer drugs targeting tumor metabolic pathways. The National Clinical Trial (NCT) numbers of anticancer drugs can be found on U.S. National Library of Medicine (https://clinicaltrials.gov/ct2/home). The information was collected until 28th March

Metabolic pathways	Target	Drug	Action mechanism(s)	Clinical stage	Cancer type	References/NCT Number
Sugar metabolism	Glucose transporter (GLUT)	Phloretin	GLUT2 inhibitor. Inhibition of glucose transport is related to the earliest steps of apoptosis	Mice/ Preclinical	Liver cancer	(Wu et al. 2009)
		Apigenin	GLUT4 inhibitor, a natural flavonoid with potential antioxidant, anti-inflammatory, and anticancer properties	Clinical Phase II	Colorectal cancer	NCT00609310
		Genistein	GLUT1 inhibitor, a natural flavonoid	Clinical Phase I/II	Colon cancer	NCT01985763
		Naringenin	Glucose transport inhibitor, a natural flavonoid	Preclinical	Colon cancer, prostate cancer	(Harmon and Patel 2004; Lim et al. 2017)
		Silybin	Glucose transport inhibitor, a natural flavonoid	Clinical Phase II	Prostate cancer	NCT00487721
					Non-small cell lung cancer (NSCLC)	NCT02146118
		Resveratrol	GLUT1 inhibitor, a plant antitoxin	Clinical Phase I	Colon cancer	NCT00256334
				Clinical Phase I/II	Liver cancer	NCT02261844
		Glufosfamide	A novel alkylating agent whose active metabolites are linked to β-D-glucose by glycosidic bonds	Clinical Phase II	Ovarian cancer	NCT00442598
	Hexokinase (HK)	2-deoxy-D-glucose (2-DG)	Glucose substitute. 2DG-6P inhibits the transformation of G6P to F6P through competitive inhibition of glucose isomerase.	Clinical Phase I/II	Prostate cancer	NCT00633087
		3-Bromopyruvate	Preventing ATP production by limiting the activity of HK in glycolysis	Mice/Preclinical	Glioma	(Bodur et al. 2016; El Sayed et al. 2012)
		Imatinib	HK inhibitor	Clinical Phase I/II	Gastrointestinal stromal tumors	NCT04138381

Table 1 (continued)

Metabolic pathways	Target	Drug	Action mechanism(s)	Clinical stage	Cancer type	References/NCT Number
	6-Phosphofructo-2-Kinase (PFKFB)	1-(4-pyridinyl)-3-(2-quinolinyl)-2-propen-1-one (PFK15)	A competitive inhibitor. PFKFB3 catalyzes the reaction to produce fructose 2, 6-diphosphate (F26BP), which is an activator of fructose 6-phosphate 1-kinase, which is a key step in glycolysis.	Mice/Preclinical	Solid tumors, hematologic malignancies	(Clem et al. 2013)
	Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	Iodoacetamide (IAA), Iodoacetate (IA)	GAPDH inhibitor	Preclinical	Glioma	(Schmidt and Dringen 2009)
		Saframycin A	Forming ternary complexes with GAPDH and DNA, which is toxic to cells	Preclinical	Cervical cancer, lung cancer	(Xing et al. 2004)
	Pyruvate kinase (PKM2)	Alkannin	PKM2 inhibitor. PKM2 is widely expressed in cancer cells and determines the final rate-limiting step that is critical to glycolysis for cancer cell proliferation and survival.	Preclinical	Breast cancer	(Chen et al. 2011)
		Shikonin	PKM2 inhibitor.	Preclinical	Breast cancer	(Li et al. 2014)
					Bladder urothelial carcinoma	NCT01968928
	Lactate dehydrogenase (LDH)	AT-101	LDHA inhibitor, which inhibits glycolysis and tumor growth, while causing cytotoxicity caused by low intracellular pH	Clinical Phase I/II	Locally advanced esophageal or GE junction cancer	NCT00561197
		NHI	LDHA inhibitor	Preclinical	Cervical cancer	(Granchi et al. 2013)
	Monocarboxylic acid transporter (MCT)	AZD3965	MCT1 inhibitor, which inhibits lactate excretion, glycolysis, and tumor growth, while causing cytotoxicity caused by low intracellular pH	Clinical Phase I	Solid tumors, diffuse large B cell lymphoma, burkitt lymphoma	NCT01791595
		Quercetin	MCT inhibitor, a natural flavonoid	Clinical Phase I	Prostate adenocarcinoma	NCT01912820

Table 1 (continued)

Metabolic pathways	Target	Drug	Action mechanism(s)	Clinical stage	Cancer type	References/NCT Number
		Luteolin	MCT inhibitor, a natural flavonoid	Clinical Phase I	Tongue carcinoma	NCT03288298
	Pyruvate dehydrogenase (PDH)	Dichloroacetate (DCA)	PDH is a rate-limiting enzyme for aerobic glucose oxidation. DCA stimulation of PDH increases the peripheral oxidation of alanine and lactic acid.	Clinical Phase II	Breast cancer, lung cancer	NCT01029925
		CPI-613	Inhibiting PDH and α-ketoglutarate dehydrogenase, disrupting mitochondrial metabolism	Clinical Phase II	Pancreatic cancer	NCT03699319
	Isocitrate dehydrogenase (IDH)	IDH305	mIDH1 inhibitor. The mutated IDH (mIDH) alters the catalytic activity of the enzyme to produce oncometabolite 2-hydroxyglutarate (2-HG), thereby regulating the methylation of histone and DNA and promoting the development of tumors.	Clinical Phase II/III	Glioma	NCT02977689
		AGI-5198	The first highly effective, selective IDH1 R132H/R132C mutant inhibitor	Cell/Preclinical	Glioma, colon cancer	(Molenaar et al. 2015)
		AG-881	mIDH1/2 inhibitor	Clinical Phase I	Glioma	NCT02492737
		Enasidenib	mIDH2 inhibitor	Clinical Phase I/II /FDA	Acute myeloid leukemia	NCT04092179
	Glucose-6-phosphate dehydrogenase (G6PDH)	RRx-001	G6PDH inhibitor. G6PDH is the oxidoreductase of PPP pathway.	Clinical Phase I	Malignant solid tumor, Lymphoma	NCT02096341 NCT01359982
				Clinical Phase II	Small cell carcinoma, NSCLC	NCT02489903
		Imatinib	G6PDH inhibitor	Clinical Phase II	Breast cancer	NCT00193180

Table 1 (continued)

Metabolic pathways	Target	Drug	Action mechanism(s)	Clinical stage	Cancer type	References/NCT Number
	6-phosphate- glucose (G-6-P)	Mannose	Accumulated in cells as mannose-6-phosphoric acid, which damages further glucose metabolism in glycolysis, TCA, PPP, and glycan synthesis	Cell/Preclinical	Ovarian cancer	(Gonzalez et al. 2018)
Amino acid metabolism	Glutaminase (GLS)	CB-839	GLS inhibitor. Glutamine is catalyzed by GLS to glutamate enter TCA. Blocking glutamine enter TCA can inhibit the progression of MYC-driven liver cancer.	Clinical Phase I/II	Lung adenocarcinoma	NCT03875313
	Phosphoglycerate dehydrogenase, (PHGDH)	CBR-5884	Inhibiting of de novo serine synthesis in cancer cells, and selective toxicity to cancer cells with high serine biosynthesis activity	Mice/Preclinical	Breast cancer	(Mullarky et al. 2016)
	Arginine succinic acid synthase (ASS1)	ADI-PEG20	ASS is the key rate-limiting enzyme for arginine synthesis, which is not expressed in some tumor tissues. ADI-PEG20 is essentially a pegylated, modified arginine deiminase that consumes arginine around the tumor.	Clinical Phase I/Listed	Lung cancer, head and neck squamous cell cancer, prostate cancer	NCT03254732
		Recombinant human arginase 1 Peg5000 (rhArgIpeg5000)	The recombinant arginase 1 depletes arginine in the tumor microenvironment.	Clinical Phase I	Lymphoma	NCT01551628
	Asparagine synthase (ASNS)	Asparaginase	Tumor cells lack ASNS and cannot synthesize the asparagine for growth. Asparaginase hydrolyzes asparagine, making the tumor cells lack asparagine, thereby inhibiting growth.	Clinical Phase II /Listed	Acute lymphocytic leukemia, acute myelogenous leukemia, acute mononuclear leukemia	NCT00854425

Table 1 (continued)

Metabolic pathways	Target	Drug	Action mechanism(s)	Clinical stage	Cancer type	References/NCT Number
	Indoleamine-2,3-dioxygenase (IDO)	Indoximod and docetaxel	IDO inhibitor. Tryptophan is one of the key amino acids linking anti-tumor immune response and immune tolerance. IDO and TDO are the key enzymes for tryptophan degradation.	Clinical Phase I	Solid tumors, NSCLC	NCT01191216 NCT02460367
	Tryptophan-2,3-dioxygenase (TDO)	Indole LM10	TDO inhibitor	Mice/Preclinical	Mast cell tumor	(Pilotte et al. 2012)
Fatty acid metabolism	Acetyl-CoA carboxylase (ACC)	Metformin	Metformin activates AMP-activated kinase (AMPK). ACC is a key enzyme in the de novo fatty acid synthesis pathway. After phosphorylation, ACC activity is lost, which is mainly regulated by AMPK.	Clinical Phase II	Prostate cancer, breast cancer	NCT03137186 NCT01266486
		ND-646	An allosteric inhibitor of ACC. Acyl-CoA group was converted into malonyl-CoA through ACC, and decreased the expression of ACC gene could induce apoptosis of cancer cells.	Mouse lung cancer model/ Preclinical	NSCLC	(Svensson et al. 2016)
	ATP citrate lyase (ACLY)	SB-204990	ACLY inhibitor. Reducing cholesterol and fatty acid synthesis	Preclinical	Lung cancer, glioblastoma, colon cancer	(Granchi 2018)
		SF1126	Novel inhibitor of PI3 kinase and mTOR	Clinical Phase I	Neuroblastoma	NCT02337309
	Fatty acid synthase (FASN)	TVB-2640, TVB-3166	Targeted inhibition of FASN can reduce palmitoylation of microtubulin, destroy microtubule tissue, and inhibit the growth of tumor cells.	Clinical Phase II	NSCLC	NCT03808558
		C93	FASN inhibitor	Mice/ Preclinical	NSCLC	(Orita et al. 2007)
		Orlistat	FASN inhibitor	Mice/ Preclinical	Melanoma	(Carvalho et al. 2008)

Table 1 (continued)

Metabolic pathways	Target	Drug	Action mechanism(s)	Clinical stage	Cancer type	References/NCT Number
	Fatty acyl-CoA synthase (ACS)	Triacsin C	ACS inhibitor. Fatty acids (FA) need to be activated into the active pool by ACS.	Cell/ Preclinical	Glioma	(Mashima et al. 2010)
		Thiazolidinediones (TZDs)	Interference with TGF-β signal transduction	Cell/ Preclinical	Breast cancer	(Jarrar and Baranova 2007)
	Carnitine palmitoyl transferase 1 (CPT1)	Etomoxir	An irreversible CPT1-specific inhibitor, preventing fatty acid transport to the mitochondrial matrix for further metabolism	Mice/ Preclinical	Glioblastoma	(Pike et al. 2011; Samudio et al. 2010)
		Ranolazine	CPT1 inhibitor	Mice/ Preclinical	Prostate cancer	(Deep and Schlaepfer 2016; Samudio et al. 2010)
Nucleic acid metabolism	Cytidine deaminase (CDA)	Different oxidation forms of 5-methylcytosine :5hmdC and 5fdC	Conversion into modified uracil inserted into DNA, causing DNA damage and eventually inducing cell to death	Cell/Preclinical	NSCLC	(Zauri et al. 2015)
		Gemcitabine	Cytosine nucleoside derivatives, which incorporate DNA into cells, also inhibit nucleotide reductase, resulting in the reduction of intracellular deoxyriboside triphosphate.	Clinical Phase II	Pancreatic cancer, small cell lung cancer	NCT04039867
	Thymidylate synthase (TS)	5-fluorouracil (5-FU)	The analogues of uracil, blocking the conversion of deoxyribonucuridine to thymidine and interfere DNA synthesis	Clinical Phase II /Listed	Colon cancer	NCT00388700
		Capecitabine	After a series of reactions, its metabolite 5-FU exerts anticancer effects	Clinical Phase IV	Breast cancer, colorectal cancer	NCT03465202
	Folic acid synthesis pathway	Pemetrexed	Folic acid synthesis pathway inhibitor, inhibiting thymidine and purine nucleotide biosynthesis	Clinical Phase I /Listed	NSCLC, pleural mesothelioma	NCT01918761
	Dihydrofolate reductase (DHFR)	Aminopterin	DHFR inhibitor	Clinical Phase II	Endometrial carcinoma	NCT00003821
	Dihydrowhey acid dehydrogenase (DHODH)	Leflunomide	DHODH inhibitor	Clinical Phase I/II	Breast cancer, multiple myeloma	NCT03709446 NCT02509052

Table 1 (continued)

Metabolic pathways	Target	Drug	Action mechanism(s)	Clinical stage	Cancer type	References/NCT Number
	PPRP amido transferase	6-thioguanine (6-TG)	Purine antagonist, converted to deoxyguanine nucleotides, incorporated into DNA to interfere the function of DNA	Clinical Phase I/II	Acute lymphoblastic leukemia, lymphoblastic lymphoma	NCT02912676
		6-thiopurine(6-MP)	Inhibiting the biosynthesis of purines	Clinical Phase II	Breast cancer, ovarian cancer	NCT01432145
				Clinical Phase IV/Listed	Acute lymphocytic leukemia	NCT03920813
	Topoisomerase II	Banoxantrone(AQ4N)	AQ4N is the prodrug of topoisomerase II inhibitor AQ4 that impedes DNA repair.	Clinical Phase I/II	Non-Hodgkin's lymphoma, chronic lymphocytic leukemia, small lymphocytic leukemia	NCT00109356
		Etoposide	Forming a drug–enzyme–DNA stable reversible complex that impedes DNA repair.	Clinical Phase III/Listed	Small cell lung cancer, malignant lymphoma, malignant germ cell tumor	NCT03305341

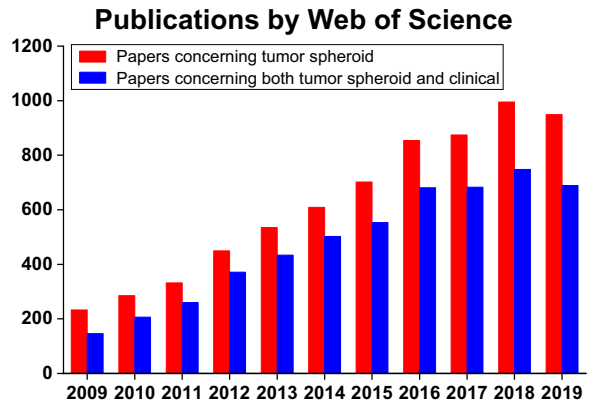

Fig. 2 The number of papers published over the past decade (2009–2019) with regard to multicellular tumor spheroid and clinical. The figure shows the result of searching either keywords "tumor spheroid" or both "tumor spheroid" and "clinical" in Web of Science databases

From metabolic perspective: metabolic disorders in tumor cells

Tumor cells require a large number of substrates and energy for the synthesis of cellular building blocks such as amino acids, nucleotides, lipids and carbohydrates (Zhu and Thompson 2019).

Sugar metabolism

Sugar metabolism in vast majority of tumor cells is pronouncedly increased for provision of carbon-skeleton elements and energy. It has been reported that glucose uptake rate can reach roughly 10 times higher in tumor cells than in normal cells (Cairns et al. 2011) and the expression of hexokinase2 (HK2) was significantly upregulated (Patra et al. 2013). Phosphoinositide 3-kinase (PI3K) can also regulate glucose uptake. PI3K signal transduction through protein kinase B (Akt) can regulate the expression of glucose transporter protein (GLUT1) and enhance glucose uptake (Courtnay et al. 2015). In the 1920s, the German physiologist Otto Warburg discovered the well-known Warburg effect (also known as aerobic glycolysis)—that even when oxygen was abundant, tumor cells do not prefer to metabolize glucose by aerobic oxidation, but by glycolysis (Liberti and Locasale 2016). Aerobic glycolysis in cancers is the combined result of mitochondrial DNA (mtDNA) mutation, oncogenes, tumor suppressors and hypoxic microenvironment. Metabolic enzymes such as phosphofructokinase (PFK) and pyruvate kinase M2 (PKM2) are important regulators of aerobic glycolysis, regulating irreversible and rate-limiting steps (Jang et al. 2013). For example, PFK activity is highly sensitive to pH and decreases with decreasing PH (Erecińska et al. 1995). As evidenced, tumors become more acidic extracellularly and more alkaline intracellularly. Higher pH in cancer cells activates PFK, which then promotes glycolysis (Cardone et al. 2005). The expression of PKM2 can allow proliferating cells to divert glucose into anabolic pathways to support the increased biosynthetic demands; in parallel, the accumulated reactive oxygen species (ROS) in tumor cells can inhibit PKM2 and thus divert glucose flux toward pentose phosphate pathway (PPP), thereby generating sufficient reducing potential for antioxidant responses (Anastasiou et al. 2011). In the presence of normally functioning mitochondria, cancer cells also use aerobic glycolysis instead of mitochondrial respiration to produce energy (Jang et al. 2013). This seems to be a waste of resources, known as overflow metabolism. However, recent studies have revealed that overflow metabolism is an adaptive mechanism because of efficient proteome resource allocation (Basan et al. 2015). Strikingly, lactic acid produced by glycolysis is often considered as metabolic waste, which exerts toxic effects on tumor cells (Martinez-Monge et al. 2019). However, a recent study has concluded that circulating lactic acid can participate in the tricarboxylic acid (TCA) cycle, and its flux is much higher than that of glucose entering the TCA cycle (Hui et al. 2017). In tumors, glucose concentrations are lower than in normal tissues, and Yun et al. found that glucose deprivation in tumors can drive mutations in the kirsten rat sarcoma (KRAS) viral oncogene pathway (Yun et al. 2009). Birsoy et al. developed a continuous culture device (Nutrostat) that simulates low glucose concentrations in tumors, and identified mtDNA mutations as potential biomarkers to identify tumors that are highly sensitive to oxidative phosphorylation (OXPHOS) inhibitors (Birsoy et al. 2014). In addition, other saccharides can also affect tumor progression. For example, Gonzalez et al. observed that tumor-cell growth was reduced and even suspended when mannose was added to cultured cells or orally given to mice xenografted with tumor. The results showed that mannose competes with glucose for transporters and affects the level of anti-apoptotic proteins in tumors, which provides new clues for cancer combination treatment (Gonzalez et al. 2018).

Amino acid metabolism

Amino acid metabolism is not only involved in protein synthesis and turnover but also correlates with nucleotide metabolism in tumor cells. As a source of nitrogen, amino acids provide the raw materials for cells to synthesize proteins, peptides and other nitrogen-containing substances. Generally, glutamine is broken down into α-ketoglutarate which can be used as the hub of other

metabolic pathways, further into saccharide, lipid or some non-essential amino acids, and it can be oxidized to carbon dioxide and water, and generate energy (Palm and Thompson 2017). In tumor cells, due to the malignant proliferation of tumor cells and the need for energy, tumor cells are in great demand for the uptake of glutamine, which is essential for many cellular functions, including biosynthesis, cell signaling, and antioxidant damage protection (Zhu and Thompson 2019). In the absence of adequate supplements of glutamine, tumor cells may undergo growth arrest. However, a recent study has shown that, in the case of glutamine deficiency, mitochondria can significantly alleviate the mortality of tumor cells by consuming aspartate instead (Alkan et al. 2018). Different cancerous cells have different amino acid metabolism. For example, there are different ways of using branched-chain amino acids (BCAAs) by PDAC and non-small cell lung carcinoma (NSCLC), which are both caused by *Kras* and *Trp53* mutations. PDAC increases tissue protein decomposition and thus BCAA concentration in blood, and elevated levels of BCAAs are associated with an over twofold increased risk of pancreatic cancer diagnosis. While in NSCLC, BCAAs can benefit tumor growth through their uptake and transamination (potentially as nitrogen source) rather than subsequent catabolism (Mayers et al. 2016; 2014). More interestingly, Spinelli et al. found that breast cancer cells could recycle waste by-products "ammonia" as a source of nitrogen to promote tumor growth (Spinelli et al. 2017). One carbon metabolism involves the folate and methionine cycles using nutrients from amino acids such as serine and glycine, glucose, glutamine and vitamins to produce lipids, nucleotides, proteins (Locasale 2013). Many studies have shown that some cancer cells rely on serine/glycine uptake to proliferate. Zhang et al. found that in NSCLC glycine decarboxylase (GLDC) induces dramatic changes in glycolysis and glycine/serine metabolism, leading to changes in pyrimidine metabolism that regulate the proliferation of cancer cells (Zhang et al. 2012). However, Labuschagne et al. found that nucleotide synthesis and cancer cell proliferation were supported by serine rather than glycine (Labuschagne et al. 2014). Chaneton et al. found that cancer cells overexpressed the PKM2, allowing more glucose-derived carbon to be introduced into serine biosynthesis to support cancer cell proliferation (Chaneton et al. 2012). Instead, Jain et al. measured 219 metabolites in NCI-60 cancer cell line and determined that glycine consumption was closely related to the expression of mitochondrial glycine biosynthesis pathway and the proliferation rate of cancer cells (Jain et al. 2012).

Lipid metabolism

Most tumors have an abnormally activated lipid metabolism that supports proliferation (Carracedo et al. 2013). Vriens et al. discovered an abnormal fatty acid—sapienate that allows cancer cells to bypass the well-known fatty acid desaturation pathway that relies on the stearoy-CoA-desaturase (SCD) to support the biosynthesis of cancer cell membranes (Vriens et al. 2019). Similarly, Jiang et al. found that in cancer cells deficient in citrate transporters (CTP), inhibition of isocitrate dehydrogenase 1(IDH1) suppresses lipogenesis from either glucose or glutamine, suggesting that IDH1 is an essential component for fatty acid synthesis in the absence of CTP (Jiang et al. 2017). Kamphorst et al. found that hypoxic cells could bypass adipogenesis from de novo and support the growth of cancer cells by scavenging unsaturated fatty acids from lysophospholipids (Kamphorst et al. 2013). Fatty acid metabolism is also an important reservoir of carbon and energy source. The β-oxidation of fatty acid is one of the most important metabolic pathways, producing acetyl-CoA toward further oxidation for provision of ATP and other precursors for tumor metabolism. Acetyl-CoA binding protein can bind to acetyl-CoA and promotes oxidation of fatty acids. It has been reported that in glioma, acetyl-CoA binding protein was overexpressed to promote the β-oxidation of fatty acids (Duman et al. 2019). Generally, fatty acids are synthesized in cytoplasm while acetyl-CoA is produced in mitochondria. Liu et al. conducted experiments on HCT116 cells and found that cells can rely on acetyl-CoA synthetase 2 (ACSS2) in cytoplasm to convert acetic acid into acetyl-CoA, thus supporting the lipogenesis. This makes it a potential target for cancer therapy (Liu et al. 2018). In addition, Camarda et al. also found that triple-negative breast cancer (TNBC) overexpressed *MYC* to increase bioenergy dependence on fatty acid oxidation (FAO), and inhibition of the FAO was a treatment for TNBC with overexpression of the oncogene *MYC* (Camarda et al. 2016).

Nucleic acid metabolism

Compared with other building blocks, nucleic acid is very special because it can hardly be taken from the outside (Zhu and Thompson 2019). Because rapid proliferation requires the synthesis of large amounts of nucleic acids, the polymerase activities of both DNA and RNA synthesis in tumor cells are higher than those in normal cells. Meanwhile, higher synthesis rates require more energy from other sources (Ertel et al. 2006). Accordingly, the nucleic acid decomposition process is also significantly reduced in rapid proliferating cells (Arsenis

et al. 1970). Current cancer treatments use antimetabolites that selectively prevent tumor cells from proliferating and cause minimal damage to normal cells, based on their ability to synthesize large amounts of nucleic acids (Parker 2009). Abnormal nucleotide metabolism may also provide new targets for cancer treatment. Bester et al. found that abnormal activation of retinoblastoma (Rb) E2F pathway (Rb-E2F), which regulates cell proliferation, leads to nucleotide deficiency that affects normal replication and genomic stability (Bester et al. 2011). Zauri et al. found that cytidine deaminase (CDA) is overexpressed in pancreatic cancer. When exposed to 5-hydroxymethyl-2′deoxycytidine (5hmdC) or 5-formy-2′deoxycytidine (5fdC), the CDA converts 5hmdC and 5fdC into modified uracil and inserts it into the DNA. It leads to the accumulation of DNA damage that eventually leads to cell death. Therefore, cancer cells which overexpress CDA can be treated with 5hmdC and 5fdC (Zauri et al. 2015a). Nucleotide production is also regulated by some carcinogenic signaling pathways. Duvel et al. found that mammalian target of rapamycin (mTOR) activation increases ribonucleogenesis. As a result, focusing on nucleotide metabolism in tumors with over-activated mTOR may have unexpected results for cancer therapy (Duvel et al. 2010).

Coenzyme metabolism

Coenzyme binds to the active site of an enzyme to catalyze a reaction (Richter 2013), and many reactions are directly influenced by specific coenzymes, mostly referred to nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleotide phosphate (NADPH) (Alberghina and Gaglio 2014). NADH transfers electrons to the electron transport chain (ETC) (Thapa and Dallmann 2020), and actively participates in mitochondrial oxidative phosphorylation and generates energy, which is the regulator of energy metabolism and redox state (Heiden et al. 2009). ROS is free radical substance that is produced in cells as normal by-products of metabolism (Cairns et al. 2011). However, when cells receive too much nutrition, the production of ROS increases. High content of ROS will cause oxidative damage, and severe oxidative stress leads to cell death (Pike et al. 2011). Cells primarily use NADPH to reduce glutathione (an antioxidant molecule) from its oxidized form (glutathione disulfide, GSSG) to its reduced form (glutathione, GSH) (Harjes et al. 2016). GSH acts as an antioxidant molecule to remove ROS and counteract oxidative damage (Mari et al. 2009). NADPH could maintain the activity and regeneration of GSH (Cairns et al. 2011); NADPH is an important coenzyme produced by PPP and one carbon unit metabolic pathway, which plays an important role in the cellular defense of oxidative stress and signal transduction (Fan et al. 2014; Jo et al. 2001; Pollak et al. 2007). In rapidly proliferating cells, glutamine feeds the TCA cycle to produce NADH and/or NADPH, which are highly needed for lipid and nucleotide biosynthesis (Tong et al. 2009). More and more studies have focused on the biological application of coenzyme perturbation in cancer treatment (Thapa and Dallmann 2020). Pike et al. found that etomox inhibited fatty acid oxidation, reduced NADPH levels, and increased ROS levels in human glioblastoma SF188 cells. This suggested that fatty acid oxidation might provide a source of NADPH that protected cancer cells from oxidative damage and death (Pike et al. 2011). Lukina et al. used autologous fluorescent metabolic coenzyme NAD(P)H as an indicator of the response of cancer cells to paclitaxel. It was also found that NAD(P)H fluorescence lifetime could noninvasively monitor drug-induced metabolic changes (Lukina et al. 2018).

Key features of MTSs

Internal structure of MTSs

Solid tumors are organ-like structures that are heterogeneous and complex (Tredan et al. 2007), mainly showing genetic and phenotypic heterogeneity. Recently, Mao et al. reported a live single-cell extractor (LSCE) based on microfluidic chip, which successfully revealed cell heterogeneity and the relationship between cell adhesion and cell activity (Mao et al. 2018a). MTSs are micron-sized heterogeneous cell aggregates with spherical geometry, which are structural and functional 3D tissues formed by one or multiple types of cells and behave closely to in vivo solid tumors (Fitzgerald et al. 2015). MTSs promote the establishment of nutrients, oxygen and signal factor gradients, and have ECM distribution (Huang and Gao 2018). Due to mass transfer limitations, MTSs usually exhibit oxygen and nutrient gradients, and accumulation of metabolic wastes. MTSs larger than 400–500 μm in diameter can mimic the characteristics of solid tumors, ranging in size from 0.5 to 1 mm^3 (Kunz-Schughart et al. 2004; Lin and Chang 2008; Mehta et al. 2012; Nath and Devi 2016). Representatively, MTSs display a concentric layered structure consisting of a necrotic core, a resting cell layer, and an outer border of proliferating cells (Mehta et al. 2012), which respectively corresponds to the necrotic core and resting cells distant from the blood vessels and the active proliferating tumor cell near the blood vessels in solid tumors (Fig. 3). MTSs retain the physiologic features of solid tumors, as well as the tissue specificity of the original cancer tissue, thus leading to the understanding of the response of tumor to chemotherapy and radiation therapy (Markovitz-Bishitz et al. 2010). More importantly, unique gene expression patterns similar to that observed in solid tumors have been

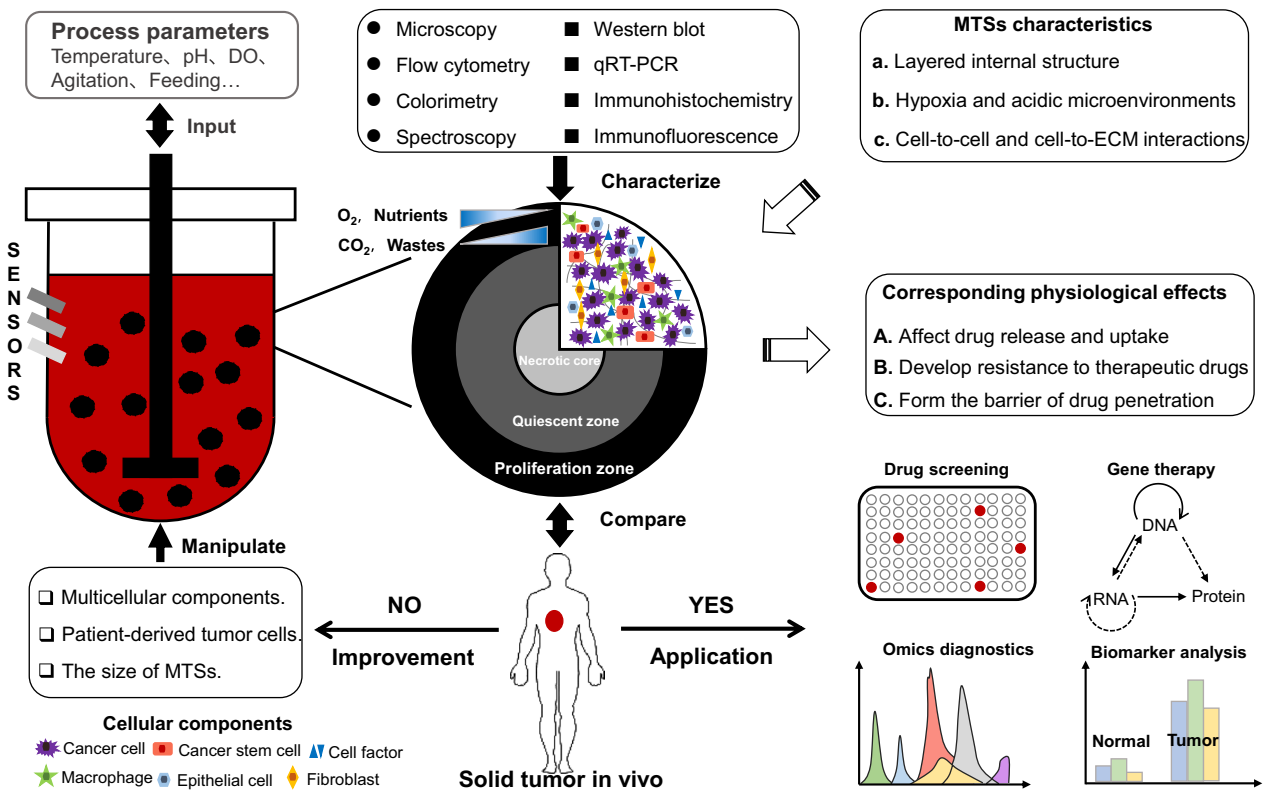

Fig. 3 Adaptable bioreactor culture system can be used for massive production of multicellular tumor spheroids serving to preclinical drug screening and evaluation. Process sensors can aid the real-time observation and regulation of process parameters, and the microenvironment surrounding the MTSs can be manipulated to allow the environment (e.g., nutrients, metabolic wastes and cell types) in the bioreactor can be as close as possible to the microenvironment of tumor in vivo. To ensure that the MTSs generated in the bioreactor can be readily applied to preclinical biomedical research, comparing phenotypic landscapes between these individualized MTSs and solid tumors in patients is a must; the resulting MTSs similar to human solid tumors could be used in drug screening, gene therapy and tumor metabolomics research

reported (Ahn et al. 2019; Däster et al. 2017; Friedrich et al. 2009).

In solid tumors, the tumor cells coexist with stromal cells, including mesenchymal stem cells (MSCs), tumor endothelial cells, tumor infiltrating lymphocytes (TILs), tumor-associated fibroblasts, blood cells, adipocytes and tumor-associated macrophages, which regulate different characteristics of tumor development (Hoffmann et al. 2015; Santo et al. 2017). For example, fibroblasts can affect morphology, invasion and metastasis of tumor cells through cell interaction and paracrine factors (Lazzari et al. 2018), and are the most popular cell type in co-culture (Dolznig et al. 2011). Endothelial cells play an important role in tumor angiogenesis (Weis and Cheresh 2011), while immune cells play a key role in the growth of tumor cells, producing a group of soluble factors that inhibit anti-tumor immune activity (Biswas and Mantovani 2010; Nunes et al. 2019). These stromal cells are non-malignant, but their secreted growth factors and cytokines interact with tumor cells. Such interaction between tumor cells and stromal cells affects the microenvironment of the tumor, promoting tumor angiogenesis, cell proliferation, invasion, metastasis, etc., and as well increasing the resistance of tumor cells to drugs (Dalton 1999). Tumor cells can also reprogram normal cells, making them become tumor-related components, such as cancer-associated fibroblasts (CAFs). CAFs may come from normal fibroblasts, induced by cancer-derived factor. It has been reported that CAF is responsible for the regulation of tumor cell invasion and tumorigenesis (Hirt et al. 2014). In addition, it can promote immune cells to express a variety of cancer-related molecules, such as chemokines, ECM protease, growth factors and cytokines, which play a key role in promoting cancer development and abnormal proliferation of stromal cells, and decrease the absorption of anticancer drugs, which helps to resist traditional chemotherapy (Azarin et al. 2015). Another important key factor produced in the matrix component is transforming growth factor-β (TGF-β). This

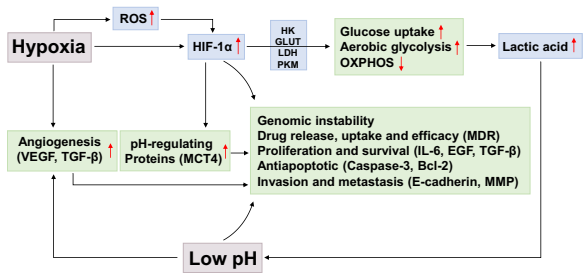

Fig. 4 The effect of hypoxia and acidic microenvironment on the occurrence and development of tumorigenesis. Red arrow denotes the increased metabolic activity. The hypoxic microenvironment in tumors would induce HIF-1α and ROS, which influence the expression of a series of metabolic enzymes, such as HK, GLUT, LDH and PKM, leading to metabolic transformation, increased glucose uptake, enhanced glycolysis, and decreased OXPHOS. The accumulated lactic acid can contribute to forming an acidic microenvironment. Hypoxia and acidic microenvironment will promote angiogenesis, tumor cell growth and apoptosis, drug resistance, and are also related to genomic instability

cytokine plays a decisive role in the membrane-bound form and activates epithelial–mesenchymal transition (EMT) in tumor cells. In addition, it also plays an immunosuppressive role, promoting regulatory T cells (Treg) production and M2 macrophage differentiation (Flavell et al. 2010). Recently, Field et al. found that lipid metabolism plays a vital role in mitochondrial integrity, which is a cell-intrinsic checkpoint for Treg suppressive function (Field et al. 2019). To better study the mechanism of drug resistance and improve drug screening efficiency, co-culture model of tumor cells and stromal cells has been developed, which can well simulate the role of the matrix in tumor tissues in vivo (Xin et al. 2019). Representative MTSs comprised of different cancer types and their ratios can be obtained by adjusting the ratio of inoculation density between tumor cells and stromal cells, preferably based on the heterogeneity found in solid tumors (Costa et al. 2016). Majety et al. found that when tumor cells co-cultured with fibroblasts, their proliferation was promoted due to the increased secretion of epidermal growth factor (EGF), hepatocyte growth factor (HGF) and interleukin-6 (IL-6). It was also found that after co-culture of tumor cells with fibroblasts, the survival of co-culture cells increased by four times than monoculture cells due to the secretion of soluble factors (Majety et al. 2015).

Hypoxia and acidic microenvironments

In solid tumors, oxygen transport becomes limited as the distance between the vasculature and the cell increases, resulting in gradients of oxygen (Hirt et al. 2014). The average diffusion limit of oxygen in normal tissues is about 100–200 μm (Carmeliet and Jain 2000). Hence, there is likely an area of hypoxia beyond this radius in solid tumors. Similar to human tumors, there are areas of normal and hypoxic in the tumor spheroid with proliferating, resting, and necrotic cells (Riedl et al. 2017). It should be noted that the hypoxia environment in the MTSs is also significant for simulating the cell heterogeneity in the solid tumors (Xin et al. 2019). Nutrients such as glucose and essential amino acids were likely to be reduced in hypoxic regions (Tredan et al. 2007). Hypoxia also induces an increase in vascular endothelial growth factor (VEGF), which is the major factor in promoting angiogenesis, causing proteomic and genomic changes in tumor cells leading to malignant progression (Fig. 4) (Gong et al. 2015; Tian et al. 2010). Relevant report has shown that the up-regulated expression of cell hypoxia inducible factor (HIF) was found in the MTSs while no expression of HIF-1α was observed in 2D cells cultured (Tian et al. 2010). HIF-1α regulates the expression of many target genes that play roles in tumor malignancy (proliferation, invasion, and metastasis) (Tian et al. 2010). HIF-1α also could induce metabolic transformation of tumor cells by regulating the expression of genes involved in glucose uptake, glycolytic pathways, and glutamine consumption (Fig. 4) (Nath and Devi 2016).

Hypoxia also promotes incomplete aerobic glycolysis and nutrient oxidation pathways, which aggravates lactic acid and produces an acidic tumor microenvironment (pH 6.5–7.2) (Alvarez-Pérez et al. 2005). HIF increases the expression of many pH-regulating proteins to which cancer cells are sensitive (McIntyre et al. 2016). Carlsson and Acker observed that the deepest part of the cell spheroid has the lowest pH and showed the worst drug uptake (Carlsson and Acker 1988). pH gradient and hypoxia in 3D solid tumor affect drug release and multidrug-resistance (MDR) gene expression patterns, and also affect drug uptake rate and efficacy (Huang and Gao 2018; Xin and Yang 2019). Hypoxia environment can support the development of resistance to therapeutic agents, making cells less sensitive to cancer drug therapy (Maeda 2001). MTSs' necrosis core produced in anoxic environment could also influence the chemical resistance of tumor cells. As a kind of central pressure sensor, p53 can regulate cell apoptosis and activate cell necrosis pathway at the response of oxidative stress (Vaseva et al. 2012). Lee et al. found that p53 protein was clustered in MTSs with a necrotic core, where 5-fluorouracil (5-FU)-induced apoptosis was significantly reduced compared to the cells without a necrotic core (Lee et al. 2010). The acidic environment in the tumor microenvironment can affect the cytotoxicity of anticancer drugs and may

inhibit the active transport of some drugs, such as doxorubicin (DOX), methotrexate (Nunes et al. 2019; Tredan et al. 2007). pH heterogeneity inhibits drug uptake rates by hindering molecular diffusion (Xin et al. 2019). It has been reported that MTSs are more resistant to DOX and radiation than traditional 2D cultures, and even small aggregates of 25–50 cells show stronger drug and radiotherapy resistance than monolayer cells (Yu et al. 2010). Däster and colleagues constructed colorectal cancer (CRC) cell spheroids of different sizes and analyzed the spatiotemporal gene expression patterns, and the sensitivity to the 5-FU. It was observed that MTSs with hypoxia and multicellular tumor necrosis areas displayed closer gene expression landscape to tumors in vivo (Däster et al. 2017). Likewise, Fan et al. used magnetic 3D bio-printing (M3DB) to generate lung (A549) and PANC tumor spheroids; the results showed that tumor spheroids were more resistant than 2D counterparts to the anticancer agent, selenite (Fan et al. 2018).

Cell-to-cell and cell-to-ECM interactions

The interactions between tumor cells, and interactions between tumor cells and various cytokines, growth factors, and ECM can affect cell sensitivity to apoptosis and response to chemotherapy (Tredan et al. 2007). Moreover, these interactions contribute to cancer progression, invasion and metastasis, which may be a new promising target for cancer treatment. Therefore, the number of research literatures focusing on cell interaction in tumor microenvironment is growing rapidly (Xin et al. 2019). Cell-to-cell interactions are more pronounced in MTSs than that in 2D cell cultures (Sakthivel et al. 2019). In 3D spheroids, all cells are in close contact that is necessary for tumor development and progression. These interactions alter signaling pathways in tumor cells and stromal cells (Riedl et al. 2017). MTSs replicate the physical communication and signaling pathways observed in solid tumors (Hanahan and Coussens 2012). Such interactions between tumor and surrounding stromal cells are also closely related to the sensitivity of anti-tumor drugs (Xin and Yang 2019). Some reports have also shown that both nonmalignant and aging cells interact with cancer cells in the tumor microenvironment, which may affect tumor metastasis and treatment response (Craig et al. 2019; Fane and Weeraratna 2019). Cell–cell and cell–ECM interactions in multilayered tumor spheroids constitute a permeability barrier through which anticancer drugs must pass (Sant and Johnston 2017). These cell–cell interactions also promote tumor growth, inducing tumor cell phenotypes and gene expression patterns that are different from those found in single-cell cultures (Huang and Gao 2018). The tumor microenvironment plays an important role in cell differentiation, which greatly affects the treatment efficiency. To simulate tumor tissue heterogeneity, a variety of MTSs based on co-culturing with fibroblasts, endothelial cells, or immune cells or multiple of them have been developed (Hirschhaeuser et al. 2010). For instance, Lazzari et al. constructed non-stented MTSs including PANC-1, fibroblasts (MRC-5), and endothelial cells (HUVEC), demonstrating that complex microenvironment reduces tumor cell chemotherapy sensitivity, which is similar to that observed in in vivo treatments. Hence, it can be used for drug screening regarding pancreatic cancer (Lazzari et al. 2018). Moreover, Costa et al. also found that when Hela and MCF-7 cell spheroids were cultured alone, there were less cells in the core of the spheroids. Solid tumors in vivo could not be simulated without the restriction of nutrients transport. However, when the cancer cells were co-cultured with human fibroblasts (hFIB), dense necrotic cores in the tumor spheroids could be observed (Costa et al. 2014).

The natural microenvironment of the tumor consists of the ECM, the vascular system and the supporting stromal cells. ECM consists of fibrous reticulum proteins (e.g., laminin, tenascin, collagen, fibronectin, and elastin) and proteoglycans (e.g., glycosaminoglycans), which fill the space around the cells and help cells connect to each other through adhesion proteins. ECM components are also involved in various cell signaling pathways (Fennema et al. 2013), promoting tumor growth and drug resistance (Santagiuliana et al. 2015). The formation of tumor blood vessels around tumor cells also requires ECM proteins and the proliferation of endothelial cells (Lazzari et al. 2018). In tumors, ECM dynamics often become abnormal and deposition of ECM proteins increases (Costa et al. 2016; Nunes et al. 2019). ECM acts on cell surface receptors and induces the expression of several anti-apoptotic genes (e.g., *Bcl-2*) that are beneficial to cell survival, promoting tumor proliferation (Gilmore et al. 2000). Extracellular molecules secreted by ECM also play an important role in the development and metastasis of tumor cells (Saglam-Metiner et al. 2019). It should be noted that not all tumor cells can form spheroids in vitro, for example, cell lines such as SK-BR-3 and suspension cell lines, even in the presence of ECM, are difficult to form MTSs (Huang and Gao 2018). Moreover, ECM plays an important role in controlling proliferation, apoptosis, metabolism and differentiation of tumor cells (Asghar et al. 2015). ECM also serves as a key barrier to the delivery of anti-tumor drugs. The collagenous scaffolds in the ECM make anticancer drugs adsorbed on it, thus affecting the delivery of drugs (Xin and Yang 2019). The physical interaction and the deposition of ECM proteins increase the density of the spheroids and reduce the penetration of the drug (Minchinton and Tannock

2006). Therefore, in the treatment of cancer, it is considered to reduce the interaction of ECM protein in tumor cells, which may promote the penetration of drugs and enhance the therapeutic effect of anticancer drugs on tumor cells. In glioma cells, when tumor cells were cultured as MTSs, the expression of ECM protein was more obvious than that in 2D culture (Glimelius et al. 1988). 2D cultured tumor cells lack the ability to correctly simulate tumor matrix heterogeneity and tumor-ECM components, so there are differences in drug sensitivity and toxicity (Saglam-Metiner et al. 2019). ECM has also been reported to play an important role in controlling the key parameters of MTSs, such as pH, oxygen and nutrient concentration gradient, cell morphology and size (Ferreira et al. 2018). Therefore, reproducing the interactions between different cells and ECM in the tumor microenvironment is critical to understanding the mechanisms of cancer (Rebelo et al. 2018). The establishment of such ECM protein environment that is reproducible and close to solid tumor in MTSs requires many operational key parameters, including various cellular components of the tumor stroma and cell type ratios, the type of medium, the size of 3D spheroid, etc. (Fig. 3). The purpose of controlling these parameters is to make the microenvironment of the MTS model as close as possible to the case of solid tumors (Ferreira et al. 2018). Mass transfer limitations caused by ECM, cell–cell interaction, and chemical gradients lead to poor drug penetration and increase cell spheroids' anticancer resistance (Tredan et al. 2007). Overall, these results highlight the importance of simulating the tumor microenvironment.

Classification and preparation of MTSs

Classification of MTSs

Single-cell spheroid model

MTSs can be divided into two modes: single cell culture and co-culture. Single-cell spheroids' culture, which is simple to operate and inexpensive, helps us understand the changes in the physiological metabolism of tumor cells under hypoxic and pH gradient conditions, as well as the effects of cell–cell and cell–matrix interactions in the radiation resistance and chemotherapy (Shield et al. 2009). As an example, Gong et al. cultured MCF-7 MTSs on homemade agarose scaffolds and evaluated the drug permeation behavior, finding that MTSs' resistance to DOX was higher than the corresponding 2D culture cells (Gong et al. 2015). However, the culture system of single-cell tumor spheroids is lacking of heterogeneous stroma, which can be well addressed by co-culturing tumor cells with immune cells, fibroblasts, mesenchymal stem cells, other stromal cells or several of them (Rodrigues et al. 2018).

Co-culture tumor spheroid model

In tumor tissues, there are various types of cells that play different roles in tumor (Huang and Gao 2018). Compared with monolayer culture, a variety of cell lines such as fibroblasts, macrophages and endothelial cells and mesenchymal stem cells when co-cultured with tumor cells would alter their phenotypic characteristics, motility, cytokines produced patterns and differentiation ability. Eventually, it may promote tumor cell proliferation and metastasis (Hauptmann et al. 1993).

Co-culture can simulate a series of intercellular interactions, such as physical support between different cells in vivo, utilization of growth factors, and circulation of metabolites. Co-culture is widely used in the MTSs, which could better mimic the response of tumor anticancer drugs (Karacali et al. 2007). Xin et al. developed a co-culture MTS model including breast tumor cell lines (MCF-7) and mouse fibroblasts (NIH-3T3) which successfully evaluated the efficacy and cytotoxicity of several anti-tumor agents, demonstrating the potential value of this 3D co-culture model for cancer drug screening (Xin and Yang 2019).

Many co-culture models have been developed; cancer stem cell (CSC) and tumor cell co-culture models contribute to tumor growth and metastasis (Burke et al. 2012). In lung cancer, the presence of macrophages promotes the metastatic phenotype due to the excretion of both matrix metalloproteinase-1 (MMP-1) and VEGF (Liu et al. 2016). Endothelial cells (ECs) are important in tumor angiogenesis and metastasis, so it may be an important target of anticancer drugs. Accordingly, Jyoti et al. optimized the liposome-wrapped topotecan low-dose chemotherapy (LDMC) which targets to tumor endothelial cells (Jyoti et al. 2015). When homotypic Caco-2 and DLD-1 MTSs were co-cultured with freshly isolated peripheral blood mononuclear cell (PBMC), immune cells were found to adhere to the spheroids, and the co-cultured MTSs were more resistant to 5-FU + oxaliplatin (FO) than the single cell-derived MTSs (Hoffmann et al. 2015). Further, Lazzari et al. developed an MTS model contained PANC-1, MRC-5 and HUVEC, which showed more resistance to drugs than the MTS co-culture tumor cells with fibroblasts or with endothelial cells (Lazzari et al. 2018). However, it is generally recommended to use up to three cell types in co-culture, as many cell types would make the system complex and difficult to process (Sakthivel et al. 2019). At present, the co-culture methods are mainly divided into three types:

i. Co-culture with direct contact: Direct-contact co-culture is simple to operate, in which stromal cells and tumor cells are uniformly mixed in a medium and inoculated into a non-adhesive multi-well

plate or a 3D scaffold to form MTSs. Although the direct-contact co-culture operation is simple and maximizes the heterogeneous cell interaction (Sakthivel et al. 2019), it is difficult to accurately control the morphology of the cell spheroid, and it is not possible to clearly distinguish the function of each cell type (Xin et al. 2019).

ii. Co-culture with semi-contact: Instead of a mixture of homogeneous cells in direct contact, MTSs comprised only of tumor cells are inoculated into a 3D scaffold embedded with stromal cells to form a semi-contact co-culture model. Weis and Cheresh inoculated human colon tumor cell lines to a non-viscous porous plate to grow into MTSs, and then co-cultured MTSs with fibroblasts in 3D collagen gel (Weis and Cheresh 2011). There is another simpler "semi-contact" co-culture, in which one type of cell is placed directly on top of a 3D scaffold mixed with another type of cell to form a semi-contact model (Xin et al. 2019). In the direct-contact model, the co-cultured cells are a uniform mixture, while the semi-contact method can overcome this shortcoming. Semi-contact co-culture is advantageous for the study of tumor invasion, which is very similar to the cell structure of tumor tissues, allowing the assessment of the contact interaction between tumor cells and surrounding stromal cells (Horie et al. 2015). However, the inoculation process is cumbersome, and it is difficult to control the shape of the spheroid and prevent physical contact of heterogeneous cell populations if the cells are co-cultured for a long period of time (Sakthivel et al. 2019).

iii. Co-culture without cell–cell contact: Non-contact co-culture tumor models are also widely used. Cells are cultured in separate 3D layers or chambers without contact between cells and physical adhesion to each other. It has better spatiotemporal control in cell interaction and any interaction is due to chemical secretion. Transwell and microfluidics are two of the most common measurement platforms for non-contact co-culture which have been used in tissue or organ engineering, drug screening and disease research (Moorst and Dass 2011; Sakthivel et al. 2019).

Recent advances in cell biology, bioengineering and tissue engineering methods have facilitated the development of MTSs (Santo et al. 2016). These methods have their own advantages and disadvantages and application fields (Table 2), promoting the rapid development of new clinical medicine, and providing an innovative platform for tumor biology research and drug screening tests (Huang et al. 2013).

Preparation of MTSs

Formation methods with scaffold

Traditional scaffold Conventional 3D cell cultures with scaffolds use solid materials that are similar to the human solid ECM, which can be characterized by charge, chemistry, hydrophobicity, porosity, surface area, stiffness, and porous interconnectivity (Ferreira et al. 2018). The control of these properties is critical for cell migration and proliferation, as well as for the exchange of nutrients, gases, and wastes, similar to what has been observed in vivo (Costa et al. 2018). These scaffolds provide proper physical support for cell growth by adhering to the surface, thus contributing to MTSs' formation and resistance to the external environment (Saglam-Metiner et al. 2019).

Traditional scaffolds are mainly divided into natural scaffolds and artificial scaffolds. The most commonly used materials in scaffolds are polymers (natural or synthetic) because of the ability to control their chemical and structural properties. Natural biopolymers include materials obtained from nature, such as silk, gelatin, alginate and ECM components (such as collagen, chitosan, hydrogels, fibrin, and hyaluronic acid) (Correia and Bissell 2012). These natural biomaterials are biocompatible and contain cell adhesion sites, endogenous chemokines and growth factors, which help improve the viability of cells. Natural materials are widely used for 3D cell culture as scaffolds. The type of hydrogel most commonly used to produce MTSs is Matrigel^{TM} (Ivascu and Kubbies 2006), a hydrogel matrix composed of basement membrane extracts obtained from Engelbreth-Holm-Swarm mice tumors. Researchers cultured breast tumor cells (MCF-7) in chitosan–collagen–alginate (CCA) scaffolds to study immunosuppression. The resulting MTSs can also be used to evaluate the resistance of anticancer drugs. It was found that the resistance to anticancer drugs in the MTS model was higher than 2D cell culture model (Wang et al. 2016). Godugu et al. used alginate scaffolds to manufacture MTSs and applied them in anticancer drug research (Godugu et al. 2013). Xin et al. co-cultured human lung adenocarcinoma cells (HCC), macrophages and lung fibroblasts in 3D collagen gel to study their synergistic effects on the secretion of MMP-1 and VEGF (Xin et al. 2019). However, since most of these natural materials are animal sources, significant batch differences, high cost and unknown ingredients are present (Sultana et al. 2015).

The 3D structure of the artificial scaffold can be made of materials such as ceramics, glass, polymers, and metals. Outstandingly, polymers are generally biologically active, biocompatible and biodegradable. For clearly

Table 2 Different techniques used for MTS formation and representative applications of the MTS in biomedical research

Methods	Concept	Advantages	Disadvantages	Cell types	Results	References
Scaffold-based	Cells adsorb and migrate into solid materials to form micro-tissues.	The simplicity of fabrication and the diversity of materials Appropriate physical support Resist the outside environment	Harvesting cells from the scaffold is difficult and possibly harmful to the cells. Suitable for tissue engineering research, not for drug screening.	Lung adenocarcinoma cells (HCC), macrophages and lung fibroblasts	Cocultivation promoted the metastatic phenotype due to the excretion of both matrix metalloproteinase-1(MMP-1) and vascular endothelial growth factor (VEGF).	(Liu et al. 2016)
				Non-small cell lung cancer (NSCLC)	Compared with 2D culture model, IC50 value of anticancer drugs in MTSs was significantly increased.	(Godugu et al. 2013)
				Breast cancer cells (MCF-7)	Compared with monolayer cells, 3D MTSs-CCA system was superior to 2D culture system in anticancer drug screening.	(Wang et al. 2016)
Hanging drop	Cells form a single cluster by dropping a cell suspension onto an inverted glass covering surface by gravity.	Mild conditions Simple materials Without shear force Easy to control the number, size and shape of spheroids	Medium changed frequently Time-consuming and labor-intensive Hard to scale-up Limited applicability in drug screening Limited culture time	Colon cancer cells (HT-29)	Encapsulation of anticancer drugs in liposomes could improve the therapeutic effect.	(Galateanu et al. 2016)
				Malignant melanoma cells (A375)	MTS was an effective tool for investigating the biological effects of oligonucleotides.	(Carver et al. 2014)
				Pulmonary epithelial cells (EPI), pulmonary vascular endothelial cells (ENDO) and human bone marrow mesenchymal stem cells (MSC)	Compared with 2D culture, the expression of ROS and ABCB1 was enhanced and drug resistance was increased.	(Lamichhane et al. 2016)
Liquid covering	Cells aggregate on non-adherent plates.	Easy to operate Low cost Large-scale production High-throughput screening of drugs	Poor consistency Static culture Difficult to guarantee cell activity	Lung cancer cell lines, breast cancer cell lines, pancreatic cancer cell lines and fibroblasts (MRC5)	After co-culture of tumor cells with fibroblasts, the survival of co-culture cells increased by four times than monoculture cells due to the secretion of soluble factors.	(Majety et al. 2015)

Table 2 (continued)

Methods	Concept	Advantages	Disadvantages	Cell types	Results	References
				Pancreatic cancer cells (PANC-1), fibroblasts (MRC-5) and endothelial cells (HUVEC)	The complex microenvironment reduced chemotherapy sensitivity. MTSs could be used in drug screening for pancreatic cancer.	(Lazzari et al. 2018)
				Breast cancer cells (MCF-7), cervical cancer cells (Hela) and primary normal human skin fibroblasts (hFIB)	When the cancer cells were co-cultured with hFIB, dense necrotic cores in the tumor spheroids could be observed.	(Costa et al. 2014)
				Breast cancer cells (MCF-7) and mouse fibroblasts (NIH-3T3)	When co-cultured with NIH-3T3, the resistance or IC50 of MCF-7 significantly increased.	(Xin and Yang 2019)
				U-87 MG glioblastoma and other 40 tumor cell lines	2D and 3D culture models exhibited different sensitivities to targeted drugs.	(Vinci et al. 2012)
				Colorectal cancer (CRC) cells	MTSs with hypoxia and multicellular tumor necrosis areas displayed closer gene expression landscape to tumors in vivo.	(Däster et al. 2017)
				Ovarian cancer cells (NCI-ADR-RES)	Targeted modified drugs were more likely to cross the permeable barrier and accumulate in spheroid.	(Perche et al. 2012)
				Epithelial ovarian cancer (EOC) cells	The response of MTSs to anticancer drugs reduced compared with monolayer culture.	(Liao et al. 2014)
				Colon cancer cells (Caco-2 and DLD-1) and Peripheral blood mononuclear cell (PBMC)	Co-cultured tumor cells were more resistant to 5-FU/Oxaliplatin (FO) than single cultured tumor cells.	(Hoffmann et al. 2015)

Table 2 (continued)

Methods	Concept	Advantages	Disadvantages	Cell types	Results	References
Micromachining wells	Cells cluster in a matrix of micromachining wells.	High reproducibility Uniform size and shape High-throughput mechanized production	High cost Specific device	Breast cancer cells (LCC6/Her-2)	Compared with traditional monolayer culture, tumor spheroids showed resistance to dose-dependent responses to DOX and anticancer drugs.	(Yu et al. 2010)
				Breast cancer cells (MCF-7)	MTSs retained the physiologic features of solid tumors, thus leading to the understanding of the response of tumor to chemotherapy and radiation therapy.	(Markovitz-Bishitz et al. 2010)
Dynamic techniques based on agitation	Cells aggregate by continuous stirring in specific reactors.	Suitable for long-term culture Easy to operate Large-scale industrialization Controllable condition	Shear force affects cells. Difficult to control shape, size and quantity Specific device	Hepatocellular carcinoma cells (Huh7) and endothelial cells (HUVEC)	The co-cultured spheroids were more resistant to anticancer drugs (Adriamycin and Sorafenib) than the monolayer cells.	(Jung et al. 2017)
				Colon cancer cells (HT-29)	Glycolysis, TCA cycle and lipid metabolism-related protein expression from inside MTSs was higher than outside MTSs.	(McMahon et al. 2012)

defined polymer scaffolds, they can accurately simulate the ECM environment and biophysical characteristics, and effectively optimize the defects of natural scaffolds, such as the variability between batches and the lack of precise control over the mechanical properties (Gill et al. 2012). However, separating cells from these materials is relatively complex, and the experimental methods used to harvest cells from the scaffold may be toxic to the cells (Tevis et al. 2017).

Emerging scaffold In addition to the use of traditional scaffold methods to culture MTSs, several emerging scaffold technologies are developed, such as 3D cell printing technology, particles and microcapsules, and organ chips. 3D bio-printing technology refers to the simultaneous printing of cells, biomaterials and scaffolds with the help of computer-assisted transfer programs. The decellularized matrix components used in bio-printing are called "bio-inks". 3D cell printing technology is often used to design and construct tissue structures, such as high-throughput analysis, tissue engineering, drug screening, and drug delivery. Especially in the field of organ transplantation and cancer model, this technique is widely used (Costa et al. 2018). However, the technique does not well construct the vascular structures in tissues, and cells using this technique often cannot grow and function properly (Fang and Eglen 2017). While most of the MTS models still lack a complete representation of tumor specificity and specific ECM of disease stage. To overcome these limitations, various studies involving ECM mimics in the form of spherical scaffolds, such as microparticles or microcapsules, have been attempted. These techniques are widely used in tissue engineering and stem cell research (Kang et al. 2014). The organ chip is a derivative technology of microfluidic chip, which serves to conduct micro-modeling of human organ structure on microfluidic cell culture chip, and to simulate the organ system activities, mechanical characteristics and physiological reactions. The biggest advantage of using this technology is to regulate the cell shape, function, location and physical and chemical microenvironment precisely (Sakalem and Ribeiro-Paes 2018). Organ chips have gradually replaced animal experiments and 2D culture for drug development and screening. The system of liver, lung, skin and kidney has been successfully developed (Kimura et al. 2018). The technology currently has broad research and development prospects, and a variety of functional organ chips are being developed gradually. However, the injection on the organ chip is mostly done by hand, with low efficiency and poor reliability, which affects the vitality of cells and the real-time detection of cell biological characteristics (Bhise et al. 2014).

In conclusion, despite the simplicity of fabrication and the diversity of materials, the cell spheroids supported by the scaffolds are often only used in tissue engineering studies. These models are not suitable for drug testing, not only because of their laborious and expensive techniques, but also because they cannot well reflect the cell growth in vivo. The concentration of drugs, growth factors and other metabolites cannot be adjusted in real time in scaffold-based tumor spheroid model. To aggravate this, many external factors interfere with the production process of scaffolds (such as electrospinning and melting molding), resulting in low repeatability of scaffolds. Most of the literatures on 3D tumor culture in vitro are based on gel scaffolds, which reflect the interaction of 3D cells in specific aspects. However, due to the presence of scaffolds, they are not very close to the microenvironment of solid tumors (Nunes et al. 2019). Therefore, the spheroids-forming method with scaffolds is gradually being replaced by the spheroids-forming method without scaffolds.

MTS formation methods without scaffold

The scaffold-free method achieves under non-adherent conditions and promotes the production of MTSs or microscopic tissues (cell aggregates) (Ferreira et al. 2018). The MTS model was originally proposed by Sutherland and colleagues in the 1970s (Sutherland et al. 1971). There are many 3D culture methods without scaffold, which are mainly divided into static culture method and dynamic culture method (Lin and Chang 2008).

There are two main types of static culture methods: the hanging drop method and the liquid covering method. Dynamic cell culture methods are divided into a rotating system and a microfluidic system depending on the device and material. The rotating systems mainly include rotating bottles, roller bottles, cyclotron oscillators, and bioreactors. By contrast, dynamic cell culture can be performed stably for a long time with good cell activity.

Hanging drop method The hanging drop method forms a single cluster by dropping a cell suspension onto the surface of an inverted glass covering surface by gravity, and then forming cell spheroids (Kelm et al. 2003). The hanging drop method is mild, cheap and easy to achieve. It is a good method to explore the conditions of spheroid generation and suitable for small-scale experiments. Compared with the stirring method, the hanging drop method has no shear force interference, and can better control the number, size and shape of cell spheroids (Mehta et al. 2012). However, the flux of this method is relatively low, so it is difficult to allow mass production for preclinical drug testing and evaluation (Froehlich et al. 2016). The cells in the microspheroids are difficult to culture for long time

due to contamination and evaporation. Therefore, the repeatability of this method should also be considered. To overcome these difficulties, the suspension drop method was combined with bio-printing technology to produce repeatable, uniform and controllable MTSs (Asghar et al. 2015). There is also a method combining suspension drop method and rotation method. Jung et al. developed and optimized a tool—spheroid-forming unit (SFU) containing a tube and filter cap, which successfully produced large and homogeneous hepatoma Huh7 cell spheroids (Jung et al. 2017).

Liquid covering method The development of liquid covering method forms normal and tumor cell spheroids, maintaining their functional activity (Hamilton et al. 2001). Cell spheroids are cultured in non-adherent porous plates, such as poly-(2-hydroxyethyl methacrylate)- or agarose-coated superhydrophobic surface porous plates, to prevent adhesion to the surface of the culture plate. This treatment promotes intercellular interaction, intercellular adhesion and aggregation, then forming MTSs and secreting ECM, which further makes these models similar to solid tumors in vivo (Yuhas et al. 1977). For example, Costa et al. produced MCF-7 and Hela multicellular tumor spheroids by liquid covering method based on agarose mulched low attached plate (Costa et al. 2014). Some use better biological materials instead of agarose to make low adhesion for better biocompatibility. For example, Vinci et al. used 96-well Ultra-low attachment (ULA) microporous plates to generate 3D tumor spheroids (Vinci et al. 2012). Ivascu and Kubbies reported a method of forming MTSs using 96-well plates coated with poly-HEMA (Ivascu and Kubbies 2006). Markovitz-Bishitz et al. have developed a porous platform, which is an array of microchambers with UV adhesive microstructure and can form a large number of cell spheroids (Markovitz-Bishitz et al. 2010). Lazzari et al. reported that three kinds of cells PANC-1, MRC-5 and HUVEC were inoculated to the coated plate of poly-HEMA at a ratio of 1:9:4 to form a tumor spheroid model (Lazzari et al. 2018). This is one of the simplest techniques available for the production of cell spheroids and is easy to operate at a lower cost. Therefore, high-throughput screening of drugs can also be carried out after successful MTS formation. The MTS models of breast and cervical cancer made by this method can be used to test the delivery system of cancer treatment (Costa et al. 2014). Nonetheless, the main disadvantage is that the consistency of cell spheroids is not easy to be controlled, and both cells and culture medium are in static state. During long-term culture, metabolites may have an impact on cell migration and activity. For instance, accumulated lactic acid may be toxic to cells and inhibit cell proliferation (Katt et al. 2016).

Micromachining wells The traditional MTS model combined with spherical structured biomaterials produces the opportunity to generalize complex cell–extracellular matrix interactions and tumor localization (Ferreira et al. 2018). The cells are clustered in matrix micromachining wells, which have the best reproducibility rate. Since the size of micromachining wells is determined, the size and shape of cells are fixed, and the production is rapid, which is suitable for large-scale cell spheroid production. However, this method is expensive and requires laboratory-specific equipment for high-throughput screening. Therefore, this method has not been widely promoted (Nunes et al. 2019).

Dynamic MTS culturing methods The static culture of MTSs, which was found in the 1950s and early 1960s, was replaced by rotating techniques which could prevent the cells from sticking to the surface of the material by continuous stirring, resulting in more efficient aggregates of cells (Chatzinikolaidou 2016). Dynamic techniques based on agitation can be divided into two categories: (i) stirred tank bioreactor, with the medium propelled internally by rotating the blades; (ii) rotating microgravity bioreactor, with a rotary cell culture system to allow a low shear-force environment. Both methods use mechanical forces to keep cells continuously suspended during the culture (Breslin and O'Driscoll 2013). Recent studies have focused on pelletizing in reactors. Jung et al. developed SFU, combined with rotary culture method, which can produce large (> 3 mm), homogeneous Huh7 spheroids, and it could well control the size (Jung et al. 2017). The advantages of the stirred tank culture system for generating 3D spheroids compared with the static conventional culture system include the ability to efficiently produce a large amount of MTSs for long-term cultivation and the simple operation, so the method is very suitable for clinical and industrial applications (Achilli et al. 2012). It is also possible to customize the cultivation parameters to induce aggregation and to allow the spheroids' culture of cell lines showing different properties and phenotypes. The production of 3D tumor cell models in stirred tank culture systems provides repeatable tools for drug screening and target validation in preclinical oncology research (Santo et al. 2016). In addition, blood flow and shear stress can affect the survival of tumor cells, and cancer cells can also use these physical forces to metastasize to distant places (Follain et al. 2019). In the stirred tank culture systems, shear and flow fields can be manipulated to simulate the action of vascular fluid. However, this method requires complex and relatively expensive instruments, such as cell spinner system or rotating cell culture system (Chan et al. 2013). Similarly, Goodwin et al. cultured BHK-21 cell MTSs under different shear stresses

in a rotating microgravity bioreactor and found that cell damage remained at a low level under simulated microgravity conditions (Goodwin et al. 1993). In addition, another dynamic spheroid-forming technology is based on microfluidics. Several different cells are co-cultured in a microfluid, and the cells are contacted with matrix proteins, collagen or Matrigel to provide nutrients and oxygen, and to remove metabolic waste. Microfluidics can control the size and shape of spheroids according to the size of fluid channel aperture, and can perform real-time imaging monitoring of the formed spheroids, which can be used for drug screening, but special devices such as micromixer, microfilter and microseparator are required (Ferreira et al. 2018). For example, Yu et al. used microfluidics to form LCC6/Her-2 breast tumor spheroids and used the model for DOX cytotoxicity test (Yu et al. 2010).

In summary, presently, the 3D tumor spheroid culture technology still cannot guarantee that the size of each tumor spheroid, the number of cells involved, the tightness and other indicators are completely the same. So, perfecting the culture method and establishing the evaluation standard are problems to be solved now and in the near future. Notably, not all tumor cells can form into spheroids, and different tumor cells have different conditions for spheroid formation. Froehlich et al. found that when inoculated 400 cells, MDA-MB-231 cells could not form into spheroids, while 5000 cells could form into compact MTSs. Similarly, for MCF-7 cells, after addition of 2% poly-HEMA on a conventional low-adhesion plate, small multiple cell clusters coated with 2% poly-HEMA would be formed; and in the V-shaped low-adhesion plate compact cell spheroids surrounded by 2% poly-HEMA can be produced (Froehlich et al. 2016).

Methods for evaluating the characteristics of MTSs

The MTSs obtained by different culture methods require a large number of techniques to study the characteristics of 3D tumor spheroids (Fig. 3), such as morphology, size, protein and gene expression of MTSs, invasion and metastasis potential of tumor cells (Costa et al. 2016). Traditional tools for analyzing MTSs are optical microscopy, scanning electron microscopy (SEM), transmission electron microscopy (TEM), flow cytometry, colorimetric and spectroscopic techniques, Western blot, qRT-PCR, and immunocytochemistry (ICC) or immunohistochemistry (IHC) staining (Asghar et al. 2015; Correia et al. 2018; Huang et al. 2012; Ma et al. 2012; Xin et al. 2019; Xin and Yang 2019).

Microscopy

Microscopy includes optical microscopy, SEM and TEM. Optical microscopy is one of the most commonly used techniques to characterize MTSs. Optical microscopes usually require digital cameras to study the growth of MTSs (Costa et al. 2016). The combination of MTSs with living cell fluorescence microscope allows the study of the subtle interactions between cancer and other cells (Wong and Searson 2017). Costa et al. used the Olympus CX41 inverted optical microscope equipped with Olympus SP-500 UZ digital camera to analyze the formation, growth and morphology of MCF-7 and HeLa MTSs at various magnifications. Images of 3D MTSs obtained at different time points were analyzed by an image analysis software, e.g., ImageJ, and they found that MCF-7 did not easily form spheroids, while HeLa MTSs increased in size over time (Costa et al. 2014). Lazzari et al. achieved the co-culture of PANC-1, MRC-5 and HUVEC cells, and used AxioObserver Z1 inverted microscope for routine tumor spheroid monitoring, which was equipped with CoolSnap HQ2 CCD camera (Lazzari et al. 2018).

Optical microscopy can be widely used to determine cell activity, invasion, adhesion, and migration (Yeon et al. 2013). However, this requires the combination with specific biomarkers to characterize the microenvironment and state of cells. Fluorescence microscope and confocal microscope are different types of optical microscope, which are used in different aspects. Fluorescence microscopy is used combined with cellular permeable dyes such as fluorescent dyes. Calcein AM and propidium iodide (PI) can be used to distinguish the survival or death of cells. Hematoxylin and eosin (H&E) staining agents can be used for histological analysis by optical microscopy (Costa et al. 2016). In addition, fluorescence microscopy is an important tool for analyzing the therapeutic effect of anticancer drugs on MTSs. Fluorescent probes are widely used cell tracking techniques. Using different fluorescent probes, it is easy to detect and distinguish co-cultured cell lines using appropriate fluorescence channels in the microscopy. When spontaneous fluorescent drugs are used (e.g., epirubicin and DOX), the dispersion of the drugs can be captured by fluorescence microscopy.

Confocal laser scanning microscopy (CLSM) can capture the 3D structure of MTSs, but its low penetration depth (about 100–300 μm) limits the imaging of large spheroids. Deep penetration imaging can be improved using a multi-photon microscope (MPM) (Graf and Boppart 2010).

The high magnification and resolution of the electron microscope (scanning or transmission) allows high-resolution images of 3D spheroids (nanoscale). Tissue morphology can be observed more clearly by SEM, which is often used for MTS observation (Costa et al. 2019; Kelm et al. 2003; Koudan et al. 2020). Koudan et al. used different types of cells to construct tissue spheroids (TSs), and images of SEM showed that

TSs would retain specific tissue structures, such as vesicles and microvilli (Koudan et al. 2020). Lee et al. used AIS 1800C electron microscope to perform SEM analysis of A549 MTSs at different magnifications and found that MTSs has microvilli in the outer layer (Lee et al. 2019). Costa et al. also observed that MCF-7:hFIB MTSs have a similar microvilli structure under the Hitachi S-2700 electron microscope (Costa et al. 2014). In addition, TEM is another electron microscope technique that is also widely used in MTSs' analysis. For example, the ultrastructure of HepG2 MTSs obtained by hanging drop method was imagined by TEM. TEM observations revealed cubic and polarized cells, which are structural features of the bile duct (Kelm and Fussenegger 2004).

Beyond that, Schnell et al. developed the hybrid microscope by adding infrared lasers and specialized microscope lenses (called interference objectives) to optical cameras. As a result, it can measure the molecular makeup of tissue to distinguish cell types which may further be used in MTSs (Schnell et al. 2020).

Flow cytometry

Flow cytometry can be used to analyze each cell individually in high speed. Moreover, multiple parameters with high precision can be obtained from one cell at the same time. However, it only works on suspended cells. Therefore, this technique cannot be used for MTSs directly. Instead, it is necessary to digest to obtain single cell suspension, and then stain for flow cytometry analysis (Carver et al. 2014; Khaitan et al. 2006). Flow cytometry can be used to evaluate the penetration of drugs with fluorescence or fluorescent labeling. Carver et al. used this method to study the permeability of oligonucleotide anticancer drugs binding with different nanocarriers such as cationic lipids and polymers (Carver et al. 2014). At present, flow cytometry is mostly used to detect cell cycle (Ivascu and Kubbies 2006; Mao et al. 2018a), activity (Ivascu and Kubbies 2006; Patra et al. 2016), apoptosis (Patra et al. 2016), and specific protein expression (Liao et al. 2014; Mao et al. 2018a), which can be applied to MTSs for drug testing and analysis. Patra et al. combined flow cytometry with microfluidic technology and proved the advantages of combining these two technologies in drug screening (Patra et al. 2016). Flow cytometry can also be used for analysis of other parameters, such as protein expression. For example, Khaitan et al. used membranectin V as a specific staining marker on human glioma MTSs to characterize apoptotic cells (Khaitan et al. 2006).

Colorimetric and spectroscopic techniques

Colorimetric and spectroscopic techniques are also often used in MTSs analysis. Colorimetry technique is used to determine the cell activity of MTSs and evaluate the therapeutic effect of anticancer drugs (Nunes et al. 2019).

Common colorimetric methods for the determination of MTSs include acid phosphatase, MTT, AlamarBlue, and CCK-8 (Kang et al. 2015; Walzl et al. 2014). Colorimetry determines the content of components by comparing or measuring the color depth of a colored solution at a specific wavelength. The principle is enzyme conversion in living cells. In general, absorption is proportional to the number of cells which are metabolically active (Riss et al. 2004). Friedrich et al. found that acid phosphatase assay (APH) was suitable for the activity detection of an MTS in 96-well plates, which did not require spheroids dissociation. In addition, it was linear and highly sensitive which could be used for rapid routine analysis of MTSs' cell activity (Friedrich et al. 2007).

Due to the inherent defects of colorimetry, some better spectroscopic techniques have been developed to replace colorimetry. McMahon et al. used MatriX-Assisted Laser Desorption Ionization (MALDI) analyze differences in protein expression level of different cell layers and found that glycolysis, TCA cycle and lipid metabolism-related protein expression from inside MTSs were higher (McMahon et al. 2012). Stable isotope tracing and isotope dilution mass spectrometry (IDMS) method has been frequently used for quantitative metabolomics analysis. Rusz et al. optimized the sample preparation method (cold methanol extraction method) for MTSs' metabolomics research, and applied liquid chromatography–mass spectrometry (LC–MS) to detect the metabolome of single spheroids efficiently, which is suitable for analysis of anticancer drug metabolism (Rusz et al. 2019). Fan et al. analyzed the differences of central carbon metabolism between MTSs and 2D culture using Ion chromatography-ultra high-resolution Fourier transform-MS (IC-UHR FT-MS) and Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) and found that carbon metabolic pathway in MTSs was much less disturbed than 2D culture after the addition of selenite (Fan et al. 2018).

Western blot and qRT-PCR

Western blot and qRT-PCR are used to evaluate protein and gene expression in MTSs, respectively. Western blot is used to detect the expression of specific proteins, and it is often used for MTSs' protein level expression detection (Badea et al. 2019; Ivascu and Kubbies 2006). The main advantage of Western blot is its high sensitivity, so it can detect protein at very low concentrations. Western blot has been used in the analysis of MTSs. For instance,

McMahon et al. confirmed the expression changes of five proteins of the stratified HT29 colon carcinoma MTS by Western blot and found that protein expression in different cell layers in MTSs was significantly different (McMahon et al. 2012). In addition to this, quantitative proteome analysis is on the rise in the field of tumor cell research. For example, Johansson et al. presented an unbiased analysis of the breast cancer (BC) proteome, quantifying 9995 proteins including BC subtypes of tumors (Johansson et al. 2019).

qRT-PCR technique quantifies gene expression by synthesizing complementary DNA transcripts from mRNA and it aids in deciphering the mechanism of tumor metastatic and chemotherapy-resistant from gene level. It was found that hypoxia-induced genes encoding, such as VEGF, integrin β1, CD44, and fibronectin increased in MTSs (Gong et al. 2015; Nishikawa et al. 2017). Liao et al. found that ovarian cancer spheroid cells overexpressed stem cell-related genes encoding such as Notch1, Nanog, Cdcp1, CD34 and Myc by PCR (Liao et al. 2014). Ahn et al. developed a spheroid-forming method that could provide vertical bidirectional flow and found that it promoted liver gene expression by qPCR compared with static and orbital shaking methods (Ahn et al. 2019).

Immunohistochemistry and Immunofluorescence

In addition to the above methods, immunohistochemistry (IHC) and immunofluorescence (IF) are also commonly used to describe the characteristics of MTSs. Serial trysinization is an option but the operation is difficult, time-consuming and laborious. IHC and IF both belong to the immune technology, which can select antibodies according to the needs. Through the chromogenic technology, usually chemical chromogenic and fluorescent chromogenic, cell state in different layers in the MTSs could be clearly displayed. These two methods are based on visualizing the binding of antigen to antibody. When the resulting report label is enzymatic, it is IHC; if it is fluorescent, it is IF (Odell and Cook 2013; Taylor et al. 2013). Through tissue sections, phenotypic differences in different cell layers of MTSs can be visually detected. For example, Ki67, GLUT1, and E-cadherin are mainly expressed in the outer layer of MTSs, and VEGF or vWF-positive cells are mainly distributed in the inside MTSs (Lee et al. 2019). Hypoxyprobe™-1 Kit has been developed for the visual detection of hypoxic areas, and can be applied to the detection of hypoxic regions in MTSs (Lamichhane et al. 2016). Vinci et al. found that the HE immunohistochemistry staining of U87 MTSs grown on ULA plates or agar was uniform and discovered differential sensitivities to targeted drugs between 2D and 3D cultures (Vinci et al. 2012). Compared with 2D culture, MTSs overexpressed HIF-1α, cleaved caspase 3 (cC3), fibrillary fibronectin (FN), intermediate filament proteins, cytokeratin-18 (CK18) and vimentin (Däster et al. 2017; Lamichhane et al. 2016). Däster et al. found a significant hypoxia gradient in the MTSs of CRC cells by immunohistochemically staining with HIF-1α (Däster et al. 2017). Abecasis et al. found that cell proliferation in the outer layer of MTSs was better in human-induced pluripotent stem cells (hiPSC) spheroids using Ki67 immunofluorescence staining (Abecasis et al. 2017).

Application of MTSs for biomedical research and clinical drug evaluation

The MTSs model has been developing rapidly in recent years, and its application in drug screening and cancer mechanism research has been growing with remarkable effects (Fig. 3). Compared with the traditional 2D cell culture model, cell proliferation, differentiation, migration and signal transduction are better simulated (Badea et al. 2019). Although the number of potential anticancer therapies was numerous in recent years, the approval rate of the entire clinical drug development process is very low, and the high attrition in the phase I clinical trial is up to 95% (Breslin and O'Driscoll 2013; Santo et al. 2016). MTSs are compact aggregates of cells, which resemble the structure of solid tumors, avascular nodules, or tumor tissues near capillaries (Friedrich et al. 2007). In recent years, MTSs have been continuously applied in the screening of tumor therapies. These models have relatively low cost and simple methods to simulate the 3D structure of tumor in vitro (Huang and Gao 2018).

Mao et al. developed a hybrid continuum/agent-based HCT116 spheroid model to simulate the significant features of the tumor microenvironment. By solving the diffusion equation and intracellular reactions, they fitted the cell growth parameters, modeled the whole system, and constructed the model describing the oxygen gradient, glucose transfer and drug diffusion (Mao et al. 2018b). Many literatures have reported that MTSs are superior to 2D models in terms of consistency evaluation between in vitro and in vivo, such as gene expression and transcription landscapes. For example, Däster et al. compared the gene expression profiles in HT29 cells cultured in monolayer (2D), MTSs and mouse models respectively with next-generation sequencing (NGS) technology, and found that the gene expression profiles of MTSs were close to those of xenografts, indicating that MTSs could better simulate the gene expression profiles in vivo (Däster et al. 2017).

MTSs can more accurately describe the effects of anticancer treatment and are widely used in the screening of therapeutic methods, such as chemotherapy (Carver et al. 2014; Chen et al. 2016; Däster et al. 2017; Galateanu

et al. 2016; Lazzari et al. 2017) and radiotherapy (Walenta and Mueller-Klieser 2016). For instance, Galateanu et al. evaluated the effects of liposomes containing folinic acid, oxaliplatin and 5-FU on rectal tumor cells in vitro using the MTS model. The survival rate of rectal tumor cells was found to be significantly reduced, suggesting that drug encapsulation in liposomes may improve the therapeutic effect (Galateanu et al. 2016). As a clinical hotspot, nanomedicines have advantages in preventing degradation, controlling release distribution, targeted transportation, etc., but the permeability is poor in tumor tissues. MTSs can better mimic the diffusion limitations of nanomedicines in vivo. There have been a variety of polymers constructed, which were evaluated in MTSs, demonstrating their role as drug carriers, such as poly (amid amine) (PAMAM), poly-L-lysine, FH-SSL-Nav, PME-(PEG-FA) (Chen et al. 2016; Lazzari et al. 2017).

MTSs can not only be used to evaluate the effect of drugs bound to the carrier, but also to study the efficacy of drugs not covered by the carrier. Oligonucleotides can be highly selective in manipulating gene expression and show promising prospects in the treatment of tumors and other diseases (Bennett and Swayze 2010). Nevertheless, they have limited penetration and distribution in tumor tissues, while cells growing in 2D monolayer do not show this restriction. Therefore, the use of MTSs compensates this deficiency and is more effective in predicting experimental results in vivo. As an example, Carver et al. evaluated the delivery modes of different oligonucleotides using MTSs, and found that the size of the delivery agent significantly affected the permeability of oligonucleotides, proving that MTS is an effective tool for investigating the biological effects of oligonucleotides (Carver et al. 2014).

In addition to single-cell component MTSs, the co-culture MTSs can also be used to study the resistance to anticancer drugs. Lamichhane et al. form a 3D co-cultured cell spheroids model by hanging drop method consisting of lung epithelial (EPI) cells, pulmonary endothelium (ENDO) cells and human bone marrow mesenchymal stem cells. Compared with 2D culture, the expression of ROS and ABCB1 (an efflux transporter associated with drug resistance) was enhanced and drug resistance was increased (Lamichhane et al. 2016). Perche et al. detected increased expression of Bcl-2 in the cell spheroids model of ovarian cancer and showed stronger drug resistance. Targeted modification (tumor cell-specific monoclonal antibody 2C5) on PEG-PE micelles loaded with DOX, demonstrating that targeted modified drugs are easier to accumulate in the cell spheroids through the permeability barrier (Perche et al. 2012). In conclusion, these results support the use of spheroids to evaluate anticancer drug delivery. The distribution of drugs in tumor is heterogeneous. To characterize the permeability distribution of anticancer drugs in tumor, immunofluorescence staining, autoradiography and other techniques for detection markers can be used to trace the distribution of drugs, thereby optimizing the distribution and the penetration of anticancer drugs in tumors (Tredan et al. 2007). In addition, it should be emphasized that resting cells in the cell spheroids have a selective toxicity to anticancer drugs against proliferating cells (Costa et al. 2018). MTSs can also be used for the study of tumor invasion and migration. For example, Liao et al. found that in ovarian cancer the malignant cell spheroid can also be shed from the primary tumor tissue. Such cell spheroids are often found in ascites of patients with malignant tumors, and their response to anticancer drugs was reduced compared with monolayer culture (Liao et al. 2014). In vivo and in vitro experiments showed that the tumor spheroids have the characteristics of cancer stem cells and play an important role in the study of recurrence, metastasis and drug resistance of ovarian cancer (Shield et al. 2009).

MTSs could be used not only for mechanism research on a laboratory scale, but also for clinical drug testing and evaluation, preferably in combination with high-throughput screening (HTS) system. Hypoxia in tumor is clinically associated with drug resistance and poor prognosis. McIntyre et al. found that treatment of various MTSs with S0859, a small molecule inhibitor of natrium-driven bicarbonate transporter, increased apoptosis in the tested cell lines (McIntyre et al. 2016). Gu et al. used A3D8, an anti-CD44 monoclonal antibody, to treat human ovarian cancer (OVCA) and found that it did inhibit MTS proliferation. It was concluded that anti-CD44 therapy might be an effective treatment for OVCA (Gu et al. 2012). In addition, researchers have developed drugs that target cancer stem cells using MTSs. Matsubara et al. found that the clinical drug rapamycin, the inhibitor of mTOR, reduced $CD133^{+}$ activity and spherogenesis of pancreatic cancer cells, which was related to the self-renewal of tumor stem cells. Therefore, they concluded that drugs targeting the mTOR pathway might be adverse to the proliferation of cancer stem cells (Matsubara et al. 2013). Although some studies have shown that the therapeutic results originated from the MTSs are similar to the models in vivo (Gunness et al. 2013), it should be aware that MTSs cannot allow a 100% recapitulation of tumor aggression and metastases in vivo, which should be, however, approximated as much as possible based on thorough analysis of patient-derived tumor tissue.

Concluding remarks and future prospects

MTSs have been developed rapidly in the past few decades. With the advancement of 3D tumor spheroid technology, the composition of tumor spheroids will become more and more complicated. MTS models with more in vivo traits can be established using co-culture model, which may obtain more solid tumor characteristics. For example, co-culture model can be made using immune cells, macrophages, mesenchymal stem cells, fibroblasts, adipocytes, endothelial cells, pericytes or multiple of them. Further, the quality of the MTS can be improved with the surrounding blood vessels, the immune system composition and oncogenic signals. The MTS model is expected to become the appropriate in vitro model of solid tumor, and presents a similar drug resistance in solid tumor environment.

Despite the existence of different methods to produce MTSs, the challenge still exists because different cancer types have different cell-to-cell interactions and heterogeneity, and each appropriate model must be developed to correspond to a particular type of cancer signature. The MTS model can be applied in the following research areas and/or application fields: (i) Precision medicine. MTSs can provide reliable information for the identification of molecular drug targets, thereby promoting the development of anticancer drugs; (ii) Tumor microenvironment. Understanding the interactions between the microenvironment of tumors facilitates the systematic study of tumor cellular phenotypes and drug metabolism; (iii) Gene therapy. Sequencing MTSs could give some clues for gene therapy; (iv) Clinical biomarker. It could provide sufficient in vitro cancer models to establish different models in individual tumors to study tumor metabolism, and find markers for clinical tumor screening; (v) Tumor invasion and metastasis. MTS provides a good platform to study the invasion and metastasis of tumor cells. Vast majority of cancers have metastasis which could increase the mortality rate. So inhibiting metastasis will be extremely important for the treatment of cancer, which may significantly improve the survival rate of cancer patients. These advances will further contribute to extracting useful biological information from 3D models and driving further development of anticancer research.

The growing need for the MTSs as preclinical model garners more attention directed toward adaptable, reproducible and scalable mass production strategies. So far, however, the mass production of MTSs is still far from complete. MTSs in larger scale bioreactors are rarely reported. In recent years, the technology of expanded culture of microorganisms and mammalian cells in bioreactors has been relatively mature. Whether this technology could be applied to the culture of MTSs is now a key issue to be considered. The focus is on the process optimization, development and sensor technology transfer. MTSs of different shapes (spherical or irregular) and sizes can be produced by manipulating the hydrodynamic conditions in the stirred tank culture system. Tumor cells are typically in a low-glucose environment due to their high glucose consumption and underdeveloped vasculature. Therefore, it has been well accepted that traditional cultured tumor cells under adequate nutrients may not completely simulate real tumor metabolism. Desirable is to make MTSs scalable in bioreactors where in vivo clinical traits, e.g., morphology and omics landscape of the tumor must be guaranteed. In these bioreactors, in vivo microenvironments such as low glucose, hypoxia or in combination can be fine-tuned via feeding strategy and gassing program. Not only the stirred tank reactor can culture single-type tumor cells, but multi-cell culture systems based on tumor cells and other stromal cells can also be established. Ideally, the resulting large number of models can be used for high-throughput drug screening for preclinical studies. Despite the current research on this area is relatively less, large-scale production of MTSs for clinical and industrial applications in bioreactors with high adaptability, high repeatability and high scalability can be anticipated in the near future.

Abbreviations

1,3-BPG: 1,3-bisphosphoglycerate; 2-DG: 2-deoxy-D-glucose; 2-HG: 2-hydroxyglutarate; 2PG: 2-phosphoglycerate; 3PG: 3-phosphoglycerate; 5-FU: 5-fluorouracil; 5fdC: 5-formy-2′deoxycytidine; 5hmdC: 5-hydroxymethyl-2′deoxycytidine; 5,10-meTHF: 5,10-methylenetetrahydrofolate; 5-PRA: 5-phosphoribasylamine; 6-TG: 6-tioguanine; 10-formyl-THF: 10-formyltetrahydrofolate; ACC: Acetyl-CoA carboxylase; ACLY: ATP citrate lyase; ACS: Fatty acyl-CoA synthase; ACSS2: Acetyl-CoA synthase; aKG: α-Ketoglutarate; Akt: Protein kinase B; AMP: Adenosine monophosphate; AMPK: AMP-activated protein kinase; APH: Acid phosphatase assay; AQ4N: Banoxantrone; Arg: Arginine; ASCT2: Solute carrier family 1 member 5; Asn: Asparagine; ASNS: Asparagine synthase; Asp: Aspartate; ASS1: Arginine succinic acid synthase; BC: Breast cancer; BCAA : Branch chain amino acid; BCAT: Branched-chain amino acid transaminase; CA9: Carbonic anhydrase IX; CAF: Cancer-associated fibroblast; CCA: Chitosan–collagen–alginate; cC3: Cleaved caspase 3; CDA: Cytidine deaminase; CH2THF: Methylenetetrahydrofolate; Cit: Citrulline; CK18: Cytokeratin-18; CLSM: Confocal laser scanning microscopy; CPT1: Carnitine palmitoyl transferase 1; CRC : Colorectal cancer; CSC: Cancer stem cell; CTP: Cytidine triphosphate; DCA: Dichloroacetate; DHAP: Dihydroxyacetone phosphate; DHF: Dihydrofolate; DHFR: Dihydrofolate reductase; DHO: Dihydroorotate; DHODH: Dihydroorotate dehydrogenase; DOX: Doxorubicin; dTMP: Deoxythymidine monophosphate; dUDP: Deoxyuridine diphosphate; dUMP: Deoxyuridine monophosphate; EC: Endothelial cell; ECM: Extracellular matrix; EGF: Epidermal growth factor; EMT: Epithelial–mesenchymal transition; E-4-P: Erythrose-4-phosphate; EPI: Epithelial cell; ETC: Electron transport chain; F-1,6-BP: Fructose-1,6-bisphosphate; F-2,6-BP: Fructose-2,6-bisphosphate; FA: Fatty acid; FA-CoA: Fatty acid-CoA; FAO: Fatty acid oxidation; FASN: Fatty acid synthase; FBPase: Fructose-1,6-bisphosphatase; FN: Fibrillary fibronectin; F-6-P: Fructose-6-phosphate; FUM: Fumarate; GAP: Glyceraldehyde-3-phosphate; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; GLDC: Glycine decarboxylase; Gln: Glutamine; GLS: Glutaminase; Glu: Glutamate; GLUT: Glucose transporter; Gly: Glycine; GMP: Guanosine monophosphate; G-6-P: Glucose-6-phosphate; G6Pase: Glucose-6-phosphatase; G6PDH: Glucose-6-phosphate dehydrogenase; HCC: Human lung adenocarcinoma; HGF: Hepatocyte growth factor; HIF: Hypoxia inducible factor; hiPSC: Human induced pluripotent stem cell; H&E: Hematoxylin and eosin; HK: Hexokinase; HTS: High-throughput screening; IA: Iodoacetate; IAA:

Iodoacetamide; ICC: Immunocytochemistry; IC-UHR FT-MS: Ion chromatography-ultra high-resolution Fourier transform-MS; ICP-MS: Inductively coupled plasma-mass spectrometry; IDH: Isocitrate dehydrogenase; IDO: Indoleamine-2, 3-dioxygenase; IHC: Immunohistochemistry; IF: Immunofluorescence; IL-6: Interleukin-6; IMP: Inosine monophosphate; hFIB: Human fibroblast; KRAS: Kirsten rat sarcoma; LC-MS: Liquid chromatography–mass spectrometry; LDH: Lactate dehydrogenase; LDMC: Low-dose chemotherapy; LSCE: Live single-cell extractor; M3DB: Magnetic 3D bio-printing; M-6-P: Mannose-6-phosphate; Mal: Malate; MALDI: MatriX-Assisted Laser Desorption Ionization; MCD: Malonyl-CoA decarboxylase; MCT: Monocarboxylic acid transporter; MDR: Multi-drug-resistance; mIDH: Mutated IDH; MMP-1: Matrix metalloproteinase-1; mtDNA: Mitochondrial DNA; mTOR: Mammalian target of rapamycin; MTS: Multicellular tumor spheroid; MSC: Mesenchymal stem cell; MPM: Multi-photon microscope; NADH: Nicotinamide adenine dinucleotide; NADPH: Nicotinamide adenine dinucleotide phosphate; NGS: Next-generation sequencing; NSCLC: Non-small cell lung carcinoma; OAA: Oxaloacetate; OMP: Orotidine monophosphate; Orn: Ornithine; OVCA: Ovarian cancer; OXPHOS: Oxidative phosphorylation; PA: Phosphatidic acid; Rb: Retinoblastoma; PBMC: Peripheral blood mononuclear cell; PDH: Pyruvate dehydrogenase; PDAC: Pancreatic ductal adenocarcinoma; PDX: Patient-Derived tumor Xenograft; PEP: Phosphoenolpyruvate; PEPCK: Phosphoenolpyruvate carboxykinase; PFK1: Phosphofructokinase 1; PFK15: 1-(4-pyridinyl)-3-(2-quinolinyl)-2-propen-1-one; PFKFB: 6-phosphofructo-2-kinase; PGI: Phosphoglucose isomerase; PHGDH: Phosphoglycerate dehydrogenase; PI: Propidium iodide; PI3K: Phosphoinositide 3-kinase; PKM2: M2 subtype of pyruvate kinase; PMI: Phosphomannose isomerase; PPP: Pentose phosphate pathway; PRPP: Phosphoribosyl pyrophosphate; PSAT: Phosphohydroxythreonine aminotransferase; PSPH: Phosphoserine phosphatase; ROS: Reactive oxygen species; RTK: Receptor tyrosine kinase; R-5-P: Ribose-5-phosphate; Ru-5-P: Ribulose-5-phosphate; SCD: Stearoy-CoA-desaturase; SEM: Scanning electron microscopy; Ser: Serine; SFU: Spheroid-forming unit; S-7-P: Eduheptulose-7-phosphate; Suc: Succinate; Suc-CoA: Succinate-CoA; TCA: Tricarboxylic acid; TDO: Tryptophan-2, 3-dioxygenase; TEM: Transmission electron microscopy; TGF-β: Transforming growth factor-β; THF: Tetrahydrofolate; TIL: Tumor infiltrating lymphocyte; TME: Tumor microenvironment; TNBC: Triple-negative breast cancer; Treg: Regulatory T cells; Trp: Tryptophane; TS: Thymidylate synthase; UDP: Uridine diphosphate; ULA: Ultra-low attachment; UMP: Uridine monophosphate; UTP: Uridine triphosphate; VEGF: Vascular endothelial growth factor; XMP: Xanthosine monophosphate; X-5-P: Xylulose-5-phosphate.

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