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The Organoid Models for Chinese Herbal Medicine Studies

Xuan Mou [a,b,c,1], *Aolin Zhang* [a,b,c,1], *Tao He* [a,b,c], *Renjie Chen* [a,b,c], *Fanfan Zhou* [d], *Tsz Ching Yeung* [e], *Chi Chiu Wang* [e], *Chao Tang* [f], *Xiaoyan Lu* [a,b,c], *Lu Li* [a,b,c,e,*], *Xiaohui Fan* [a,b,c,*]

[a] Pharmaceutical Informatics Institute, College of Pharmaceutical Sciences, Zhejiang University, Hangzhou, 310058, China;

[b] Innovation Center in Zhejiang University, State Key Laboratory of Component-Based Chinese Medicine, Hangzhou, 310058, China;

[c] Future Health Laboratory, Innovation Center of Yangtze River Delta, Zhejiang University, Jiaxing, 314100, China;

[d] School of Pharmacy, The University of Sydney, Sydney, NSW 2006, Australia;

[e] Department of Obstetrics and Gynaecology; Li Ka Shing Institute of Health Sciences; School of Biomedical Sciences; and Sichuan University-Chinese University of Hong Kong Joint Reproductive Medicine Laboratory, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong;

[f] National Clinical Research Center for Child Health of the Children's Hospital, Zhejiang University School of Medicine, Hangzhou, 310052, China.

[1] Xuan Mou and Aolin Zhang contributed to the manuscript equally.

* Corresponding authors:

Lu Li, E-mail: luciali@zju.edu.cn; Xiaohui Fan, E-mail: fanxh@zju.edu.cn.

Fax: 86-571-88208596, Address: College of Pharmaceutical Sciences, Pharmaceutical Informatics Institute, Zhejiang University, #866 Yuhangtang Road, Hangzhou 310058, China.

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Abstract: Organoids are the aggregation of three-dimensional cells generated from pluripotent stem cells or adult stem cells *in vitro*. With many advantages over cell models and animal models, organoids have gradually been used in drug and clinical medical research in recent years. Chinese herbal medicine (CHM) is characterized by its multi-target and multi-pathway treatment method; however, there is no commonly accepted study method regarding its efficacy and mechanism. In this review, we summarize the important applications of organoid models in pharmacodynamic mechanism study, efficacy and safety evaluation and personalized medicine of CHM, providing with the theoretical basis for its development and innovation.

Keywords: organoids, Chinese herbal medicine, three-dimensional model, *in vitro* experiments

1. Introduction

Organoids are tissue analogs with specific spatial organization created through three-dimensional stem cell cultivation *in vitro* [1]. It is a miniature *in vitro* organ model that closely resembles the properties of actual *in vivo* organs. Organoids are derived from either adult stem cells (ASCs) or pluripotent stem cells (PSCs). During organoids culture, stem cells proliferate and differentiate into organ-representative cell types in an environment with multiple signaling factors, mimicking the natural environment *in vivo*. Hence, organoids can be applied in various species and tissues.

Advances in stem cell technology have driven the development of organoids. The field of organoids has made remarkable progress in the past decade. Intestinal

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organoids were first constructed in the laboratory of Hans Clevers, the originator of organoids, in 2009 [2]. In 2013, brain organoids developed from human PSCs were successfully cultivated [3]. Subsequently, various organoids originating from different germ layers were gradually developed [4]. Compared to conventional two-dimensional cell culture models, organoids have more complex and diverse cell types with a more stable genome, mimicking and reflecting the interaction between cells and the extracellular environment. Up to now, various kinds of organoids have been established for numerous potential applications, such as drug screening [5].

Compared with traditional animal models, organoids avoid the differences between species and thereby simulate human traits more accurately [6]. Of all animal models, nonhuman primates are most closely related to humans, making experimental monkeys as suitable models for evaluating drugs. However, monkey experiments require a long duration and high cost. When compared, drug screening and organoids evaluation requires shorter time and lower research cost. Apart from the time and cost factor, regarding animal protection, guidelines were issued to reduce animal testing. For instance, the European Union issued a directive in 2010 to reduce animal testing [7]. Recently, the U.S. Senate passed the FDA Modernization Act 2.0 to reduce the testing of new drugs on dogs, primates, and other animals. At present, the most likely alternative to animal testing technology is organoids, which can well simulate three-dimensional organs in the body *in vitro* [8].

ASC-derived organoid cultures are typically established by embedding isolated adult stem cells, or single-cell suspensions, into extracellular matrix hydrogels. PSCs,

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including induced stem cells and embryonic stem cells, can also take advantage of their self-renewal and differentiation capabilities to generate organoids [5]. In addition, for tumor organoids, tumor cells (containing cancer stem cells) isolated from tumor tissues of patients are cultured in a microenvironment similar to *in vivo* after adding certain growth factors and small molecules, which can be stably expanded in the *in vitro* culture system. Growth factors are important substances that need to be added to organoid cultures. Growth factors direct stem cell differentiation by regulating various signaling pathways. For most organoid types, growth factors that need to be added include Wnt, R-spondin-1, and others [2,3]. The type of organoids needed for specific experiments should be determined according to the sample source, experimental content, and conditions.

Chinese herbal medicine (CHM) is regarded as one of the world's greatest treasures. Due to its impressive curative effects and negligible side effects, CHM has become increasingly popular for treating specific disorders, such as cardiovascular disease [9], COVID-19 (Corona Virus Disease 2019) [10], and cancer [11]. Unlike chemical drugs of a single chemical compound that in most cases act on a specific target, CHMs are usually prescribed in the form of ‘formulae’, that is, a combination of herbs. As the herbs target on different organ, the incidence of drug resistance in CHM is relatively lows.

CHM is characterized to be multi-target and multi-pathway, inducing many limitations in its study through conventional animal and cell models. Of note, it is effective to use organoids to avoid deviations in experimental results caused by

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differences between species in animal models. More importantly, organoids make up for the lack of integrity and heterogeneity in the cell culture model. Owing to the great work in the past decades, organoids have emerged to play an important role in regulating disease occurrence and development, hunting for drug targets, and conducting drug screening and safety evaluation [12]. In view of the above, in this paper, we summarize the application of organoids in CHM research in the view of efficacy, drug screening, safety and underlying mechanisms, and put forward solutions and prospects for existing problems, for instance the deficiency of structures and cell types, and batch variation (Figure 1).

2. Efficacy evaluation

Organoids play a great role in the evaluation of drug efficacy, providing references for later clinical medication. There have been many reports on the research of organoids in western medicine [13]. Although there are relatively few reports on CHM (Table 1), organoids still have great development potential.

Organoids are rich in cell types and contain certain tissue structures that can be utilized to create disease models by modifying culture conditions, gene editing, and obtaining cell sources from patients, in order to evaluate the efficacy of CHM treatment.

Fan *et al*. assessed the mechanism and pharmacodynamic impact of the CHM extraction Guanxinning Injection (GXNI) using an organoid model of cardiac hypertrophy [14]. The model was made of cardiac fibroblasts, cardiomyocytes, and

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endothelial cells. Cells in the model were cardiac-like in shape with extracellular matrix components and exhibited spontaneous, rhythmic contractile and diastolic activities. In terms of human organoids, Du *et al*. created a blood-brain barrier (BBB) organoid oxygen glucose deprivation model to investigate the protective effect of GXNI on BBB dysfunction induced by ischemic encephalopathy, which is useful for the development of central nervous system targeted drugs [15]. These are spheroids formed by various types of cells, and the cells are recombined through the secreted cytoplasmic matrix. They can simulate tissue characteristics in vivo but may lack other cell types.

In addition, normal organoids derived from stem cells can also be established for drug intervention to study the protective effect of CHM on specific organs. Chen *et al*. established intestinal organoids and found that glycyrrhetinic acid can increase the level of human antigen R and downstream proliferation associated nuclear antigen Ki67, in order to promote the development of intestinal organoids and maintain intestinal homeostasis [16]. Wang *et al*. ascertained the impact of polysaccharide extracts of the CHM formula Sijunzi Decoction on the growth of intestinal organoids [17]. They used the dry crypt cell mass of mouse small intestine to cultivate into intestinal organoids *in vitro*, observed and characterized the morphological traits and protein expression of intestinal organoids. Adult stem cells are isolated from mouse tissues and cultured to obtain organoids, which can produce a large number of organoids in a short time, and the operation is simple. But there are species differences between mouse-derived organoids and human organs.

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In the research of liver protection, Wu *et al*. added the CHM product cholesterol+ MIX (Chinese pending patent number: ZL 201810211144.X) in the formation process of hepatobiliary organoids. They found that the organoids have better secretion ability and drug metabolism ability, and have a longer survival time after transplantation *in vivo*, which provides a new direction for further study [18]. In this study, human induced pluripotent stem cells (hiPSCs) are induced to differentiate into different types of organoids. HiPSCs are readily accessible cell types, and organoids derived from them have greater potential for translation into clinical applications. However, this type of organoid has a relatively long culture time.

3. Drug screening

Organoids can be cultured in a relatively short period of time in large quantities, with a high genomic stability. Therefore, organoids have been gradually applied to drug screening [19]. Colorectal cancer organoids were established to evaluate the effects of several toxic CHM monomer components on their activity, including triptolide, cantharidin, and bufalin. At the same time, colorectal normal organoids were established to evaluate their toxicity and verify the feasibility of anti-cancer methods [20]. Other studies used patient-derived colorectal cancer organoids and found that celastrol could inhibit their growth, and the inhibitory effect of celastrol was stronger than that of the positive drug L-OHP [21]. These studies provide a new methodological reference for screening the anti-cancer activity of CHM. Human tissues are isolated, stem cells are extracted, and organoids are cultured in these

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studies. Organoids with donor characteristics can be grown in a relatively short period of time. But donor samples are scarce, and some types of organoids cannot be generated from human tissues.

In addition, the establishment of organoid biobanks plays an important role in CHM screening, especially for cancer [22]. Li *et al*. established living non-small cell lung cancer organoids biobanks for drug research, and these tumor organoids show similar pathological features to primary tumors [23]. They screened some CHM monomers, including chelerythrine chloride, cantharidin, harmine, berberine and betaine, and found that the latter three had anti-cancer activity to lung cancer organoids.

Patient derived cancer cells (PDCs) and patient derived xenotransplantation mice (PDXs) are often used as tumor models in drug screening. However, PDCs lack diversity in cell types and environments, and PDXs have a low transplant success rate and require long culture time [24, 25]. When compared, tumor organoids can maintain the genetic characteristics and individual heterogeneity of patients' tumors more efficiently, which is a good model for drug screening and is helpful for individualized drug use.

CHM has a significant impact on cancer treatment as a complementary and alternative therapy [26]. Many studies have shown that the combination of CHM and chemotherapy drugs can enhance the anti-tumor effect of chemotherapy drugs through a variety of molecular mechanisms and also overcome the resistance of molecularly targeted drugs [27]. Although chemotherapy is widely used, it has great toxicity and side effects on normal tissues. The use of CHM to mobilize the internal resistance to

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disease can reduce the toxic side effects of western medicine and achieve the effect of overall regulation [28]. In addition, CHM therapy attaches importance to the individualized medical care that Chinese Medicine Practitioners (CMPs) adjust the combination of herbs and dosage of medications based on the patient's specific body constitution. Organoids originating from human cells or tissues, can preserve donor heterogeneity, especially tumor organoids. The idea of using the organoid model to evaluate the impact of drug therapy and screen drugs coincides with the treatment concept of CHM.

4. Safety evaluation

In recent years, CHM has been widely applied and its safety has also received more attention. It is challenging to research the safety of CHM due to the complexity of its components and the limitations of conventional models. Where cell models lack intercellular connections and animal models require a long drug administration process, organoids are potential efficient models for the safety evaluation of CHM.

Organoids are used to access hepatotoxicity. Liver is the main organ of metabolism and is prone to drug-induced injury. It has been reported that many CHMs have potential hepatotoxicity, leading to serious clinical adverse events, such as liver fibrosis [29]. Previous studies used PSCs-induced hepatocytes to evaluate the hepatotoxicity of CHM, and found that the toxicity pattern of such hepatocytes is similar to that of human primary cultured hepatocytes [30, 31]. Compared with liver cells cultured *in vitro*, liver organoids induced by PSCs are more similar to the human

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tissue microenvironment, and are thus more suitable for hepatotoxicity assessment. Li *et al*. used droplet overlapping method to construct liver organoids [32]. Combined with high-intensity imaging, it was found that the toxicity of 2,3,5,4′-tetrahydroxy-trans-stilbene-2-O-β-glucoside (trans-SG, ingredients of Polygonum multiflorum (He Shou Wu, *Fallopia multiflora*)) was significantly lower than that of its cis-isomer (cis-SG), which was consistent with the previous animal results. Zhu *et al*. combined 3D printing and microfluidic chip technology to obtain liver organoids derived from human cells, and evaluated the hepatotoxicity of commercially available CHM injections [33]. It is found that the evaluation results are more accurate than those of 2D cell model.

Kidney is the main excretory organ of the human body, which is vulnerable to drugs. At present, renal toxicity of CHM is mainly evaluated by animal models based on biomarkers and histopathological results. Kidney organoids overcome the shortcomings of animal models, such as being time-consuming. Gu *et al*. used human-induced PSCs to obtain renal organoids, and applied this organoid to evaluate the nephrotoxicity of Phytolacca saponins [34]. It was found that the application of organoids can reduce the time and amount of drug required.

Cardiotoxicity is an important indicator that must be considered before clinical trials of newly invented drugs [35]. Recently, various CHMs, for instance Aconite (Wu Tou, *Aconitum carmichaeli*) [36], Tripterygium wilfordii (Lei Gong Teng, *Tripterygium wilfordii Hook. f.*) [37], and Oleander (Jia Zhu Tao, *Nerium oleander L.*) [38], are reported with their cardiotoxicity. Liu *et al*. summarized the application and

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advantages of cardiac stereoscopic cell models and cardiac organoids in the evaluation of CHM cardiotoxicity [39]. They proposed that the combination of cardiac organoids with automatic patch clamp technology and high-connotation cell image analysis technology is more effective than traditional models for CHM cardiac safety research.

Neurotoxicity is also one of the possible toxicities caused by CHM. For neurotoxicity evaluation, previous *in vitro* models were mainly neurons and glial cells derived from human neural stem cells [40], but these models lack 3D structures and may not accurately summarize the key events of neural development. When compared, brain organoids mimic more structure and neural functions of human brain [41], which can be used for neurotoxicity evaluation to solve the clinical difficulty of obtaining human brain samples. Using brain organoids, Huang *et al*. found that cadmium exposure induced neuronal apoptosis, inhibited the proliferation of neural progenitor cells, and impaired ciliogenesis [42]. In terms of CHM, our team is using brain organoids to evaluate the neurotoxicity of some CHM treatments for neurological diseases.

5. Mechanism study

Organoids, as 3D culture models *in vitro*, have more complicated structure and biological functions. They can be used to better characterize the CHM efficacy or toxicity mechanisms by using real-time quantitative polymerase chain reaction (qPCR), western blot, and various imaging technology [43]. Xu *et al*. established human patient derived colorectal cancer organoids, undergo flow cytometry analysis,

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and found that atractylenolide I can enhance T cell cytotoxicity and improve tumor response to immunotherapy [44]. Yan *et al*. established intestinal organoids through qPCR, and found that St. John's wort (Hypericum perforatum) extract and its active component hyperforin can activate progesterone X receptor and inhibited NFκB activation for the prevention and treatment of inflammatory bowel disease [45]. Through the combination of fluorescent probe and high-intension imaging technique, it was found that the damage mechanism of cis-SG hepatotoxicity was mainly mitochondrial damage [46]. It was proved that combined with high intentionality imaging, organoids could predict the toxicity of CHM and explain the mechanism in a low-cost and high-throughput manner.

CHM is known for its multi-component, multi-target and diverse modes of action. It is difficult to interpret the molecular mechanism of CHM, and find the differences in gene expression of different cells caused by the intervention of CHM. Organoid models are helpful to analyze the molecular mechanisms and action targets of CHM on disease treatment from a systematic perspective regarding genomics, transcriptomics, proteomics and metabolomics.

Single-cell RNA sequencing (scRNA-seq) can not only map the single-cell profiles of developing and mature organoids, but also reveal the potential mechanisms of disease by identifying the cell types expressing disease-related genes. Moreover, it provides higher resolution experimental data for the study of the action targets of CHM, and further builds a ‘component-target-pathway’ spatial regulatory network [47]. At present, there are some western medicine researches applying the combination of

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scRNA-seq and organoid technology [48], but no research regarding its application on CHM has been published. Our team has recently successfully constructed brain organoids and applied scRNA-seq to study the efficacy and mechanism of Chinese herbal compound GY-1. Besides, our team has also elucidated the dynamic regulation of immune cells on the repair process of myocardial infarction using scRNA-seq, and further explained the specific mechanism of Tanshinone IIA in the treatment of myocardial infarction [49].

In addition, other omics techniques also play an important role in organoid research. Proteomics and metabonomics are two examples. Proteomics studies the specific proteins in organisms from an overall perspective, which can quantitatively observe the changes in functional pathways and biological processes with high precision and sensitivity [50]. Metabonomics provides more comprehensive phenotypic characteristics and physiological interactions of diseases. Compared with 2D cell culture, 3D culture can more efficiently simulate the physiological environment *in vivo*, so it is more suitable for combined research with metabonomics [51, 52].

6. Challenges and opportunities

6.1 Existing problems

Even though organoids are superior to cell and animal models, they also have certain limitations at the moment. Firstly, unlike the internal environment, organoids have no vascular structure and no connections between tissues, which is incompatible with the CM holistic dialectical theory. Secondly, there are large differences between batches

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when using organoids to detect the efficacy and toxicity of CHM, and it is difficult to obtain similar experimental results. Moreover, there is no reference standard for clinical drug use, and it is challenging to match the dose of organoid pharmaceuticals to the dose of the human body. At present, more and more researches have combined organoid models with other technologies to address these issues, as will be discussed below.

6.2 Future prospect

Compared with western medicine studies, there are relatively few reports on organoids in CHM studies, but they have great potential for development in this area. First of all, dialectical treatment is one of the main concepts of CHM, which fully reflects the characteristics of individualized treatment. CHM treatment is an ancient form of precision medicine, while organoids are the embodiment of modern precision medicine. In terms of individualized precise treatment, organoid studies are consistent with the concept of CHM. In addition, the treatment of CHM has a wealth of clinical cases, and the combination of CHM and organoid studies is conducive to the treatment of more difficult and miscellaneous diseases. Moreover, there are a large number of CHMs, including compounds, single drugs, active ingredients, and so on, providing a large number of choices for organoid studies.

CHM has the characteristics of holistic body regulation, so it is not enough to reveal its mechanism of action from the local perspective, such as a single organ. Although there are many cell types and certain tissue structures in organoids, the interaction between organoids and the surrounding environment cannot be fully simulated, which

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is different from the real situation *in vivo*, thus resulting in the inability to reveal the holistic CHM mechanism. To better mimic the cell microenvironment, organoids are occasionally co-cultured with different cell types or microbes. For instance, the team led by Hans Clevers at Nature Protocols described the microinjection approach for co-culture of intestinal organoids and microbes. These intestinal organoids are derived from adult stem cells and provide fully differentiated and ancestral epithelial cell types, so their co-culture with microorganisms facilitates the study of host-microbe interactions [53]. In the study of cancer, Cattaneo *et al*. co-cultured tumor organoids and peripheral blood lymphocytes to expand tumor-reactive T cells from peripheral blood, which is conducive to evaluating their reactivity and lethality to tumor cells [54]. In addition, some studies have found that the co-culture of specific cells in tumor microenvironment (TME) with tumor organoids can partially simulate some characteristics of TME [55].

No vascularization of organoids has always been a problem. Organoid vascularization is attempted in organoid transplantation to mice or rats [56, 57]. Another emerging method is to co-culture organoids with human umbilical vein endothelial cells, but there is a big gap between the time and space of early development *in vivo*. Recently, Salmon *et al*. obtained a neurovascular organoid by controlling the physical interaction of endothelial cells, pericytes and organoids spatially through microfluidic chips through three-dimensional printing and microfluidic technology [58]. This study achieved a highly synchronized vascularization process of organoids in time and space, and provided a new approach for the study of tissue vascularization *in vitro*.

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Another challenge of organoid construction is the large batch variation. Existing studies have shown that the three-dimensional bioprinting of organoids has greatly improved with a very low coefficient of size difference of only 1-4% [59]. Three-dimensional bioprinting technology enabled a high-throughput organoid construction in terms of biomimetic quality and production speed. Lawlor *et al.* demonstrated that automatic construction of renal organoids can be achieved using three-dimensional bioprinting, with morphological component cell types and gene expression levels consistent with those of manual construction [60].

7. Conclusion

Due to its intricacy, CHM has long lacked an appropriate research model. Organoids are useful for the investigation of CHM because of their special benefits. Future studies, however, will need to address the shortcomings of current organoids in CHM research, such as the accuracy of CMH dosage between clinical dosage and the large variation of organoid batches. With the improvement of organoid technologies, organoids will be better applied in the study of the efficacy and safety of CHM, and may gradually replace other conventional research models in the future.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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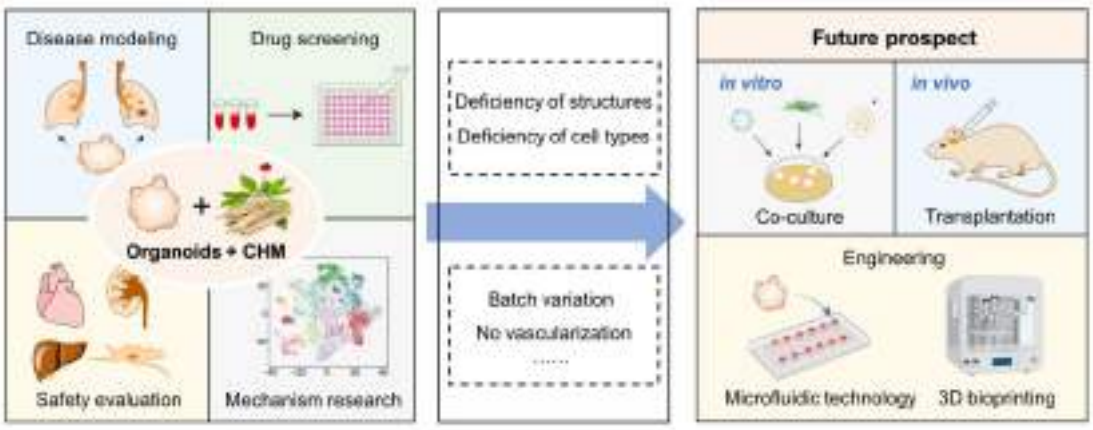

Figure 1 | Application and prospect of organoids in the research of CHM.

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Table 1 | Application of organoids in efficacy evaluation and drug screening of

Type of organoids	Species	Origin	Models of disease	CHM
Cardiac organoids	Rats	Cardiac fibroblasts (CFs), cardiomyocytes (CMs), and endothelial cells (ECs)	Myocardial hypertrophy	Guanxining Injection (GXNI)
BBB organoids	Human	Human brain microvascular endothelial cells (HBMEC), human brain astrocytes (HA) and human brain vascular pericytes (HBVP)	Oxygen-glucose deprivation/reoxygenation (OGD/R)	Guanxining Injection (GXNI)
Small intestinal organoids	Mice	Intestinal crypt stem cells	Normal	Glycyrrhetinic ac
Small intestinal organoids	Mice	Intestinal crypt stem cells	Normal	Sijunzi Decoction
Small intestinal organoids	Mice	Intestinal crypt stem cells	Normal	St. John's wort extract and hyperforin
Hepatobiliary organoids	Human	Human induced pluripotent stem cell (hiPSC)	Normal	Cholesterol+ MIX (Chinese pending patent number: Z

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Patient-derived organoids	Human	Tissues from patients with colorectal cancer	Colorectal cancer	Triptolide, cantharidin, and bufalin
Patient-derived organoids	Human	Tissues from patients with colorectal cancer	Colorectal cancer	Celastrol
Patient-derived organoids	Human	Tissues from patients with non-small cell lung cancer	Non-small cell lung cancer	Chelerythrine chloride, cantharidin, harmine, berberin and betaine