

Review Article

Trends in the development of human stem cell-based non-animal drug testing models

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ABSTRACT *In vivo* animal models are limited in their ability to mimic the extremely complex systems of the human body, and there is increasing disquiet about the ethics of animal research. Many authorities in different geographical areas are considering implementing a ban on animal testing, including testing for cosmetics and pharmaceuticals. Therefore, there is a need for research into systems that can replicate the responses of laboratory animals and simulate environments similar to the human body in a laboratory. An *in vitro* two-dimensional cell culture model is widely used, because such a system is relatively inexpensive, easy to implement, and can gather considerable amounts of reference data. However, these models lack a real physiological extracellular environment. Recent advances in stem cell biology, tissue engineering, and microfabrication techniques have facilitated the development of various 3D cell culture models. These include multicellular spheroids, organoids, and organs-on-chips, each of which has its own advantages and limitations. Organoids are organ-specific cell clusters created by aggregating cells derived from pluripotent, adult, and cancer stem cells. Patient-derived organoids can be used as models of human disease in a culture dish. Biomimetic organ chips are models that replicate the physiological and mechanical functions of human organs. Many organoids and organ-on-a-chips have been developed for drug screening and testing, so competition for patents between countries is also intensifying. We analyzed the scientific and technological trends underlying these cutting-edge models, which are developed for use as non-animal models for testing safety and efficacy at the nonclinical stages of drug development.

INTRODUCTION

The rapid progress of science and technology related to the drug development has resulted in numerous new drug candidates using new techniques. However, the number of new licensed products has been declining, despite the expenditure of large amounts of money on R&D [1,2]. This decline is partly because the U.S. Food and Drug Administration (FDA)'s screening standards have been strengthened since 2000, but the technologies for assessment of safety and efficacy used in the development of new drug products take time to reflect the technological advances.

Experimental animals are useful models in pre-clinical tests, but the tests are time-consuming and costly. In addition, many pharmaceutical companies in various countries, including those developing chemicals and cosmetics, have recently banned animal testing due to ethical issues and errors in predictions due to differences between species [3,4]. Therefore, the need for the development of new non-animal test models is increasing.

With the recent rapid development of stem cell technology, many studies seeking to differentiate human stem cells into specific cells that make up organs and use them for nonclinical testing have been carried out around the world. *In vitro* two-dimen-



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sional cell culture models are used early in nonclinical testing, because they generate large amounts of basic data, which can be compared and analyzed, and are relatively inexpensive and easy to use [5,6]. However, there are limits to present a real physiological cell environment under the conditions of incubation in a static environment. Three-dimensional (3D) cell culture models, have been proposed as an ideal way to imitate the form and function of human organs, although such models are not yet complete [7]. Thus, there are increasing attempts to use *in vitro* models made using 3D culture methods for safety and efficacy testing of drug candidates [8,9], and competition among countries for patents is getting fiercer. In this review, we survey the trends in development of human stem cell-derived 3D culture models, organoids, and organ-on-a-chip models, which are being developed as non-animal models for use in drug development.

DEVELOPMENT OF NON-CLINICAL TESTING MODELS USING ORGANOID

Organoids (also known as organ-like organisms) are organ-specific cell clusters created by aggregating cells separated from stem cells or organs in 3D culture. They are collections of cells with a 3D structure containing organ-specific cells. Organoids can self-organize; that is, they can be spatially organized in a form similar to the actual organ. They can reproduce organ-specific functions, and form systems. Depending on the cell type of origin, it is possible to distinguish between pluripotent stem cell-derived organoids, adult stem cell-derived organoids, and cancer cell-derived organoids (Fig. 1).

Pluripotent stem cell-derived organoids

Many studies have been carried out into the production of organoids using 3D culture techniques with various growth factors and an extracellular matrix from embryonic and induced pluripotent stem cells.

As Lancaster and Knoblich [10] published in *Science* in 2014, there has been considerable research into the production of various organoids, including intestines, kidneys, and brains. The cellular signals related to the differentiation of each type of organoid have also been studied. For example, in the case of intestinal organoids, the pluripotent stem cells (PSCs) were treated with the proteins Wnt3a and Fgf4 and then cultured in a supporting scaffold (such as Matrigel), whereas in the case of kidney organoids, the PSCs were treated with the BMP4, Fgf9, and retinoic acid (RA) to generate kidney tissue [10-13].

In the journal *Cell*, in a paper published in 2006 [14], the signaling system related to differentiation into organoids in each organ has been investigated, and many studies have succeeded in producing organoids including intestines, liver, brain, kidneys, lungs, and pancreatic organoids. These organoids have been validated

using gene expression analysis, protein expression analysis, and a functional analysis that can determine each organ-specific characteristic function. Proven organoids have been used in research to establish models of diseases such as cancer, cystic fibrosis, virus and bacterial infections. Also, the organoids are used to inducing mutations, profiling of transcripts, transplantation studies, CRISPR/Cas9 gene correction, and other studies [15-17]. Liver organoids have been used in Alagille syndrome and cystic fibrosis-associated liver disease modeling, and colon organoids have been used to model inflammatory bowel disease [18-21]. Pancreatic organoids have been used for cancer modeling, pulmonary organoids for cystic fibrosis, and brain organoids have been used in investigations into autism and microcephaly [22-28].

Adult stem cell-derived organoids

Adult stem cell-derived organoids are derived from organ-specific stem cells present in each organ in the body. They have the ability to differentiate into the cells that form each organ [29]. Many studies have been conducted on various adult stem cell-derived organoids. There have been reports of systematic follow-up studies through the induction of tamoxifen in relation to tongue cancer, using tongue-stem cell-derived organoids, and many studies have been conducted into the establishment and utilization of intestinal organoids, using Lgr5⁺ stem cells [11,30]. Gastric tissue-derived organoids are composed of Lgr5⁺ stem cells, mucous neck cells, pit cells, and enteroendocrine cells, and the corpus is composed of chief cells, neck mucus cells, and spring cells [31]. These

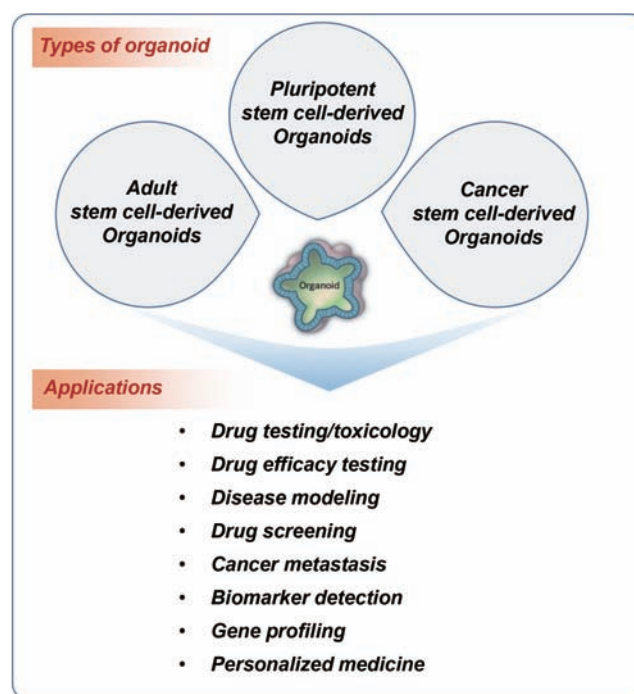


Fig. 1. Types of organoids depending on the originated cells and their applications.

organoids are used in a variety of studies related to transcript profiling, mesenchymal cells, co-culture studies, infections by adenoviruses or retroviruses, and cancer modeling [31,32]. In addition, pancreatic organoids, prostate organoids, and small intestine organoids are used in cancer modeling [33-37]. The esophageal tissue-derived organoids are used in modeling of Barrett's esophagus [38]. Pluripotent stem-derived organoid and patient-derived disease organoids have been established for the development of therapeutic agents and the study of disease mechanism [39]. In Fig. 2, we summarize the established organoids for human diseases in liver [21,40-44], lung [45], brain [24,27,28,46-49], lingual [30], colon [38,50,51], intestine [33,37,52,53], ovary [54], retina [55], kidney [56,57], esophagus [58], heart [59], breast [60], stomach [52,61], and pancreas [26,62,63].

Cancer cell-derived organoids

Cancer cell-derived organoids are referred to as tumor organoids (tumoroids), and are made using a 3D culture method *in vitro* using tumor tissue obtained from animal models or cancer patients [64]. This type of organoid is expected to have a variety of characteristics that appear due to the tumor microenvironment and individual characteristics of cancer. Research into anti-cancer screening using tumor organoids is being actively carried out. Hans Clevers' research team at the Hubrecht Institute in Utrecht, the Netherlands, established 22 tumor organoid biosample banks from the tissues of 20 patients with colorectal cancer [65]. Using these organoids, 83 anti-cancer drugs are being examined to identify the specific genes that are upregulated in

response to each drug. This high-efficiency drug screening is expected to be useful in the use of personalized precision medicine using patient-derived organoids [66,67]. The Calvin Kuo's group at Stanford produced models by introducing two to four cancer mutations in normal colon, gastrointestinal, and pancreatic organoids with tumor-like phenotypes. These models are expected to be valuable for finding new tumor-inducing mutants and verifying their function [68,69]. In the Republic of Korea, the Asan Medical Center and a joint research team are building a biobank of liver cancer, stomach cancer, colon cancer, lung cancer, and pancreatic cancer organoids (<http://brc.amc.seoul.kr/>).

Global scientific trends in organoid research

In order to investigate the trends in global organoid research, organoid-related publications were analyzed by Scopus, a leading journal search site (<https://www.scopus.com/>). We searched for nonclinical tests using organoids in the ten years from 2008 to 2019, and found 195 documents. The rankings by year, country, and institution were investigated for the 195 documents searched. According to the number of papers released on a yearly basis, the use of nonclinical testing technology using organoids increased sharply between 2016 and 2019 (Fig. 3). The top 10 countries were the United States of America, followed by the United Kingdom, the Netherlands, Germany, Japan, China, Italy, Australia, Austria, and the Republic of Korea (Fig. 4). The top five institutions were the University Medical Center Utrecht, the Memorial Sloan-Kettering Cancer Center, Harvard Medical School, Hubrecht Laboratory KNAW Netherlands Institute for Developmental Biology,

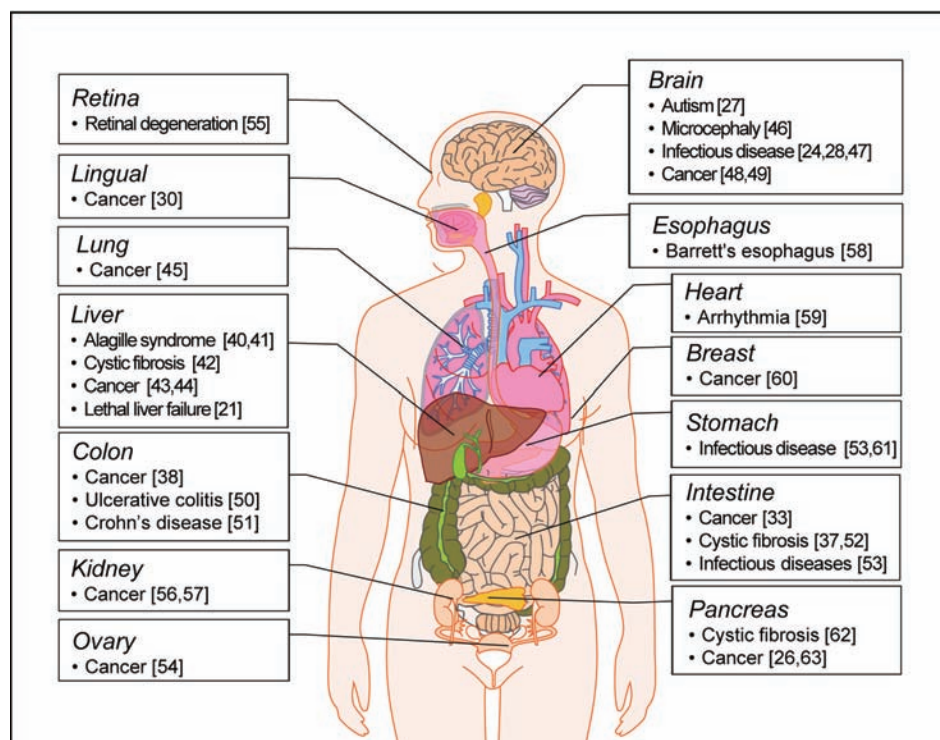


Fig. 2. Establishment of human disease modeling using organoids. The human disease organoids currently established from the pluripotent stem cells and tissues. References are indicated in brackets.

and Erasmus MC (Fig. 5).

Global technology trends with respect to organoids for drug screening technology

To analyze technology patent trends related to "drug screening technology using organoids", the WIPSON Patent search DB (<https://www.wipson.com/>) was used to search for the disclosed/registered patents in the Republic of Korea (KR), the United States of America (US), Europe (EP), Japan (JP), and China (CN), which were submitted before 10th September, 2017. A total of 870 patents were screened. We performed filtering to eliminate noise for

870 preprocessed data retrieved. The noise rejection criteria were set as follows: records i) with respect to tissue transplantation, ii) with respect to plant cultivation, iii) with respect to cancer treatment constructions, iv) with respect to cellular construction, and v) with respect to stem cell differentiation, were judged and filtered as noise. Next, the effective patents were selected by dividing the methods by tissue generation, 3D cell culture method, and tissue or 3D cell culture device/system, using organoids for (1) drug screening, (2) safety evaluation, or (3) efficacy evaluation of drug candidates. In particular, organoids created from all organs were searched. Even if no information related to drug screening was available within the scope of the search, the patent was iden-

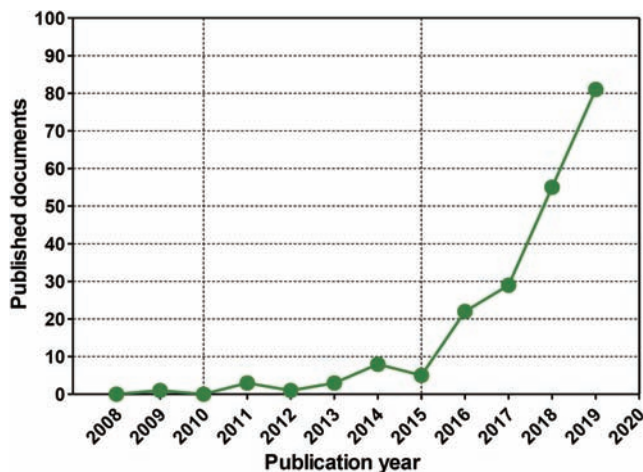


Fig. 3. Status of documents related to nonclinical testing using organoids by year. Global scientific trends in organoid research. A summary of the publications on organoids that are indexed in Scopus according to publication output by year from 2008 to 2019 (Searched date: December 13, 2019).

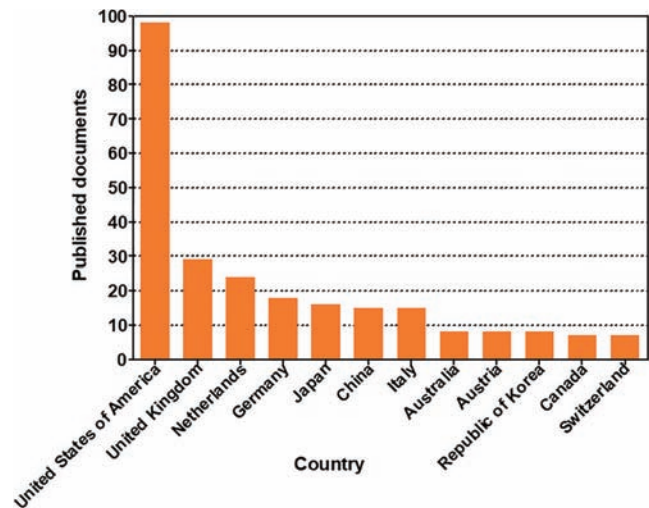


Fig. 4. Status of documents related to nonclinical testing using organoids by country. A summary of the publications on organoids that are indexed in Scopus according to the 12 most frequent affiliation countries of the authors from 2008 to 2019.

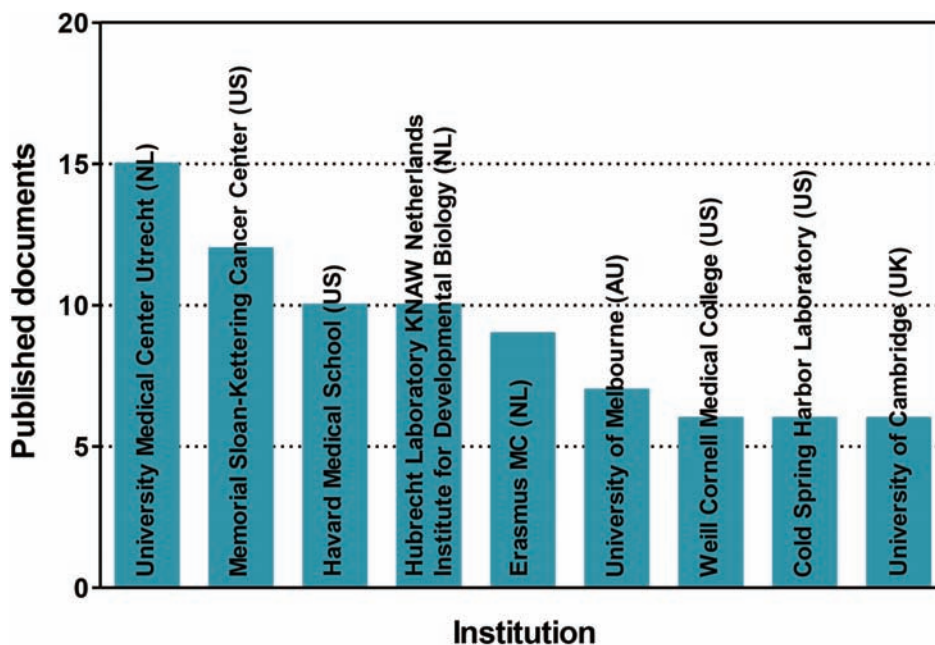


Fig. 5. Status of documents related to nonclinical testing using organoids by institution. A summary of the publications on organoids that are indexed in Scopus according to the 9 most frequent affiliation countries of the authors from 2008 to 2019. NL, the Netherlands; US, the United States of America; AU, Australia; UK, the United Kingdom.

tified as valid patent if it stated that the organoid could be applied to drug screening.

The patent classification resulted in a total of 335 patents related to drug screening using organoids (Fig. 6). The highest percentage of patents was related to 3D cell culture, a total of 188 cases. Patents related to 3D cell culture represented the largest number in 2014, and the number has been decreasing since 2015. There was a total of 144 patents related to tissue generation. In most countries, the number of patent applications has increased since 2009. The lowest number of patents, thirty-three, were related to devices and systems involving tissue generation or 3D cell culture. In 2011, there were many applications filed in the United States of America, the Republic of Korea, and China.

DEVELOPMENT OF NON-CLINICAL TESTING MODELS USING BIOMIMETIC CHIPS

Biomimetic organ/tissue chip technology

The biomimetic organ chip is a model that replicates the physiological and mechanical functions of human organs. Fine connections between living human cells are manipulated to control the organ's long-term characteristics or internal cell size, using microfluidic chip technology derived from the semiconductor industry. We summarize the advantages and disadvantages of 2D cell culture models, 3D cell culture models, and microfluidic chip

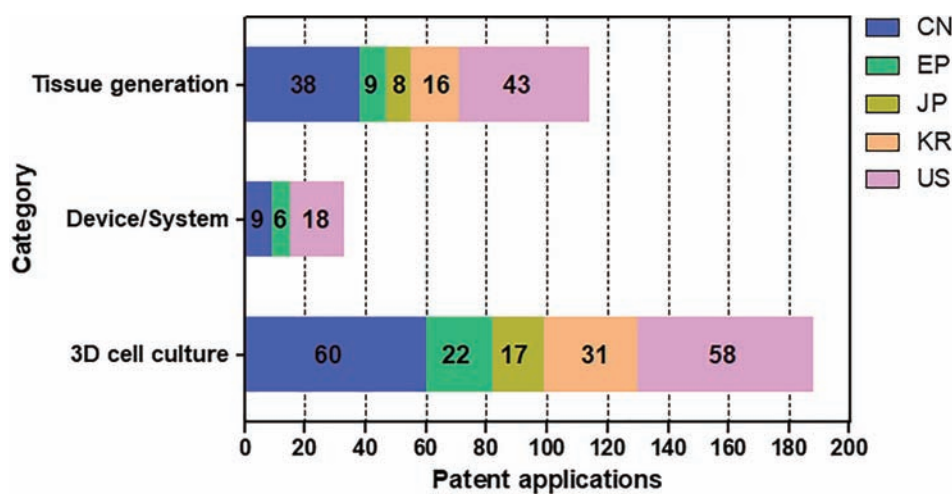


Fig. 6. Number of patent applications by category and country of organoids for a drug screening. The effective patent classification resulted in a total of 335 patents related to a drug screening using organoids. They were divided tissue generation, 3D cell culture method, and tissue or 3D cell culture device/system, using organoids for drug screening, safety evaluation, and efficacy evaluation of drug candidates. The Republic of Korea (KR), the United States of America (US), Europe (EP), Japan (JP), and China (CN).

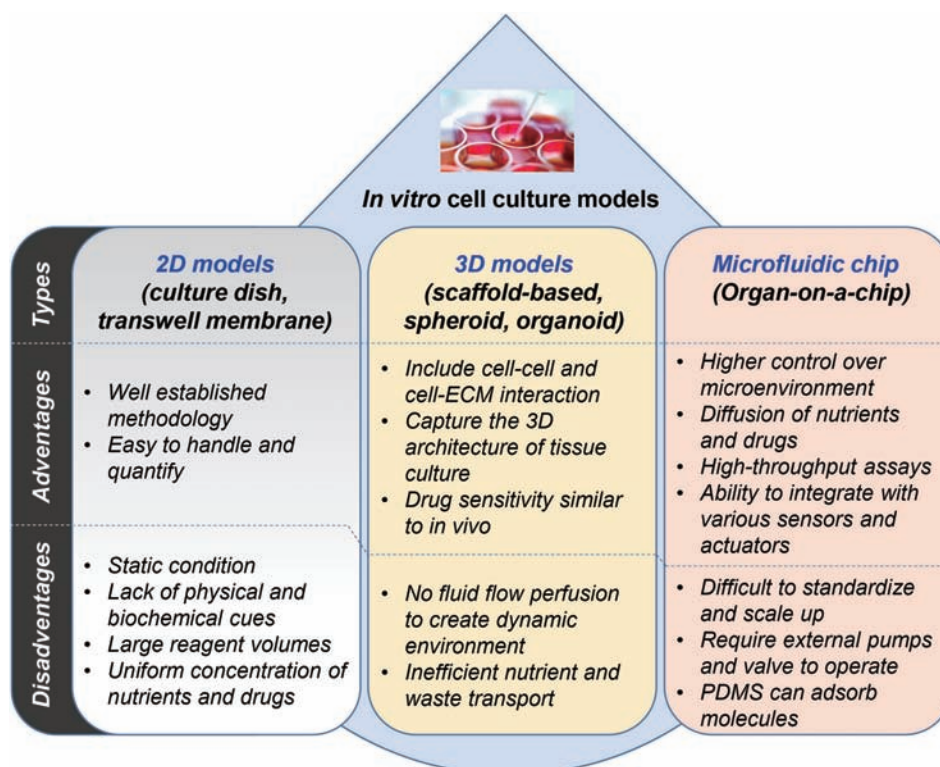


Fig. 7. Comparison of in vitro cell culture models. Summarization of advantages/disadvantages in various cell culture systems with 2D models, 3D models, and microfluidics models.

models in Fig. 7.

Experiments that were not possible using only cell culture models are possible, including the study of metabolites produced, exchanged, and consumed within tissues, the study of developmental biological signaling systems, and the study of interactions between tissues simulating the dynamics of blood circulation. These experiments are expected to contribute to reduce the gap between *in vitro* and *in vivo* experimental environments. In addition, organs-on-a-chip can be used as a candidate drug screening platform at the nonclinical testing stage by allowing drug reactions to be monitored in a similar way to human physiology [70,71].

Research into the development of microfluidic chip platforms

for drug screening has been carried out in a variety of countries, such as the United States of America and China. At the International University of Florida in the US, a biosensor that can measure nanomaterial toxicity at the cellular level in real-time can obtain quantitative information about multiple samples at a time [72]. The National Center for Advanced Translational Science (NCATS) has been developing tissue-on-a-chip for drug screening programs since 2011, in collaboration with the National Institutes of Health, the Defense Development, and the FDA [73]. In October 2016 NCATS announced a plan to develop tissue chips for disease modeling and efficacy testing, that could support further development of human disease tissue chip models imitating the pathology of major human organs and tissues. The

Table 1. Biomimetic organ-on-a-chip developed for drug screening

Organ/system	Type of chips	Characteristics
Brain	Brain development on a chip	Multiple types of brain cells including neurons, astrocytes, and oligodendrocytes, and endothelial cells are cultured in the chip system
	Blood-brain barrier on a chip	The brain cells and blood vessels are separated by a membrane that acts like the blood-brain barrier
Heart	Heart on a chip	The human iPSC-derived heart tissue in the chip is beating as a heart in the human body
Muscle	Muscle on a chip	The muscle cells to multiply and self-organize into bio-engineered muscle bundles
Lungs	Lungs on a chip	The bronchial smooth muscle cells (SMCs) based on an airway are in a chip to establish a healthy airway
		The healthy bronchial SMCs treated with a chemical (IL-13) to establish an asthmatic airway
Liver	Liver on a chip	A liver on a chip consisted with hepatocytes, stellate cells, Kupffer cells, and endothelial cells
	Liver and heart on a chip	The human heart and liver tissues derived from hiPSC are on a chip
	Liver, heart and microvessels on a chip	The iPSC-derived liver cells added to a special biomaterial to create a liver module and integrated with similar modules with cardiac cells or microvasculature
Kidneys	Kidney on a chip	The tiny human blood vessels were placed on a device that mimics the environment inside the human kidney
Gastrointestinal system	Gut on a chip	It consists of gut tissue and nerves that stimulate the action of smooth muscle that controls gut motility
Female reproductive system	Female reproductive tract on a chip	The top layer of the chip consists of multiple components that individually support the cervix, fallopian tubes, uterus and ovaries, which are integrated on the platform. The bottom two layers hold the tiny channels and ports that mimic the human environment
Blood vessels	Blood vessels on a chip	The bioengineered vessels can deliver oxygen and nutrients into the tissue, and contract and dilate in the human body do to help regulate body temperature and metabolism
	Microvessels on a chip	Functional microvessels derived from iPSC form networks capable of fluid flow and deliver oxygen and nutrients to tissues
Fat (adipose)	Adipose tissue on a chip	Human adipose tissue is on a 3D chip system
Skin	Skin on a chip	The iPSCs-derived skin cells in a 3D chip have transformed into different types of skin cells and organized themselves into layers as actual human skin
Disease models	Metastatic breast cancer in the liver	The breast cancer cells are placed on the liver chip system seeded to create a model of metastatic breast cancer in the liver
	Rare heart and muscle condition	The cardiac chips using iPSCs from a patient with Barth syndrome displayed a weakened heartbeat and poorly organized structure that mimics a Barth syndrome patient's heart
	Tumor with blood vessels	The tumor chip includes functioning blood vessels, which tumors in the body need to stay alive

Various kinds of tissue-on-a-chips for drug screening programs developed by the Center for Advanced Translational Science (NCATS), US. hiPSC, human induced pluripotent stem cell. The information on the table was prepared by referring to the contents posted on the NCATS website (<https://ncats.nih.gov/tissuechip/chip/>) in August 2020.

goals of this new plan are (1) to support the development of *in vitro* disease models using primary tissue or patient-derived induced pluripotent stem cells in a tissue/organ chip platform, (2) to conduct preliminary tests to determine whether these models are related to real diseases, and (3) to carry out the validation of candidate drugs [74]. To date, the NCATS has developed many tissues-on-a-chips including brain [75], heart [76], muscle [77], lung [78], and liver [79] for a variety of drug screening programs. The tissue-on-a-chips for drug screening programs developed by the NCATS were summarized in Table 1.

The Chinese Academy of Sciences has developed an effective cell capture method for cytotoxicity experiments. Cells captured in the eight Micronet structures (microsieves) contained in each chamber can be tested under the same conditions for each group of toxicity tests [80]. The Biotechnology Research Institute, National Research Institute, National Research Council Canada has developed a cell-based chip that consists of eight culture chambers, and can detect the reactivity of cells according to the concentration and treatment time of an administered drug through impedance measurements [81]. At Simon Fraser University in Canada, cells obtained from the same host were also used to form cell retention structures in fine panels to conduct safety assessments of drugs from single cells, in order to minimize the sensitivity of each cell to The Dalian Institute of China produces HTS sensors, and at the same time processes various drugs at different concentrations to analyze the physiological response of cells using tubes [82]. The University of Leipzig, the Center for Biomedical Sciences and the Center for Biomedical Science in Germany, has developed electrochemical microcavity chips that can capture cells and allow 3D cell culture and drug testing without the adhesion of cells and electrodes [83]. It is possible to shorten the clinical trial process of anticancer drugs by modelling the metastasis process of cancer cells by providing a similar environment *in vivo*. Boston Children's Hospital and Harvard University's Wyss Institute developed a lung-on-a-chip similar to human lung structures [84]. This chip can be used in the differentiation and culture of stem cells, and is expected to be useful in the screening of lung-related disease drugs. Donghua University in China developed a chip that is stable for neurotransmitter transmission signals during neuron differentiation using graphene, a substance with high electrical conductivity, and can reproduce soft tissue *in vivo* through cross-connections with poly-dimethylacrylamide [85]. The chip can recognize not only the differentiation of nerve cells but also the efficiency of differentiation and the signal transmission system between neurons through electrical signals [86]. The University of Münster, Germany, has developed a biosensor to measure the toxicity of material in real-time using specific changes in the form of cells caused by impedance [87].

In the U.S., a sensor was developed which changes in response to electrochemical resistance values mammalian cells are cultured the chip is injected with water containing toxins. The chip is used not only in the pharmaceutical sector but also in the envi-

ronment, food, and military industries due to its convenience and portability [88].

In the Republic of Korea, the Korea Institute of Science and Technology has developed a sensor that measures and detects micro-wavelengths using multi-electrode array network activity between neurons [89]. The chip has the advantage of producing a lot of results under a variety of experimental conditions, and can be utilized in a variety of fields, such as the evaluation of safety and usability and the cell culture status of a drug. Sogang University produced an electrochemical biosensor capable of screening drugs with high sensitivity by fixing cells directly on a gold electrode [90].

Among the companies and schools currently studying organ-specific biomimetic organ-on-a-chips, the number of companies and schools developing skin chips is the largest (Fig. 8), because they can be used in the cosmetics industry.

Global technology trends in biomimetic organs-on-a-chip

To search for patents in the field of biomimetic chips, search keywords were organized and searched using chip-related synonyms, including keywords related to stem cells, organoids, human organs, or spheroids. In addition, five categories of organ- and disease-specific chips were classified: heart, liver, brain, lung, and multi-organ.

The annual trend of applications for effective biomimetic chip patents is 59 cases (94%) for multi-organ and 3 cases (5%) for lung, 1 case of liver (1%), and 0 cases of heart (0%), and brain (0%) (Fig. 9A). The overall trend is increasing. The number of patents centered on multi-organ technology increased significantly between 2012 and 2015 (Fig. 9B). There are many technologies that

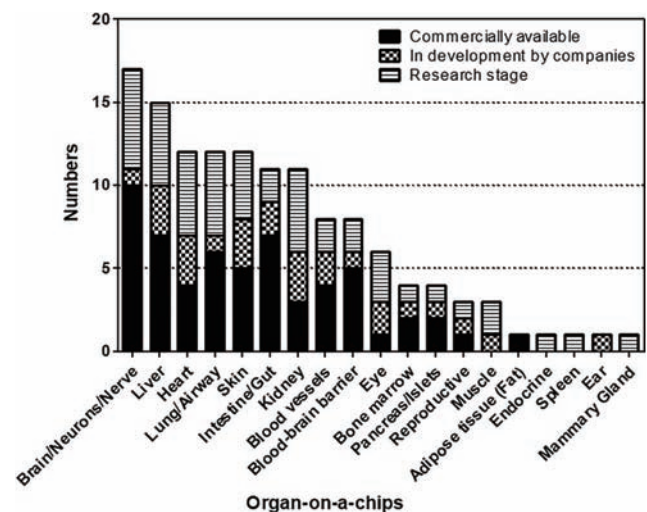


Fig. 8. Number of companies and schools developing organ-on-a-chips. Excerpted from the *Organs-on-chips: From Technologies to Market*, YOLE (April, 2017) and modified the graph format.

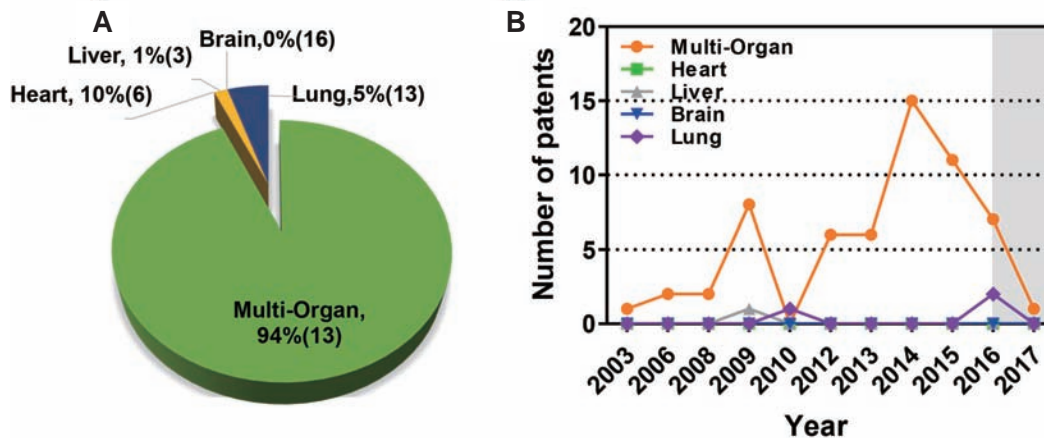


Fig. 9. Global technology trends of biomimetic organ-on-a-chip by organ. (A) Percentage of patents for biomimetic organ chips by type of organ from 2003 to 2017. (B) The annual trend of applications for effective biomimetic chip patents by organ types. The gray section represents the unpublished section of the patent.

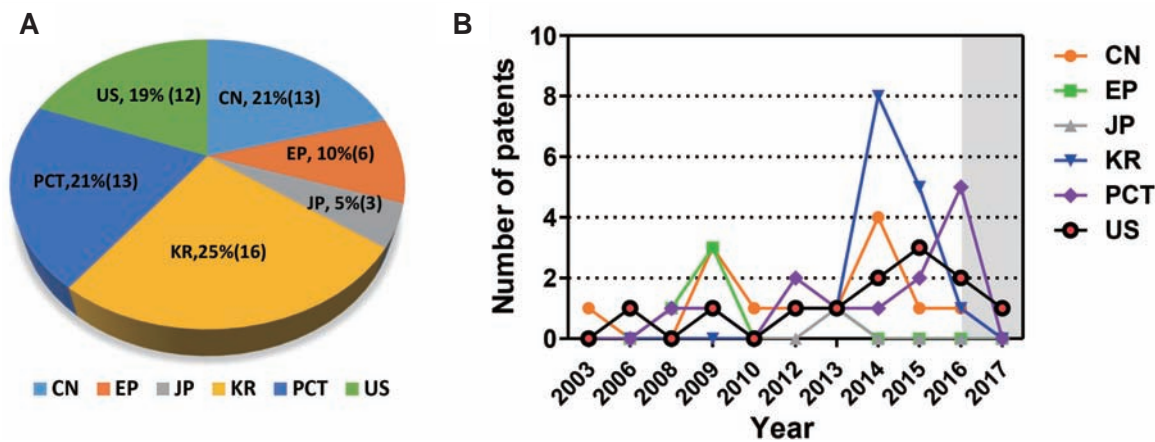


Fig. 10. Patent applications trend by country and year. (A) Percentage of patents for biomimetic organ chips by country from 2003 to 2017. (B) The annual trend of applications for effective biomimetic chip patents by country. The gray section represents the unpublished section of the patent. CN, China; EP, Europe; JP, Japan; KR, Republic of Korea; PCT, Patent Cooperation Treaty; US, United States of America.

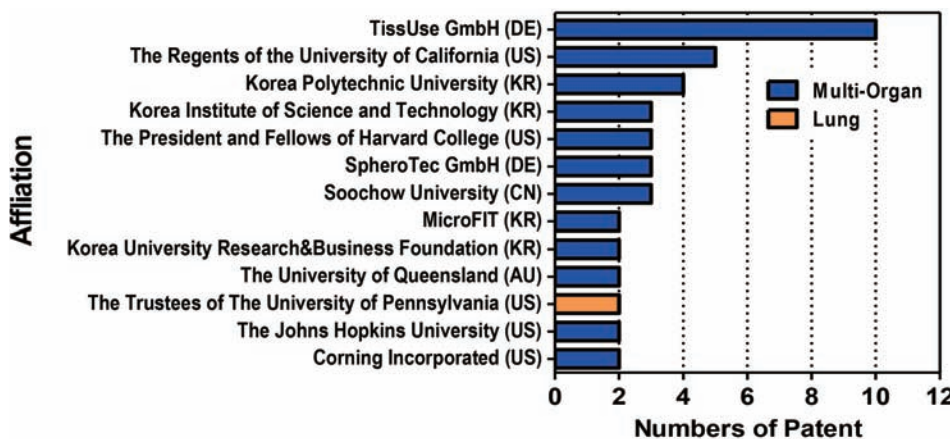


Fig. 11. Number of patent applications by institution. A summary of the patents on organ-related chip that are indexed in WIPS ON search site according to the 10 most frequent affiliation (13 institutions are reported due to a tie for tenth place). DE, Germany; AU, Australia; CN, China; KR, Republic of Korea; US, United States of America.

correspond to multi-organ as chip-oriented technology, rather than being limited to specific diseases. Overall, multi-organ technology accounted for more patents than those related to the heart,

liver, brain, and lungs.

Among valid patents for biomimetic organ-on-a-chip are 16 cases (25%) in the Republic of Korea, 13 cases (21%) in China, 13

cases (21%) from the Patent Cooperation Treaty (PCT), 12 cases (19%) in the U.S., 6 cases (9%) in Europe, and 3 cases (5%) in Japan (Fig. 10A). In particular, the number of patents filed in the Republic of Korea (KR) increased significantly in 2014, which can be seen as due to a high level of chip-making technology based on this country's excellence in electronic and material technologies. Recent trends are seen as active applications for China, and the United States of America in 2015, and the PCT in 2016 (Fig. 10B). As shown in Fig. 11, TissUse GmbH in Germany is the institute with the largest number of patent applications, followed by the Regents of the University of California in the U.S. and the Korea Polytechnic University in the Republic of Korea.

CONCLUSIONS

An organoid simulates each organ's environment in the complex human body or animal, and has similar characteristics to the actual organ. Organoids can produce more accurate results than two-dimensional cell models when assessing the reactivity of drugs. In addition, cell death, viability, division, and ligands according to the movement of molecules, can produce information such as the interactions of receptors, the activity of the genome and proteins, and organoid structure. Combining these advantages with tissue engineering, biomimetic tissue chips and biomimetic organs-on-a-chip are rapidly being developed. Organoids are available for alternative toxicity studies in non-clinical trials, in keeping with animal use regulations, and are more predictable at a lower cost than animal models. It is possible to overcome the differences between heterogeneous drug reactions that exist between humans and animals.

However, there are still challenges to overcome in order to develop organoids and biomimetic organs-on-a-chip. In order for this model to be utilized in drug evaluation technology, standards are needed for the management of cells, production technology, and quality evaluation of the chip models by the organ/drug, and there is a need to verify the test methods using known control drugs. If the regulations related to human stem cells and biomimetic organs-on-a-chip are set at a national level, it will be possible to test the potential effects of various substances, such as pre-clinical drugs, in the field of drug development, using non-animal models.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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