Stem Cells International

Stem Cell-Derived Organoids

Lead Guest Editor: Yujing Li
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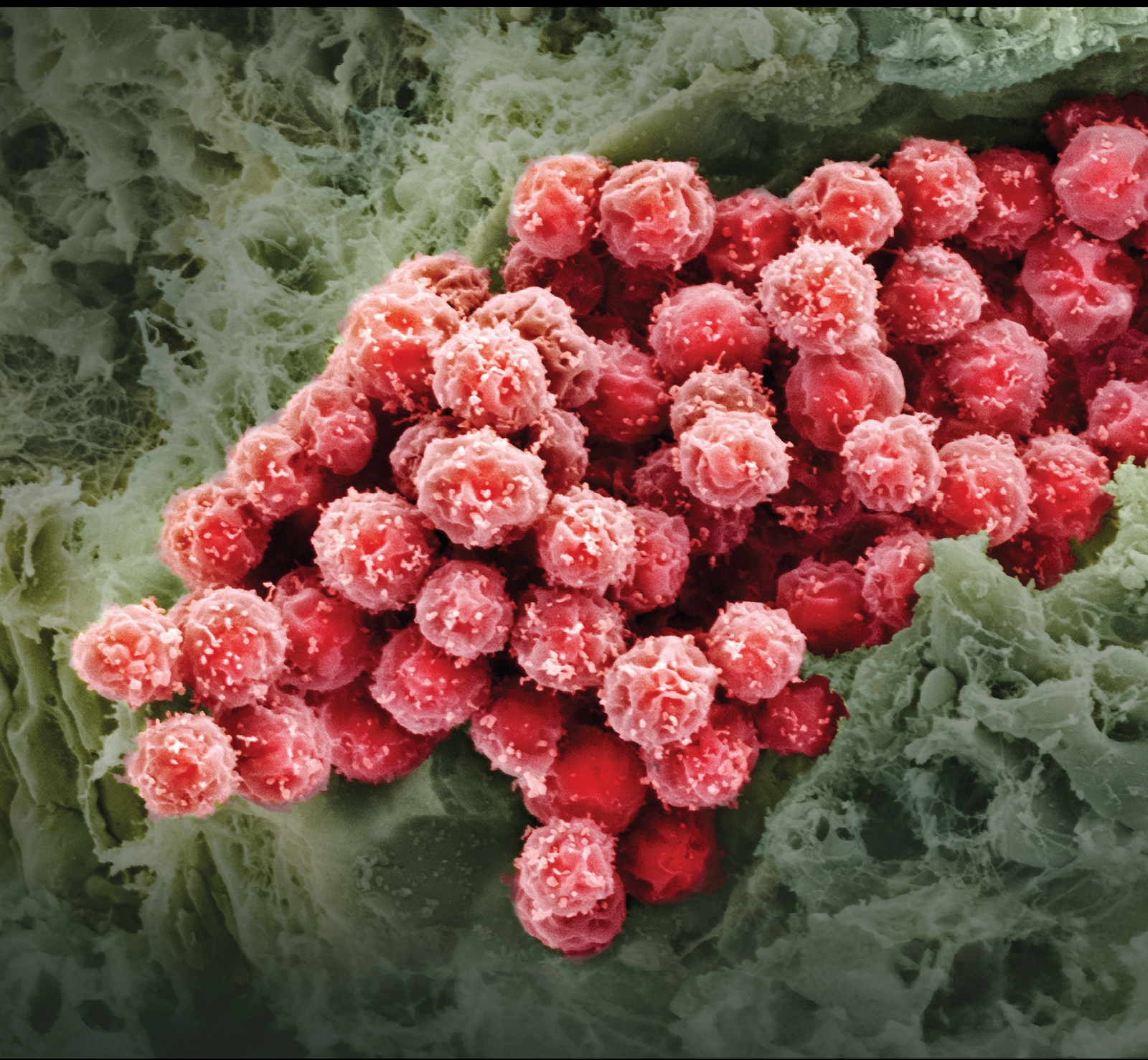

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Editorial

Editorial for Special Issue: "Stem Cell-Derived Organoids"

Yujing Li,[1] **Xuekun Li**,[2] **Yunhee Kang**,[1] **and Shu Jiang**[3]

[1]*Department of Human Genetics, Emory University School of Medicine, 615 Michael Street, Atlanta, GA 30047, USA*
[2]*The Institute of Translational Medicine and The Children's Hospital, School of Medicine, Zhejiang University, Kaixuan Road 268, Hangzhou, Zhejiang 310029, China*
[3]*1089 Chaguang Road, Nanshan District, Shenzhen City 518055, Guangdong Province, China*

Correspondence should be addressed to Yujing Li; yli29@emory.edu

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Conventionally, 2D-cultured cells and animal models, such as Drosophila, Zebrafish, and mice, have played a vital role in understanding the developmental and pathophysiological mechanisms underlying human diseases. However, the shortcomings of these models, including the lack of spatial information and genetic/physiological disparities between animals and humans, hinder the translation of research findings into clinical applications, particularly for complex and chronic diseases. This disparity becomes apparent in the underwhelming results of clinical trials, specifically those investigating mGluR5 antagonists for fragile X syndrome, which were based on insights gained from animal model research.

To overcome the limitations mentioned above, organoids have emerged as a promising alternative. Organoids are miniature and simplified versions of organs generated *in vitro*, faithfully recapitulating the microanatomy of their *in vivo* counterparts. By starting from human embryonic stem cells or human induced pluripotent stem cells, researchers can develop organoids that closely mimic human organ development, providing invaluable platforms for biomedical studies, investigations into genetic disorders and diseases, drug screening and development, and regenerative medicine.

Thus far, various types of organoids have been successfully generated, representing a wide range of organs, including the brain, lung, intestine, stomach, liver, pancreas, kidney, and more. Despite the initial advancements in 3D culture systems, there are still existing engineering and conceptual challenges that hinder the efficiency and quality of organoids in accurately mimicking human organ development—an essential aspect for therapeutic purposes. Key limitations in current *in vitro* culture systems include the lack of vascularization, cellular diversity, tissue maturity, and overall functionality.

Recent advancements have brought about exciting developments in the establishment of functional vascular-like networks within organoids. These breakthroughs have been achieved through various approaches, including the addition of vascular endothelial growth factor and wingless-related integration site 7a (Wnt7a) to the culture media, coculturing with endothelial cells (ECs), culturing organoids on ECs beds, transplanting them into vascularized animal tissues, or employing genetic engineering techniques such as inducing the expression of human ETS variant 2. The vascularized organoids could more accurately mimic features of their counterparts *in vivo*, exhibiting better organization, reduced hypoxia and apoptosis. This breakthrough sheds light on the generation of organoids with higher efficiency and greater resemblance to human organogenesis.

These breakthroughs have served as a catalyst for scientists to redirect their attention from animal models to human-centric models, with the objective of studying pathological mechanisms in the context of human genetic disorders and diseases with greater precision. As a result, it is of paramount importance to initiate a special issue that specifically highlights the advancements made in the generation and utilization of organoids derived from stem cells.

In this special issue, a total of 10 articles were published, including four original research and six review articles covering topics on the generation techniques of human organoids and their diverse applications in biomedical studies.

The intestine plays crucial roles in the digestive system and is highly prone to various diseases caused by enteric pathogens. However, studying the pathophysiology of these pathogens within host cells using animal models has been extremely challenging. Fortunately, the emergence of human intestinal organoids (HIOs) holds great promise as they

faithfully mimic key features of the human intestinal mucosa. This collection comprises four articles that specifically center around HIOs. It includes two research articles authored by Bruegge et al. and Kandilogiannakis et al., as well as two review articles by Hentschel et al. and Hisa et al. Bruegge et al.'s article investigated bacterial stimulation experiments and revealed that cryopreserved HIOs were not suitable replacements for animal models. This was attributed to their premature properties and heightened proinflammatory response to bacterial infection. On the other hand, Kandilogiannakis et al. determined that early passages of HIOs provided optimal opportunities to study inflammatory and fibrotic responses during HIO development. Regarding the review articles, Hentschel et al. provided a comprehensive update on HIO-based tissue engineering. This included insights into the latest pathophysiological characteristics observed in infected HIOs, as well as an exploration of the limitations associated with this innovative *in vitro* model. Hsia et al., on the other hand, focused on the advancements made in the transplantation of HIOs and various other organoids, considering the limitations posed by the restricted available space.

Exosomes, as vital components of the paracrine pathway, play a pivotal role in intercellular communication and the transfer of genetic material. Their involvement in bone injury repair is significant, and their relevance in tissue engineering has been increasingly acknowledged.

Shao et al. found that exosomes derived from mesenchymal stem cells (MSCs) obtained from the infrapatellar fat pad enhance the proliferation of rabbit chondrocytes. Interestingly, exosomes derived from MSCs that were pretreated with KGN (wingless-related integration site 7a (Wnt7a)) exhibited an even more remarkable stimulation of proliferation. Moreover, the combined treatments demonstrated significant potential in facilitating the repair of articular cartilage damage, thus illuminating a promising strategy for future therapeutic applications.

Zhang et al. offer an up-to-date and comprehensive overview of the methodological aspects involved in the generation of adipose-derived stem cells (ADSCs). Additionally, it emphasizes significant advancements, perspectives, and challenges pertaining to the preclinical and clinical applications of ADSCs in the field of regenerative medicine.

Zhao et al. highlight the advances in salivary gland organoids, discussing both the advancements and limitations associated with their development. The authors address the promising potential of salivary gland organoids in the field of regenerative medicine.

In addition, two independent review articles by Ma et al. and Xu et al. focus on the recent progress made in the generation methodology and applications of human brain organoids. These articles cover a wide range of topics, including basic biological studies, disease modeling, and high-throughput drug screening using brain organoids. These reviews provide valuable insights into the current state of research and the potential of human brain organoids in various areas of study.

As the articles in this issue highlight, human organoids offer fundamental advantages over *in vitro* cells and animal models in biomedical research, and they hold immense promise for potential clinical therapy and regenerative medicine. Continued development and innovation in the generation of human organoids will enable them to replicate the features of their *in vivo* counterparts more accurately. By ensuring high quality, organoids can significantly contribute to the understanding of the physiopathology underlying human diseases, enhance the efficiency of drug screening, advance regenerative medicine, and provide a novel avenue for clinical therapy.

The authors of this special issue hold a strong desire that the research and review articles published here will provide valuable and convenient information for readers interested in this closely related research field, both technically and scientifically.

Conflicts of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Authors' Contributions

YL drafted and UK and XL revised the editorial. All authors approved the final version.

Yujing Li
Xuekun Li
Yunhee Kang
Shu Jiang

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Review Article

Human Brain Organoid: A Versatile Tool for Modeling Neurodegeneration Diseases and for Drug Screening

Cuili Ma,[1] Hwanwook Seong,[2] Xiaowei Li,[3] Xiao Yu,[4] Shunliang Xu,[5] and Yujing Li[2]

[1]*Department of Internal Medicine, Qingdao Agricultural University Associated Hospital, Qingdao 266109, Shandong Province, China*
[2]*Department of Human Genetics, Emory University School of Medicine, 615 Michael St, Atlanta, GA30322, USA*
[3]*School of Life Sciences, Nanjing University, 163 Xianlindadao Rd, Nanjing 210023, China*
[4]*Department of Neurology and Department of Nutrition, The Second Hospital Cheeloo College of Medicine, Shandong University, 247 Beiyuan Road, Jinan 250033, Shandong Province, China*
[5]*Department of Neurology, The Second Hospital Cheeloo College of Medicine, Shandong University, 247 Beiyuan Road, Jinan 250033, Shandong Province, China*

Correspondence should be addressed to Shunliang Xu; slxu@live.com and Yujing Li; yli29@emory.edu

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Clinical trials serve as the fundamental prerequisite for clinical therapy of human disease, which is primarily based on biomedical studies in animal models. Undoubtedly, animal models have made a significant contribution to gaining insight into the developmental and pathophysiological understanding of human diseases. However, none of the existing animal models could efficiently simulate the development of human organs and systems due to a lack of spatial information; the discrepancy in genetic, anatomic, and physiological basis between animals and humans limits detailed investigation. Therefore, the translational efficiency of the research outcomes in clinical applications was significantly weakened, especially for some complex, chronic, and intractable diseases. For example, the clinical trials for human fragile X syndrome (FXS) solely based on animal models have failed such as mGluR5 antagonists. To mimic the development of human organs more faithfully and efficiently translate in vitro biomedical studies to clinical trials, extensive attention to organoids derived from stem cells contributes to a deeper understanding of this research. The organoids are a miniaturized version of an organ generated in vitro, partially recapitulating key features of human organ development. As such, the organoids open a novel avenue for in vitro models of human disease, advantageous over the existing animal models. The invention of organoids has brought an innovative breakthrough in regeneration medicine. The organoid-derived human tissues or organs could potentially function as invaluable platforms for biomedical studies, pathological investigation of human diseases, and drug screening. Importantly, the study of regeneration medicine and the development of therapeutic strategies for human diseases could be conducted in a dish, facilitating in vitro analysis and experimentation. Thus far, the pilot breakthrough has been made in the generation of numerous types of organoids representing different human organs. Most of these human organoids have been employed for in vitro biomedical study and drug screening. However, the efficiency and quality of the organoids in recapitulating the development of human organs have been hindered by engineering and conceptual challenges. The efficiency and quality of the organoids are essential for downstream applications. In this article, we highlight the application in the modeling of human neurodegenerative diseases (NDDs) such as FXS, Alzheimer's disease (AD), Parkinson's disease (PD), and autistic spectrum disorders (ASD), and organoid-based drug screening. Additionally, challenges and weaknesses especially for limits of the brain organoid models in modeling late onset NDDs such as AD and PD., and future perspectives regarding human brain organoids are addressed.

1. Introduction

Due to the inaccessibility of human organs/tissues/systems, biomedical studies on human development and disease pathophysiology are conducted only in animal models or cell-based in vitro assays. Animal models, particularly mouse models, have contributed significantly to gaining insight into the developmental and pathophysiological understanding of human diseases. However, the large evolutionary distance between mice and humans led to a discrepancy in genetic, anatomic, and physiological aspects between animal models and humans. This makes the existing animal models unable to efficiently simulate the development of human organs and systems at the anatomical and pathophysiological levels. Given that clinical trials serve as the fundamental prerequisite for human disease therapy, all the clinical trials designed and conducted on animal models significantly weaken the translational efficiency from the basic biomedical research outcomes to clinical therapy. To mimic the development of human organs more faithfully and facilitate translational efficiency from benchwork to clinical trials, in vitro organoid models derived from the stem cells have been established as a more effective and reliable platform relative to animal models. Organoids are characterized as miniaturized and simplified versions of an organ generated in vitro but could recapitulate key features of human organ development. As such, organoids pave a novel avenue for in vitro human disease models, an invaluable platform for biomedical studies and pathological investigation of human diseases. Meanwhile, human organoids have been applied for drug screening and successful achievements have been made. Drug development has been acknowledged as the key issue for advancing clinical therapy, particularly for diseases such as cancer, heart diseases, and neurological disorders. Therefore, a rapid and reliable drug screening strategy is essential to identify "hit" compounds prior to assessing the metabolic and toxicologic mechanisms both in vitro and in vivo. To develop an efficient operating system for primary screening, the cell-based efficacy and toxicity assay has been extensively used. However, the cell-based drug assays fail to recapitulate the response of human organs or systems to the compound agents, leading to a large-scale loss of resources in drug discovery and a low rate of successful cases.

As the headquarter of the human body, the brain, structurally composed of a complex architecture, performs cognitive functions to orchestrate the normal functions of all the systems and organs via extraordinarily intricate networks [1–3]. Developmental abnormality in the structure of the brain could lead to severe neurological or psychiatric disorders. While the limited access to primary patient brain tissues provides the main source of gaining insight into disease pathology, the information it represents contributes to an understanding of consequential mechanisms [4]. Vertebrate animal models such as mice provide important resources for the dissection of the developmental mechanisms and pathogenesis of disorders [5]. However, when we evaluate the significance of the discovery made in the mouse for translation to clinical application, it is important to remember the dramatic evolutionary distance between levels of mammals and humans as well as the subtle differences of the nervous system in both morphology and complexity [6]. It is plausible to say that the animal models fail to recapitulate the numerous key features unique to the development of the human nervous system and neurological disorders.

Central nervous system (CNS) diseases are much more complicated relative to other organ disorders, leading to lower efficiency for modeling CNS diseases via animal models. Faithfully mimicking the human brain's development, function, and susceptibility to disease by using organoids could significantly enhance translation efficiency from biomedical studies to clinical trials [7, 8]. Architecturally, the 3-D brain organoid consists of a majority of all known human brain cell types such as progenitor, neuronal and glial cells [9–14].

Thus far, the pilot breakthrough has been made in the generation of numerous types of organoids representing different human organs as reviewed recently (Table 1) [15–20]. These brain organoids could partially recapitulate some aspects of human brain genesis and development, potentially modeling developmental neurological disorders, such as FXS, Alzheimer's disease (AD), Parkinson's disease (PD), and autistic spectrum disorders (ASD) (Table 2). In addition, brain organoids were also employed to model Schizophrenia [21, 22], Down syndrome [23], Lissencephaly [24–27], Rett syndrome [28], and Timothy syndrome [29]. Brain organoids have also been utilized for modeling parental alcohol and drug abuse [30, 31]. The organoids generated from the patient with Niemann-Pick disease type C (NPC), a neurodegenerative lysosomal storage disorder caused by genetic mutations could mimic the phenotype of the NPC patients [30]. Some cortical organoids were used for modeling the impairment of molecular subtype specifications caused by ectopically activating cellular stress pathways under the conditions of cell stress [32]. Some of the brain organoids were used for modeling the key features of ionizing radiation-induced DNA damage in human neurons to understand the repair mechanisms [33]. Some patient-derived glioma cerebral organoids have been developed for disease modeling to understand glioma biology and predict responses to chemotherapy drugs [34–37]. In this paper, we focus on the recent advances in human brain organoid models for neurological disorders including AD, PD, ASD, FXS as well as organoid-based drug screening. Additionally, we also discuss the challenges, weaknesses, and future perspectives of organoid research.

2. Brain Organoid-Based Modeling of Neurological Disorders

2.1. Modelling of AD Using Brain Organoids Derived from AD Patients. AD is a late-onset (at age over 65) disorder that is not caused by natural aging. It accounts for 60-80% of dementia cases associated with progressive memory loss and other cognitive abilities. Pathologically, AD is characterized by the accumulation of protein aggregates, tau plaques, and synaptic dysfunctions. Relative to cell and animal models, human cerebral organoids can efficiently mimic the

Table 1: Advances in generation of organoids.

(a)

Culture medium/supplement/culture strategy	Regions/ organoids type	Key features	References
SHH, FGF8	Midbrain simBOs	High efficiency, high homogeneity, easy to specify	[58]
CHIR99021	Midbrain-like MLOs	Robust generation Homogenous distribution of mDAs, other neuronal subtypes, and functional glial cells, such as astrocytes and oligodendryocytes	[61]
WNT3A and mixed medium with 1 : 1 of fresh and supernatant derived from interfollicular epidermal SCs	Epidermal organoids	Functional with polarity	[120]
RSPO1, WNT3A, WNT7A	Endometrial organoids	Endometrial disease facilitate growth of endometrial disease organoids and the long-term expansion	[121]
WNT and nodal antagonists	Hippal/CB organoids	Original differentiation method Low efficiency of O2 and nutrient diffusion	[122]
Dkk1 and LeftyA Floating culture with 40% O2 and 5% CO2 CHIR 99021, BMP4	Hipp/cortex organoids		[10, 13, 24119–121]

(b)

Culture device	Key features	References
1. Spinning bioreactor	High cost and require a high volume of culture medium	[122]
2. Multiple-well culture plates with orbital shakers	Reducing the cost and consumption of the culture medium successful generation of cerebral organoids	[123, 124]
3. Miniaturized multiwell spinning bioreactor	Facilitate the establishment of brain region-specific organoids that mimic the dorsal forebrain, midbrain, and hypothalamus	[14, 95]
4. Collagen hydrogel systems	Consisting of interconnected excitatory and inhibitory neurons with supportive astrocytes and oligodendrocytes fiber for bioengineered organoids A highly interconnected neuronal network established in organoids at a macroscale tissue format. More importantly, the engineered organoids share structural and functional similarities with the fetal brain, potentially allowing for the study of neuronal plasticity and modeling of disease	[8]
5. Carbon fibers (CFs) for midbrain organoids	The porosity, microstructure, or stability CF scaffolds could improve efficiency in iPSC differentiation within organoids relative to the PLGA scaffolds. The midbrain organoids generated in the CF scaffolds could more efficiently enhance terminal differentiation and the survival of midbrain dopaminergic (mDA) neurons.	[59]
6. Brain organoids	The modified hydro-Matrigel with an interpenetrating network (IPN) of alginate has been employed to maintain the mechanical microenvironment for brain organoids, conferring the viable growth environment with the characteristic formation of neuroepithelial buds.	[125, 126]
7. Brain organoids	The platform of "tissue-like" cyborg stretchable mesh nanoelectronics were invented to provide seamless and noninvasive coupling of electrodes to neurons within developing brain organoids, enabling continuous recording of single-cell action without interruption to brain organoid development	[127]

(c)

Coculture of organoids	Key features	References
Co-culture of cancer organoids with other non-tumor cells	Tumor organoids could get other cell types of cells and tissues	[128, 129]

Table 1: Continued.

Coculture of organoids	Key features	References
Vascularization of organoids		
1. Direct transplantation of the brain organoids into mouse brains		[32, 34, 130]
2. Coculture of brain organoids with epithelial cells followed by transplantation into mouse brains		[131]
3. Genetic operation-based vascularization	Expression of human ETS variant 2 (ETV2) in human cortical organoids (hCOs), led to generation of the functional vascular-like vessels in the vascularized hCOs (vhCOs), improving organization, alleviating hypoxia, and reducing apoptosis	[132]
4. BVO cells infiltrate into brain organoids	High efficiency to generate vascularized human brain organoids	[133]
5. The microfluidic chips-based coculture with epithelial cells		[134]
6. Vascularized spheroid using an injection-molded microfluidic chip	By coculturing the spheroids derived from induced neural stem cells (iNSCs) with perfusable blood vessels, the vascularized spheroid was generated. The vascularized spheroid network significantly improved spheroid differentiation and reduced apoptosis.	[99]

(d)

Differentiation methods		
Unguided strategy	Generation of brain organoids with mixed cell lineages of forebrain, midbrain, hindbrain, and retina, enabling the organoids to grow with minimum external interference High variability and heterogeneity	[11, 31, 95]
Guided strategy	Directed differentiation to generate brain region-specific organoids, such as cerebral cortex, hippocampus, midbrain, and cerebellum	[10, 13, 14, 119, 135, 136]
Fused culture technologies for integration of different regions of the organoids	More closely resembling the complexity of the brain in identity, architecture, and interaction manners enhanced the formation of microcircuits with the local excitatory neurons	[123, 124, 135]
Long-term propagation, storage, and regrowth following the frozen and thaw cycles	CRISPR-Cas9-based knock-in of the mutant $KRAS^{G12D}$ allele into human colon $APC^{-/-}$ organoids	[115, 137]
Application of 3D printing technology in	Enabled an engineered organ to maintain the spatial arrangement	[39, 134, 138, 139]
Organoids-on-a-chip based approach to	Could remove the dead cells via connecting with an external pumping	[140]
Generate the tube-shaped epithelial organoids	System, extending tissue lifespan and enabling the colonization of organoid tubes with microorganisms to model the host–microorganism interactions	
Generation of microglia cell-containing microglia cerebral organoids	Microglia were naturally developed in cerebral organoids and displayed similar characteristic ramified morphology as in normal fetal brains.	[106, 141]
Generation of microglia-containing hCOs (mhCOs)	Microglia-containing hCOs (mhCOs) were generated via overexpression of the myeloid-specific transcription factor PU.1 in cortical organoids. The mhCOs have become an efficient tool for functional investigation of microglia in neurodevelopmental and neurodegenerative disorders, such as AD	[108]

key features of the human brain. Therefore, many *in vitro* organoids models have been developed for AD modeling ([38–40] [37, 41–45].

Most brain organoids for AD modeling harbor familial patient-specific genomic backgrounds including mutations, deletions, and insertions. Importantly, most of these AD patient-derived brain organoids are familial isogenic lines and are matched with normal brain organoids to serve as controls (Figures 1(a) and 1(b)). These organoids could partially recapitulate the key pathological features of the AD patient's brain on a molecular, cellular, and network-connectivity basis. Therefore, these organoids function as pilots for understanding the pathophysiological mechanisms on a patient-specific basis. Furthermore, these organoids could be employed as a platform for drug screening. It is highly expected that the drugs identified and validated to increase neuronal activity could contribute to therapy based on the patient-specific personalized medicine. The advances in AD organoid models within the recent two years are highlighted below.

Table 2: Current brain organoids for modeling of neurological disorders.

Organoid type/brain regions	Disease	References	Main discovery
Human forebrain organoid	FXS organoids with	[6]	Dysregulated neurogenesis, neuronal maturation, and neuronal excitability in the forebrain loss of FMRP. Inhibition of the PI3K signaling could rescue developmental deficit of the FXS forebrain organoids
Cortical brain organoid cortical organoids	FXS	[73]	Increased number of glial cells, and bigger organoid size compared to normal person
Cortical brain organoid	FXS	[74]	FXS organoids bear higher percentage of $Ki67^{+}SOX2^{+}$ proliferative cells PI3K functions as a key driver of downstream dysregulation of both translations and cell proliferation in early NPCs.
Cerebral organoid	ALS/FTD	[142]	Recapitulates mature cortical architecture and early molecular pathology of C9ORF72 ALS/FTD. Increased levels of the autophagy signaling protein P62 in astroglia. Accumulation of DNA damage, poly(GA), and nuclear pyknosis in deep layer neurons
Sensorimotor organoid	ALS	[143]	Diversity of neuronal derivatives, such as motor, sensory neurons, astrocytes, and mesodermal derivatives, including vasculature, microglia, and skeletal muscle. The NMJs connect the motor neurons and skeletal muscle, but the NMJs were defected in ALS organoids. Altered ability for deriving the NMJ synapse and cell diversity that exert autonomous and noncell autonomous effects on motor neurons
Schizophrenia (Scz) cerebral		[144]	In the Scz organoids, the progenitor survival significantly changed led to disruption of neurogenesis, ultimately generating fewer neurons within developing cortical fields compared to the normal organoids.
Cerebral organoids (iCOs)	AD	[145]	Miniaturized AD pathological models and CRISPR-Cas9-edited isogenic lines established a high-content screening (HCS) system, and the FDA-approved drugs were tested for the blood–brain barrier-permeability
Cerebral organoids whole brain	AD	[38]	The organoids from patients affected by familial AD or DS displayed pathological features of AD, such as accumulation of structures like amyloid plaques and neurofibrillary tangles, but nondetectable in the control organoids.
Cerebral organoids whole brain	AD	[53]	Significant apoptosis, impaired synaptic integrity, enhanced stress granules and disrupted RNA metabolism were detected in cerebral organoids (CO) with *APOE* $\varepsilon4/\varepsilon4$ genotype from AD patients. Conversion of *APOE4* to *APOE3* ameliorated the *APOE4*-associated phenotypes in Cos from AD patients. *APOE4*-related degenerative pathways were assumed to contribute to AD pathogenesis.
Cerebral organoids whole brain	AD	[56]	CKD-504, a highly BBB-penetrating HDAC6 inhibitor, significantly reduced tau via acetylation in AD patient-iPSC-derived brain organoids, dramatically attenuating pathological tau and ultimately rescuing the synaptic pathologies
Cerebral organoids whole brain	AD	[48]	Cerebral organoids (Cos) generated from PITRM1-KO iPSCs recapitulated AD pathological features such as the accumulation of protein aggregates, tau pathology, and neuronal cell death. ScRNA-seq discovered mitochondrial function defect in all cell types in COs with PITRM1-KO. PITRM1-linked neurodegeneration caused by defects of mitochondrial presequence processing induce an early activation of UPRmt, supporting a mechanistic link between mitochondrial function and common neurodegenerative proteinopathies.
Cerebral organoids whole brain	AD	[46]	Compared with the isogenic control organoids, AD organoids with PSEN2N141I mutation recapitulated an AD-like pathology at the molecular, cellular, and network level, such as a higher $A\beta42/A\beta40$ ratio and enhanced neuronal hyperactivity. Altogether suggests these isogenic organoids as a promising tool for the pathological study of AD.
Cerebral organoids whole brain	AD	[54]	An episomal plasmid vector derived from EBV based simple and versatile genetic engineering was employed to efficiently generate organoids harboring a normal tau protein with fluorescent tag vs. a mutant genetic form (P301S) of tau that leads to fronto-temporal dementia. The harbored plasmid did not affect differentiation, and the isogenic organoid lines were stable for more than 30 passages expressing either normal or mutant form. The cerebral organoids manifested hyperphosphorylation of the tau protein, a pathologically relevant phenotype, contributing to disease

Table 2: Continued.

Organoid type/brain regions	Disease	References	Main discovery
			modeling, personalized medicine and potentially translating to clinical therapeutics.
Cerebral organoids whole brain	AD	[55]	The enhanced spontaneous action potentials, slow oscillatory events (~1 Hz), and hypersynchronous network activity were detected in the AD neuronal organoids. The dual-allosteric NMDAR antagonist NitroSynapsin, revoked the hyperactivity, but the FDA-approved drug did not, suggesting the AD organoid models could be efficient tool for drug screening and modeling of the related synaptic damage in AD.
Cortical organoids cortex	AD	[51]	Time and spatial patterns of tau expression at a molecular level was compared during brain development using the iPSC-derived cortical organoids and developing human brains. Neuronal maturation led to the dramatic elevation of tau mRNAs, while low expression levels were observed in SVZ radial glia and deep white matter intermediate progenitors. This system could help further study on the pathophysiological mechanism of triggering and enhancing tau expression, simplifying the identification of therapeutic targets for tauopathy-based neurodevelopmental disorders.
Human midbrain-like organoids (hMLOs)	Early-onset PD	[66]	DNAJC6 mutation vs. CRISPR-Cas9 manifestation of key PD features, pathologic neurodevelopment defects, DNAJC6- mediated endocytosis defect, impairment of the WNT-LMX1A signal during the mDA neuron development reduced *LMX1A* expression during development, generation of vulnerable mDA neurons
Midbrain organoids	PD	[57]	The first organoid model for an idiopathic form of PD and healthy volunteers were generated by the Sendai viral vector mediated transduction. The mature organoids manifested statistical differences in the expression levels of neuronal early and late markers between organoids from PD patient and healthy volunteer. Altogether suggests that PD human organoids could be potentially suitable for modeling PD and cellular interactions within the human brain.
Midbrain organoids	PD	[63]	Isogenic 3D midbrain organoids with or without a PD-associated LRRK2 G2019S mutation recapitulate the pathological hallmarks observed in patients with LRRK2-associated PD. The protein-protein interaction network in mutant organoids revealed that TXNIP, a thiol-oxidoreductase, is essential for development of LRRK2-associated Parkinson's disease in a 3D environment. Altogether suggests the potential of 3D organoid for modeling sporadic PD in advancing therapeutic discovery.
simBOs	PD	[58]	Simplified brain organoids (simBOs), composed of mature neurons and astroglial cells were rapidly generated in 2 weeks and have more homogeneous properties. The SimBOs facilitates the conversion of pNSCs to mature neuronal systems in the context of neurotransmitter release, synaptic vesicle formation, ion channels, calcium signaling, axonal guidance, extracellular matrix organization, and cell cycle. The simBOs could easily be specified into midbrain-like simBOs by treatment with Shh and FGF8. Midbrain-like simBOs from a PD patient (LRRK2G2019S)-derived pNSCs manifested disease-associated phenotypes in terms of increased LRRK2 activity, decreased dopaminergic neurons, and increased autophagy. Treatment with the LRRK2 inhibitor, PFE-360, relieved the phenotype of Parkinson's disease in midbrain-like simBOs. Taken together, these approaches could be applied to large-scale disease models and alternative drug-testing platforms.
Midbrain organoids	PD	[69]	The patient-based midbrain organoid model of PARK7-linked PD was created, and aberrant U1-dependent splicing was detected, causing a drastic reduction in DJ-1 protein and, consequently, mitochondrial dysfunction. Targeting defective exon skipping with genetically engineered U1-snRNA recovered DJ-1 protein expression in neuronal precursor cells and differentiated neurons. Combinatorial treatment with the small-molecule compounds rectifier of aberrant splicing (RECTAS) and phenylbutyric acid, could restore DJ-1 protein and mitochondrial dysfunction in patient-derived fibroblasts as well as dopaminergic neuronal cell loss in mutant midbrain organoids. Therefore, this system could

Table 2: Continued.

Organoid type/brain regions	Disease	References	Main discovery
			become an alternative strategy to restore cellular abnormalities in in vitro models of PD and provides a proof of concept for neuroprotection based on precision medicine strategies in PD.
Midbrain organoids	PD	[59]	The physicochemical properties of carbon fibers (CFs) scaffolds make CFs more advantageous over the conventionally applied PLGA scaffold in improving the efficiency of iPSC differentiation within organoids. The organoid generated using CFs scaffolds were used for screening genes that expressed during the organoids differentiation at crucial stage of brain development. Correlation between PITX3, one of the essential factors for terminal differentiation and the survival of mDA neurons, and TH gene expression was detected. Thus, it is plausible to suggest that organoids containing mDA neurons formed on CFs could be suitable for investigation of the midbrain-associated NDD such as PD.
Midbrain organoids	PD	[60]	A fast and robust method to generate human midbrain organoids and incorporate microglia together with astrocytes into the organoids. These ratio-defined and three cell type-based organoids are suitable for the study on toxicology and pathophysiology of the CNS.
Midbrain organoids	PD	[70]	A midbrain PD organoid model was generated and applied to test and characterize the neurotoxic effect on dopaminergic neurons via a machine learning-based analytical method. This approach has been used for HCI cell profiling and toxicity evaluation in midbrain organoids treated with/without 6-OHDA, the neurotoxic compound. This platform could be employed for modeling PD and drug screening to identify the neurotoxic compounds
Midbrain organoids	PD	[61]	The homogeneous midbrain-like organoids (MOs) were generated with mature architecture of midbrain dopaminergic (mDA) neurons, other neuronal subtypes, and functional glial cells such as astrocytes and oligodendrocytes but no microglias. The MLOs are extremely sensitive to 1-methyl-4-phenyl-1,-2,3,6-tetrahydropyridine that conferred the mDA neuron-specific cell death.
Midbrain organoids	PD	[72]	The midbrain organoids generated by Renner et al., could recapitulate architecture, size, cellular composition, homogeneous morphology, aggregate-wide synchronized neural activity, and global gene expression. These midbrain organoids have been employed to create a scalable and HTS-compatible platform for drug screening and evaluation with criteria of HCI and RNA-seq at the single-cell level, generating the reproducible prediction of the drug effects on neurological disorders of PD.
Cerebral organoids	Schizophrenia	[21]	Cerebral organoids of four controls and three schizophrenia patients to model the first trimester of in utero brain development. It was found that progression of the cortical malformation was associated with aberrant FGFR1 signaling
Forebrain organoids	Schizophrenia	[22]	Schizophrenia patient derived forebrain organoids to model human brain development. It was found that disrupting DISC1/Ndel1 complex formation contributes to brain development of schizophrenia patient
Telencephalic organoids	ASD	[12]	The cerebral telencephalic organoids generated from affected families were utilized for modeling the idiopathic ASD for the first time with organoids from the unaffected family members as control. Molecularly, the altered gene expression network could contribute to the pathogenesis of ASD such as the enhanced expression of FOXG1, which leads to overproduction of GABAergic inhibitory neurons. Cellularly, the synaptic growth, cell cycle, and imbalance in GABAergic/glutamatergic neuron differentiation were significantly altered in the ASD organoids.
Cerebral organoids	ASD	[34]	Human cerebral organoids carrying the mutations of Rab39b, a small GTPase associated with X-linked macrocephaly, ASD, and intellectual disability, respectively. Cellularly, the proliferation of NPCs was promoted but the differentiation was impaired in the RAB39b mutant cerebral organoids, and ultimately the size of the organoids, whereby resembling the trait of ASD. These organoids have provided a cellular and molecular platform to study the pathophysiology of ASD and drug screening.

Table 2: Continued.

Organoid type/brain regions	Disease	References	Main discovery
Cerebral organoids	ASD	[68]	Cortical organoids (mCOs) from CNTNAP2 KO mouse dysregulated generation of the GABAergic inhibitory neurons at cellular level and the transcriptional network involved in GABAergic neurogenesis at molecular level. And the dysregulations could be rescued by treatment with retigabine, an antiepileptic drug, indicating the potential Cntnap2 as a therapeutic target for clinical therapy of ASD

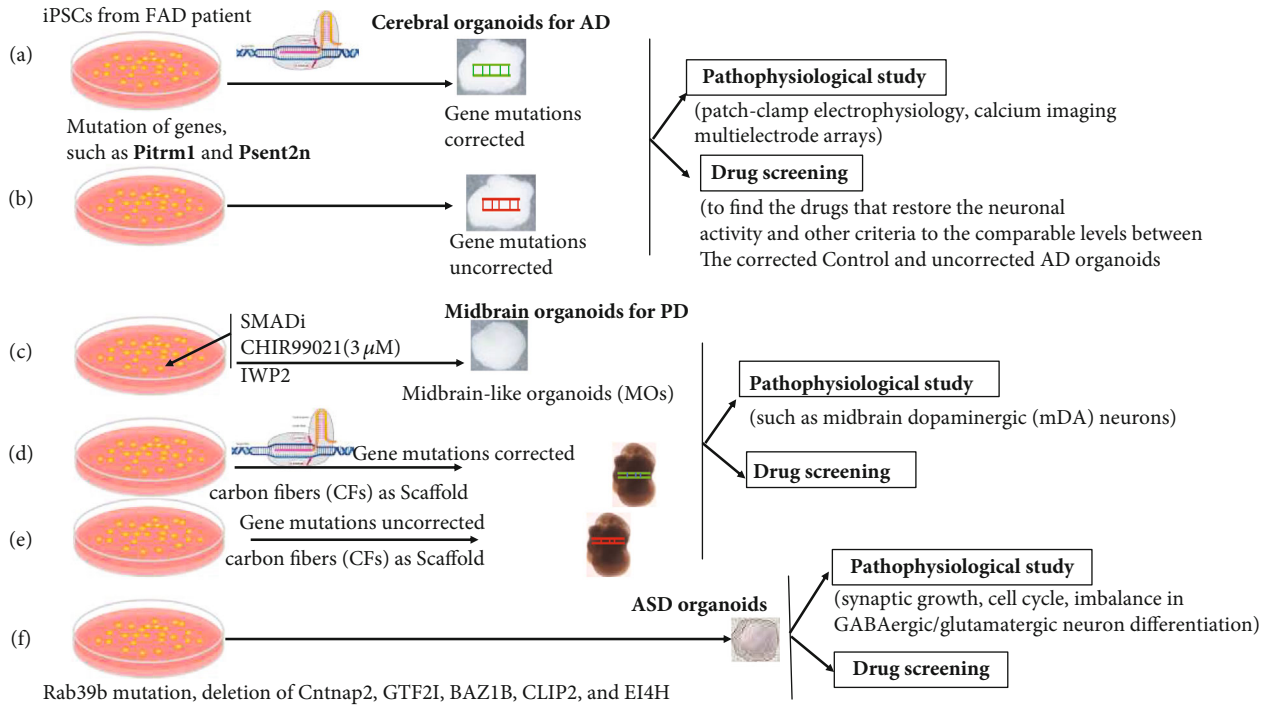

Figure 1: The AD, PD, and ASD isogenic organoids derived from the patient iPSCs where their mutated genes were corrected via CRISPR-CAS9 based genome editing as well as from the iPSCs where their mutated genes were uncorrected. These isogenic organoids could recapitulate the key pathophysiological features. A-B) iPSCs and the organoids from FAD patients. a) Gene mutation was corrected via CRISPR-CAS9 based genome editing in the iPSCs derived from FAD patients and the organoids from the FAD iPSCs; b) Both iPSCs and the organoids are identical except for the uncorrected gene mutation. C-E) iPSCs and organoids from PD patients. c) mid-brain-like organoids (MOs) generated with the addition of SMADi, CHIR99021 at 3uM, and IWP2 in the culture medium; d) Gene mutation was corrected via CRISPR-CAS9 based genome editing in the iPSCs derived from PD patient and the organoids from the PD iPSCs; e) Both iPSCs and the organoids are identical except for the uncorrected gene mutation. The organoids were generated using the carbon fibers (CFs) as scaffolds in both D) and E). f) iPSCs and the organoids from ASD patients with Rab39b mutation, deletion of Cntnap2, GTF2I, BAZ1B, CLIP2, and EI4H, but no mutation corrected organoids were available currently. The panels described the key features of the organoids, and the panels showed the potential pathophysiological study and drug screenings.

2.1.1. PSEN2N Mutation. The self-organizing cerebral organoids were generated from a familial AD patient with *PSEN2N* mutation and control organoids from an identical genetic background without *PSEN2N* mutation by genome editing. Treatment of these organoids with drugs that increase neuronal activity could facilitate the synchronization of high-frequency networks bursting at a comparable level in both control and AD organoids. Thus, these organoids can potentially become promising tools for AD pathological studies and a platform for drug screening [46].

2.1.2. BIN1 Gene Mutation. The *BIN1* KO organoids displayed the phenotype of early endosome narrowing, which could be rescued by the expression of BIN1iso1 but not BIN1iso9. Given that BIN1iso1 overexpression could enlarge the early endosomes and lead to neurodegeneration in human induced neurons (hiNs), it is plausible to suggest that the AD susceptibility gene *BIN1* could become an early biomarker for AD pathology [47].

2.1.3. Mitochondrial Protease PITRM1-KO. Cerebral organoids derived from Pitrilysin metallopeptidase 1 (PITRM1)-KO iPSCs could recapitulate the pathological features of AD, such as the accumulation of protein aggregates, tau plaques, and synaptic dysfunctions. PITRM1 is a mitochondrial protease, and its deficiency causes a slow-

progressing neurological disorder with a similar syndrome to AD, linking the mitochondrial function to the pathogenesis of common neurodegeneration [48].

2.1.4. Mouse IFITM3-KO. Inflammatory cytokines induce the expression of IFITM3, a γ-secretase in neurons and astrocytes, which bind to γ-secretase and upregulate its activity, thereby increasing the production of amyloid-β. The expression of IFITM3 is increased with aging and in mouse models that express familial AD genes. Furthermore, knockout of IFITM3 reduces γ-secretase activity and the subsequent formation of amyloid plaques in a transgenic mouse model (5xFAD). The IFITM3 protein is upregulated in tissue samples from a subset of late-onset AD patients who exhibit higher γ-secretase activity. The quantity of IFITM3 in the γ-secretase complex possesses a strong positive correlation with γ-secretase activity in the late-onset AD patient samples. This discovery suggests that γ-secretase is modulated by neuroinflammation via IFITM3, thereby increasing the risk for AD pathogenesis [49].

2.1.5. Mimicking Blood-Brain Barrier (BBB) Breakdown. To simulate the serum exposure consequence of BBB breakdown in AD patients, brain organoids from sporadic AD patients were exposed to human serum. AD-like pathologies were observed, such as magnified Aβ aggregates and elevated phosphorylated p-Tau levels, synaptic loss, and neural network damage [50].

2.1.6. Spatiotemporal Expression of Tau. Given the significant contribution of tau to the pathogenesis of AD, the spatiotemporal expression of tau has been mapped during brain development using iPSC-derived cortical organoids. While tau expression was detected in radial glia, neuronal maturation led to the dramatic elevation of tau mRNAs by using single-cell RNA sequencing, RNA in situ hybridization, and IHC. Spatially, low expression levels were observed in SVZ radial glia and deep white matter intermediate progenitors. This discovery could pave the way for further studies on the pathophysiological mechanisms of triggering and enhancing tau expression, simplifying the identification of therapeutic targets for tauopathy-based neurodevelopmental disorders [51].

2.1.7. BACE2 Mutation. Control or the BACE2 loss of function mutation (BACE2G446R) human brain organoids were used to investigate the contribution of BACE2 to AD pathogenesis. BACE2 was predominantly expressed in the ventricular zone and cortical plate of the organoids, and the expression levels were gradually elevated during the maturation of organoids. Furthermore, compared to control organoids, the mutant organoids displayed significantly enhanced apoptosis and elevated levels of Aβ oligomers, representing the AD-associated phenotypes [52].

2.1.8. ε4/ε4 Genotype. The cerebral organoids generated from iPSCs derived from APOE ε3/ε3 or ε4/ε4 genotypes could recapitulate the APOE4-related phenotypes. To be specific, significant apoptosis and detrimental synaptic dysfunction were detected in the AD patient organoids. Furthermore, elevated Aβ and phosphorylated tau levels relative to the healthy subject-derived cerebral organoids were detected. Accordingly, conversion of APOE4 to APOE3 partially reversed the APOE4-associated phenotypes in cerebral organoids from AD patients. Molecularly, enhanced stress granules and irregular genes were linked to AD phenotypes. Thus, it could be inferred that the APOE4 may contribute to late-onset AD pathogenesis [53].

2.1.9. Tau P301S Mutation. A new method was established recently for the generation of isogenic cerebral organoids for modeling AD with controlled genetic variables and mutation(s) in a specific gene by using an episomal plasmid vector derived from the Epstein-Barr virus (EBV). It turns out that this vector-based method could facilitate the easy and powerful generation of the isogenic cerebral organoids by avoiding the complexity and incompatibility offered by conventional genetic engineering and the CRISPR-Cas9 technology. More importantly, the isogenic cell lines generated from wild-type tau versus its mutant harboring the genetic form P301S were stable for more than 30 passages in terms of genetic and pathophysiological features. Thus, this strategy could make the generation of isogenic organoids easy and robust, facilitating the study of disease pathology, personalized medicine, and drug screening for clinical therapy [54].

2.1.10. Mutations of PSEN1 M146V, APP^{swe}, and PSEN1 ΔE9. In a separate study, organoids harboring familial AD mutations against their wild-type (WT) isogenic controls were employed as a platform for drug screening to identify drugs functional for therapy of hyperexcitability, subsequent extensive synapse loss, and cognitive dysfunction. The physiological assays based on this platform led to the identification of NitroSynapsin, a dual-allosteric NMDAR antagonist, that could eradicate the hyperactivity and rebalance the aberrant neural networks. Thus, this platform could be promising for large-scale screening to identify drugs for therapy of hyperexcitability and synaptic damage in AD patients [55].

2.1.11. Histone Deacetylase-6 Inhibitor Partially Reverses the Phenotype of AD Organoids. Treatment of AD animal model (ADLP^{APT}) brains and AD patient-derived brain organoids with CKD-504, a histone deacetylase-6 (HDAC6) inhibitor, could significantly degrade pathological tau plaques. Mechanistically, the inhibitor CKD-504 leads to the enhanced acetylation of tau, thereby recruiting chaperone proteins such as Hsp40, Hsp70, and Hsp110 to form complexes. The acetylated complexes with HSPs could bind to UBE2O and RNF14, the novel tau E3 ligases, degrading pathological tau via proteasomal pathways. This discovery could be translated into a clinical therapy for AD [56].

2.2. Modeling of PD Using Midbrain Organoids Derived from PD Patients. PD is another complicated progressive nervous system disorder. Almost all the PD modeling information originated from animal models before human brain organoids were available. Recently, midbrain organoids have been generated by improving the conventional strategy

[57–61]. These midbrain organoids are of significant interest for modeling PD as they generate dopaminergic neurons expressing markers of Substantia Nigra identity, the most vulnerable to degeneration [62]. Studies showed that PD organoids could catch the key pathophysiological features of PD, suggesting their potential for pathological study and drug screening to identify the compounds for clinical therapy (Figures 1(c)–1(e)).

2.2.1. Organoids from the Idiopathic Form of PD Patients. The first midbrain organoids were generated from iPSCs of the idiopathic form of PD patients, reprogrammed with the aid of non-integrating Sendai viral vectors. The mature organoids could simulate the expression of early and late neuronal markers as well as the statistical differences in the expression levels of these markers between the organoids from PD patients and healthy people. Therefore, it is highly expected that these organoids could be promising for modeling the idiopathic form of PD and in vitro pathological studies [57].

2.2.2. The Isogenic Organoids Derived from Familial PD Patients. The isogenic midbrain organoids were derived from PD patients harboring a genomic mutation in LRRK2 G2019S and were employed for the pathogenic study. The key pathological features observed in the LRRK2-associated sporadic PD patient brains were also detected in the isogenic midbrain organoids. Molecularly, protein-protein interaction network assays have enabled the identification of TXNIP, a thiol-oxidoreductase, as a key contributor to the development of LRRK2-associated PD in the LRRK2 mutant organoids. Thus, these isogenic PD organoids provide a platform for pathological study as well as drug screening for clinical therapy of the LRRK2-associated sporadic PD [63]. More recently, human midbrain organoids derived from healthy individuals against their isogenic LRRK2-p.Gly2019-Ser-mutant counterparts were compared to determine if the in vitro model simulates the in vivo equivalents from the aspects of developmental pathways and cellular events. It turns out that the midbrain organoids could model the early developmental stage of PD [64]. Midbrain organoids carrying the biallelic mutations of the *PINK1* gene from the patients and from the corrected cell lines by genome editing were employed for modeling PD. Compared to the corrected organoids, the patient organoids recapitulate the key PD features, consistent with the fact that mutation of the *PINK1* gene alone is sufficient to cause PD. Using both types of midbrain organoids as a platform, the selected compounds from the mouse model were tested [65]. In a separate study, human mid-brain-like organoids (hMLOs) harboring control or mutant DNAJC6 were generated to model the early-onset PD caused by a *DNAJC6* mutation. It turned out that the mutant hMLOs could recapitulate the key pathogenic features, thereby serving as a tool to investigate the pathology [66]. To decipher the role of the Bridging Integrator 1 (*BIN1*) gene in AD pathogenesis, the induced human cerebral organoids and neurons (hiNs) were generated with *BIN1* knock-out (KO).

Lewy body-like inclusions, one of the key features of PD, were observed in the human midbrain-like organoids (hMLOs) derived from $GBA1^{-/-}$ and SNCA overexpressing isogenic ESCs, suggesting that the hMLOs could recapitulate the underlying mechanisms for progressive Lewy body formation [67]. Most regular brain organoids bear the limitations of heterogeneity and long-term differentiation. To overcome these shortcomings, the simplified brain organoids (simBOs) composed of mature neurons and astroglial cells were generated from the hPSC-derived primitive neural stem cells (pNSCs). The midbrain-like simBOs bear several advantages over the traditional brain organoids such as rapid generation, high homogeneity, and easy specification into midbrain-like organoids via treatment with Shh and FGF8. The simBOs generated from a PD patient with a mutation of LRRK2 demonstrated typical symptoms like upregulated LRRK2 activity, down-regulated dopaminergic neurons, and enhanced autophagy. Moreover, treatment of simBOs with PFE-360, an LRRK2 inhibitor, could relieve the abnormalities, suggesting the potentiality of simBOs serving as PD models and alternative platforms for drug-testing and screening [68].

2.2.3. The PARK7-linked PD Organoids. The midbrain organoids derived from the Ibrahim Boussaad1 PD (PARK7-linked PD) patient, a highly heterogeneous neurodegenerative disorder, have been applied for the characterization of aberrant RNA splicing. It turned out that U1 splicing site mutations were enriched in sporadic PD patients, leading to a significant reduction of DJ-1 proteins and causing consequential mitochondrial dysfunction. The organoid-based drug testing has enabled the identification of certain compounds such as phenylbutyric acid as well as the genetically engineered U1-snRNA. These compound hits have been further tested and validated to be effective in reversing mis-splicing, mitochondrial dysfunction, and dopaminergic neuron loss in the mutant midbrain organoids. This could be an efficient alternative strategy for precision medicine to treat sporadic PD by molecularly targeting the splicing abnormality to rectify cellular mitochondrial dysfunction [69].

2.2.4. PD Organoids for Toxicology Study. A robust method has been developed to generate human organoids and incorporate microglia together with astrocytes into the organoids for studying toxicology and pathophysiology of the CNS. This type of organoid has been employed to test the PD model toxicants and will be promising for drug screening in the future [60]. More PD organoid models were established to test and characterize the neurotoxic effects on dopaminergic neurons via a machine learning-based analytical method. This approach has been used for the high content calcium image-based (HCI) cell profiling and toxicity evaluation in midbrain organoids treated with/without 6-OHDA, a neurotoxic compound. This platform could be employed for modeling PD and drug screening to identify the neurotoxic compounds ([70]. Further improvement was made for the generation of midbrain organoids to avoid the inherent shortcomings including batch-to-batch variability and the presence of a necrotic core. The midbrain

organoids simulate some key features of midbrain development like dopaminergic neuron and astrocyte differentiation. This strategy is efficiently suitable for pathological studies on toxin-induced PD [71].

The MOSs generated with the improved protocol by Kwak et al., are homogeneous with mature architecture of midbrain dopaminergic (mDA) neurons, other neuronal subtypes, and functional glial cells such as astrocytes and oligodendrocytes. More importantly, these MLOs are extremely sensitive to 1-methyl-4-phenyl-1, -2,3,6-tetrahydropyridine demonstrating mDA neuron-specific cell death. Thus, the MOs could serve as a platform for the in vitro study of PD pathology as well as drug screening for PD therapy [61]. Renner et al. developed midbrain organoids that claimed to be able to faithfully recapitulate the main characters such as architecture, size, cellular composition, homogeneous morphology, aggregate-wide synchronized neural activity, and global gene expression. They then employed the midbrain organoids to create a scalable and HTS-compatible platform in standard 96-well-plates for drug screening and evaluation with the criteria of HCI and RNA-seq at the single-cell level. By automating the entire workflow from generation to analysis, the intra- and inter-batch reproducibility was enhanced as demonstrated by RNA-seq and HCI. It turned out that this platform could be automated to generate the reproducible prediction of the drug effects on neurological disorders such as PD at the single-cell level albeit within a complex organoid environment [72].

2.2.5. Organoids Generated through a Carbon-Based Scaffold for Modeling PD. To overcome necrosis within the organoids during the long-lasting cultures, carbon fibers (CFs) have been employed as a new type of scaffold to generate midbrain organoids in replacement of the conventional polylactide-co-glycolide copolymer (PLGA) scaffold. Physiochemically, the porosity, microstructure, or stability of CF scaffolds could improve efficiency in iPSC differentiation within organoids relative to the PLGA scaffolds. The midbrain organoids generated in the CF scaffolds could more efficiently recapitulate the midbrain development evidenced by the expression of key regulator genes such as *PITX3* for terminal differentiation and the survival of midbrain dopaminergic (mDA) neurons. This strategy is promising for the establishment of the organoids in modeling neurodegenerative diseases associated with the midbrain such as PD and drug screening platforms [59].

2.3. Modeling of Fragile X Syndrome (FXS). Fragile X syndrome (FXS) is one of the NDDs with key features of intellectual disability and sensory deficits caused by a loss of FMRP, a multi-functional RNA binding protein. Compared to the in vitro brain organoid models for other NDDs, so far only three independent research laboratories reported brain organoid models for FXS [6, 73, 74]). Human forebrain organoids were generated from the iPSCs derived from healthy control and FXS patients, respectively, to model FXS in vitro [6]. It turns out that dysregulation was observed in neurogenesis, neuronal maturation, and excitability of the FXS organoids as compared to healthy organoids. A different group has generated cortical organoid models for FXS by knocking out the FMR1 gene [73]. Cellular and molecular tests confirmed the alteration of gene expression, aberrant differentiation, increased number of glial cells, enhanced spontaneous network activity, and depolarizing GABAergic transmission compared to the healthy counterpart. However, so far, the FXS-derived organoids have been not tested for drug screening.

2.4. Modeling of ASD Using Brain Organoids Derived from ASD Patients. Autism Spectrum Disorder (ASD) is caused by early neuron developmental dysfunction and lasts for the entirety of life, lacking clear etiology and genetic basis, but is linked to abnormal social communication and behaviors [75, 76] [11, 12, 75, 77, 78]. The incidence of ASD is approximately 1 in 59 children and 1% of the global population according to the CDC statement (https://www.afhu.org/2017/10/23/what-to-know-about-asd/?gclid=EAIaIQobChMI-oD087qQ7gIVh56zCh11og-UEAAYBCAAEgKxmPD_BwE). The organoids derived from ASD patients have been employed for modeling, pathological studies, and drug screening [34, 76].

The telencephalic organoids from the affected families were generated for modeling the idiopathic ASD for the first time using these cerebral organoids. Relative to organoids from the unaffected family members, significant cellular alterations were detected in the ASD organoids including synaptic growth, cell cycle function, and imbalance in GABAergic/glutamatergic neuron differentiation. Molecularly, the altered gene expression network could contribute to the pathogenesis of ASD. For instance, the enhanced expression of FOXG1 leads to the overproduction of GABAergic inhibitory neurons [12]. To investigate the metabolic pathway networks that contribute to ASD pathogenesis, human cerebral organoids were produced to harbor mutations of Rab39b, a small GTPase associated with X-linked macrocephaly, ASD, and intellectual disability. The enhanced proliferation and impaired differentiation of neural progenitor cells (NPCs) were observed in the *RAB39b* mutant cerebral organoids, leading to an enlarged size of the organoids that resemble the trait of ASD. At the molecular level, the interaction between *RAB39b* and PI3K components was confirmed by the promotion of the PI3K–AKT–mTOR signaling in NPCs of the Rab39b mutant cerebral organoids (Figure 1(f)). Furthermore, the enlarged organoid sizes and NPC over-proliferation caused by Rab39b mutation were rescued by the inhibition of AKT signaling, providing a platform to study the pathology of ASD and drug screening [79]. To further investigate the mechanism of ASD at a cellular and molecular level, mouse cortical organoids (mCOs) were generated from the KO of contactin-associated protein-like 2 (CNTNAP2), a member of the neurexin protein family. At the cellular level, defective generation of the GABAergic inhibitory neurons was observed in the KO mCOs. Consistently, at the molecular level, the dysregulated transcriptional network involved in GABAergic neurogenesis was demonstrated at the neural progenitor stage without Cntnap2. Furthermore, the dysregulations in the KO mCOs at the cellular and molecular levels could be rescued by treatment with retigabine, an antiepileptic drug, suggesting that Cntnap2 could serve as a therapeutic target for clinical therapy of ASD [68].

Microduplication at 7q11.23 (7Dup), harboring 26–28 genes, is marked to be a highly associated genetic mutation relevant to ASD. The cortical neurons derived from this microdeletion offer unique opportunities for translational studies at the genetic and pathological levels as well as for drug screening to identify drug efficacy in therapy. Williams–Beuren syndrome (WBS), characterized by hyper sociability and language strengths, is caused by microdeletion with several genes located within the deleted region such as *GTF2I*, *BAZ1B*, *CLIP2* and *EIF4H*. These have been acknowledged as potentially crucial contributors to the pathogenesis of WBS.

The cortical glutamatergic neurons derived from the WBS patients were employed for a large-scale drug screening to identify the hits from a small molecule compound library consisting of potential reagents for CNS, epigenetic modulators, and function-unknown compounds. By comparing the transcriptional alteration of the WBS interval genes, three histone deacetylase inhibitors (HDACi) were identified and further validated at the levels of both mRNA and protein to downregulate the expression of *GTF2I* with a prevalent pathogenic role [80].

More recently, cerebral cortical organoids were generated from iPSCs carrying the mutations in *KMT5B*, *ARID1B,* and *CHD8*, three ASD risk genes or the wild-type genes for modeling of ASD. These organoids were used to identify aberrant cell-type-specific neurodevelopment shared across ASD risk genes and investigate the underlying mechanisms of these genes in their contribution to ASD pathology [81]. Given the complexity of neurological disorders and the limitations of the animal models in the pathological study, human brain organoids will play vital roles in modeling disorders and drug screening procedures [82].

3. Advances in Organoid-Based Drug Screening

A workflow for drug screening using serum-free embryoid bodies (SFEBs) derived from hiPSCs for scalable high-throughput screening (HTS) has been developed. The screening was conducted with criteria of multi-electrode arrays (MEAs) to show the firing and burst rates determined by single-cell HCI to assess the number of excitatory neurons, demonstrating a high degree of consistency and reliability. Thus, the SFEBs could serve as a platform for HTS to enumerate the high variation in cortical organoids. Although this strategy is time-consuming, it could serve as an efficient starting point for phenotypic drug screening [83]. The current brain organoids resemble the early stage developing brain; developing brains are more sensitive to toxic exposure relative to fully developed brains [83]. Therefore, the brain organoids could serve as an ideal platform for screening developmental neurotoxicity. Brain organoids have been applied for modeling early-stage neurotoxicity screening. With this platform irreplaceable by in vivo animal models or cell-based screening [84], large-scale chemicals in use and potential drugs in the future could be determined. Thus, this strategy opens a new avenue for evaluating toxicants by determining if members of the compounds library potentially belong to developmental neurotoxicants. Successful studies have been conducted to identify drugs and heavy metal chemicals as developmental neurotoxicants [84–86].

Due to the selective permeability of drugs to the brain, BBB impairment or dysfunction in many types of NDDs contribute to pathogenesis [87–89]. Therefore, the BBB serves as one of the key structures for drug discovery for the therapy of human NDDs [87], indicating a potential first target for new drugs to enter the brain. Most of the current organoid models are single tissue or organ-based, failing to orchestrate multiple different relevant tissues or organs let alone the system levels. Given the evolutionary distance between humans and mice, the discrepancy between BBB function and brain microvascular endothelial cells (BMECs) dampens the simulation of animal models to humans [90]. To mimic the human CNS and circulation system-level interactions, several physiologically relevant BBB-on-a-chip models have been established, composed of brain neural/organoids, the BBB, and a vascular side separated by a porous membrane [146–150], several of which are reported to model drug penetrability accurately [91–93] (Figure 2).

Recently, human CNS barrier-forming organoids (CBFOs) were established from the choroid plexus (ChP), a protective epithelial BBB by which the cerebrospinal fluid (CSF) is produced. The CSF is a vital liquid that provides nutrients and signaling molecules to help remove toxic waste products to aid in the survival and maturation of the brain. ChP selectively permeabilizes entry of the molecules to avoid free entry of toxic molecules or drugs from the blood. The human ChP-CSF organoids recapitulate the main traits of the ChP. On one hand, the ChP-CSF organoids could secrete the CSF-like fluid to mimic in vivo CSF. On the other hand, the ChP-CSF organoids have a restrictive barrier that exhibits the same selective permeability to small molecules in vitro as the ChP in vivo. The ChP-CSF organoids could progressively mature over time under in vitro conditions. Molecularly, ChP-CSF organoids bear a high degree of similarity to the ChP in vivo at the transcriptomic and proteomic levels. Combined transcriptomic and proteomic analysis at the single-cell level leads to the identification of key human CSF components undetected but produced by epithelial subtypes. More importantly, the ChP-CSF organoids can be employed to predict the permeability of new compounds into the CNS [94]. Thus, the new CBFOs-on-a-chip model may successfully simulate the selective permeability of drugs into the brain, thereby functioning as a platform to carry out drug screening for easy translation into clinical therapy of NDDs (Figure 2).

Shortcomings of the current approaches for organoid-based drug screening.

Human organoids have been acknowledged as a relatively ideal versatile tool for modeling human diseases, in vitro pathological studies, and drug screening. However, the current drawbacks at the level of organoids hampered the reliability and the efficiency of drug screening. At the organoid level, the quantity and quality of organoids significantly impact the drug screening efficiency and reproducibility. Due to the low generation efficiency of organoids, the scale of the organoids has become one of the bottleneck limiting factors for the efficiency and reproducibility of the drug screening. In addition to the scale of the organoids, the quality of the organoids including the capacity of simulating their

parental organs or disease phenotypes dramatically affect the reliability of the drug screening. At the systemic level, one of the main fatal shortcomings in most organoid models consists of the ignorance of the drug's interaction with multiple tissues/organs/systems in vivo. The issue is particularly important for NDDs due to the existence of the BBB.

4. Future Perspectives

Significant achievements have been made in the generation of human organoids, particularly in the brain and cancer-specific organoids. These organoids generated by utilizing current strategies could recapitulate key features of the human brain, making it possible for in vitro studies on neurodevelopment and modeling of NDDs. The organoid-based small- or large-scale drug screening processes proved to be promising with some compound hits being identified and validated for therapy. However, to a larger extent, these brain organoids are incomparable to human brains both architecturally and functionally. Therefore, the generation of organoids and the organoid-based study remain in the infancy stage. Among other issues, overcoming the limitations to generating high-quality organoids has been the top priority. The basic requirement is to enable the organoids to faithfully recapitulate key features of the brain region(s). To effectively mimic the human brain, further characterization and comparison of the human fetal, postnatal, adult, and aging brains at structural, cellular, and molecular levels is indispensable [95–98]. Although the current brain organoids at different ages could partially recapitulate the developing stage of their in vivo brain counterparts, they bear some shortcomings for modeling neurological disorders such as NDDs:

(1) Vascularization. Currently, most organoids lack 3D vascular networks limiting neurogenesis, proliferation, differentiation, apoptosis of organoids, and long-term culture, leading to a low efficiency in recapitulating the late stages of human brain development. This issue could be partially ameliorated by vascularization via genetically engineered induction of ETV2, co-culture with epithelial cells, or by graft of human brain organoids into mouse brains. However, the capacity of oxygen, nutrient supply, and metabolic clean-up provided by this alone remains to be insufficient. More recently, several strategies for improving brain organoid vascularization were invented. The first method consists of co-culturing neuronal spheroids with perfusable blood vessels. The vascularized neuronal spheres could efficiently enhance proliferation, differentiation, and reduce apoptosis [99]. The second method is comprised of the separate generation of vessel organoids and brain organoids followed by a co-culture of two types of organoids. Increased number of neural progenitors, functional BBB-like structures, and active microglial cells were observed in the fused/vascularized brain organoids. Therefore, the fused organoids enable us to investigate interactions between immune and non-immune cells as well as neuronal and non-neuronal cells in vitro [100]. Thus, these two strategies could serve as a better tool to simulate brain development and model neurological disorders. However, some concerns remain to be against this strategy. Effectively improving the quality and distribution of vessels in organoids in concert with biomaterials and microfluidic system-based technology could be promising in this regard

(2) Most brain organoids usually represent early fetal brain development, whereas some NDDs such as AD and PD are usually late-onset. Thus, the application of organoids for modeling late-onset NDD-associated aging progression such as PD is limited. Fortunately, human cortical organoids could mature to 250~300 days postnatal, parallel to in vivo development and maintenance of in vivo developmental milestones. Furthermore, the genes critically involved in neurodevelopment and NDD risks were mapped to in vitro gene expression trajectories. This suggests that human cortical organoids hold the potential for long-term cultures, which parallels in vivo developmental progression and maturation [101]. Therefore, appropriately maintaining the long-term maturation of human cortical organoids and avoiding necrosis and abnormalities during the culture are essential to generating brain organoids that match key postnatal transitions for modeling NDDs

(3) Lacking microglia, the key player in the developing brain, in the current brain organoids has been an essential drawback for modeling NDDs, limiting the application of brain organoids. Co-cultures are the conventional strategy for integrating microglia into brain organoids. Indeed, the human microglia could be integrated into human midbrain organoids [71, 102–104]. Xu et al. developed a new protocol for generating brain region-specific microglia-containing organoids by co-culturing at a proper time point [105]. Bodnar et al. developed a protocol to generate microglia-containing CO (MCO) by a novel technique for embryoid body (EB) production directly from iPSCs combined with orbital shaking cultures. However, the microglia ratio remains low (~7%) [106]. Interestingly, it was observed that during cultures, erythromyeloid progenitors migrating to brain organoids could gradually develop into microglia-like cells [107]. Recently, a protocol was developed for the generation of microglia-containing hCOs (mhCOs) via the overexpression of myeloid-specific transcription factor PU.1 in cortical organoids without co-culture. The mhCOs have become an efficient tool for functional investigation of microglia in neurodevelopmental and neurodegenerative disorders such as AD [108]. Given that microglia could not emerge natively inside organoids using the previous methods, this novel strategy has been a breakthrough for microglia generation in brain organoids.

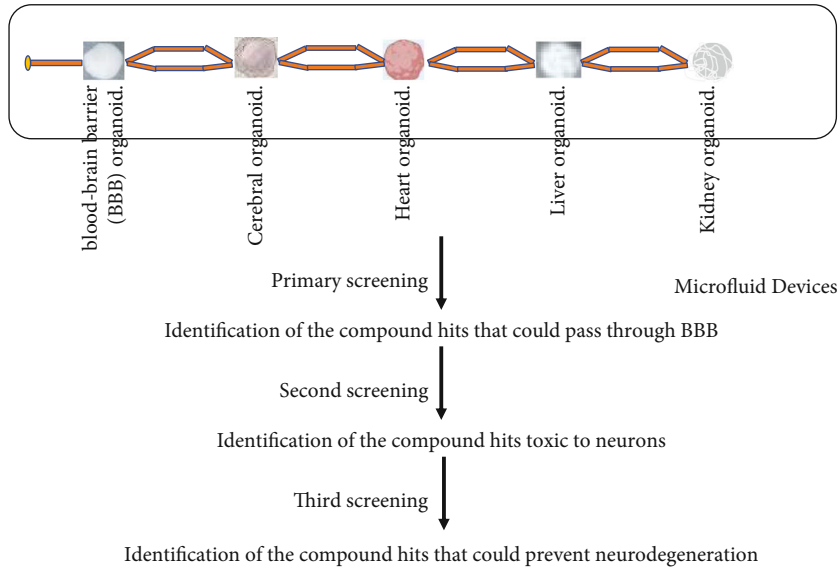

Figure 2: The body-on-a-chip-based drug screening flowchart. The different tissues/organs-specific organoids were arranged in order with BBB organoids in the first place followed by cerebral organoids and other organoids that recapitulate their corresponding tissues/organs in a body-on-a-chip device. Several successive screening processes could be conducted starting from the primary screening to identify the compounds that could pass the blood-brain-barriers (BBB) followed by the second and third rounds of screenings to identify the compound hits that are toxic to neurons and that could rescue neurodegenerations, respectively.

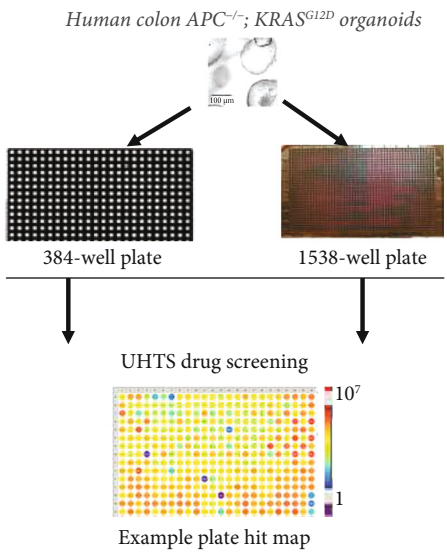

Figure 3: The cryo-preserved human colon organoids with $APC^{-/-}$; $KRAS^{G12D}$ mutation could confer the long storage and re-grown upon cryo-recovery for expansion to make the culture operation alike for the cell lines. This strategy could significantly overcome the bottleneck limitation of the organoid supply for the ultra-high-throughput screening (UHTS) in 384-well and 1538-well plates.

However, further improvement in the protocol is required to maintain native microglia emergence with a controllable microglia ratio. These brain organoids will be essential for modeling NDDs, in vitro pathological studies, and drug screening

(4) The limited size and heterogeneity of the current brain organoids offer inefficient representative capacity to their in vivo counterparts. On the other hand, separately generated organoids that represent different brain regions could be assembled to generate whole-brain organoids to recapitulate the entire brain more faithfully as compared to their separate counterparts [109, 110]. Thus, breakthroughs in the generation of the fused organoids open a new window to investigate the crosstalk at the inter- brain-region and the inter-organ levels. However, the assembly of the whole brain organoids is still at the infancy stage; many technical issues need to be resolved such as guiding border formation and interconnection of the separate tissues

Assembloids generated from the co-culture of different brain regions of organoids have been employed to investigate the internal interactions between the brain regions but fails to offer the tool for understanding signal transduction from the brain to the whole body. In addition, assembloids stem from the fusion of human organoids bearing the shortcomings of high heterogeneity and variable reproducibility. Ao et al. developed a simple and versatile acoustofluidic method to partially overcome these disadvantages by a controllable spatial arrangement of organoids [111]. Recently, a breakthrough was made in the generation of engineered brain-spinal cord assembloids (eBSA) by co-culturing cerebral organoids (COs) and motor neuron spheroids (MNSs) [100]. The eBSA connects COs and MNSs to recapitulate the brain-spinal cord connection. Potentially, the eBSA could serve as a platform to screen and validate neurochemical stimulus signal transduction. In addition, the accumulation of knowledge regarding

the neural signal transfer from the CNS to the peripheral nerve system (PNS) will provide a better understanding of controlling muscle actuators within the nervous system.

(5) Under in vitro culture conditions, the growth and maturation of the brain organoids are time-consuming. To overcome this issue, pharmacological strategies have been proposed for accelerating growth and maturation. However, these pharmacological strategies may potentially result in the alteration of intrinsic differentiation processes programmed naturally, interfering with the recapitulation of the resulting brain organoids to their in vivo counterparts. Efforts have been made to genetically induce aging [112–114]. However, these genetic operations should be further improved and validated to determine if the resulting brain organoids are reliable in terms of faithfully modeling pathological features, particularly disease-associated aging

(6) Large scale drug screening has been carried out in cancer organoids, and some compounds have been successfully identified for further assessment and validation. However, limited information is available for the brain organoid-based high throughput drug screening, but some previously tested compounds and current clinical drugs were tested in brain organoids that could model AD, PD, and ASD, respectively. Key issues for brain organoid-based drug screening is reliability and efficiency, particularly for the organoids for NDD modeling. Many factors affect the efficiency and reliability of brain organoid-based drug screening. Currently, most drug tests were conducted on the region-specific organoids. The variability of the organoids derived by the self-organization of neuronal cells hinders the efficiency, reliability, and availability of personal medicine. The whole-brain organoids assembled from separately generated organoids representing different brain regions could be more reliable for drug screening. The recent development of the BBB organoids could efficiently prescreen the permeability of new compounds passing through the BBB first before functional screening for a potential therapy for neurological disorders

Another issue for large-scale drug screening using cerebral organoids is developing long-term storage and culture operations alike for the cell lines. Although no cerebral organoids were reported to have this property, success of the colon $APC^{-/-;}$ $KRAS^{G12D}$ organoids [115] shed light on the development of cerebral organoids with this property (Figure 3). It is highly expected that the brain organoids with long-term storage and culture operations alike for the cell lines will be developed in the future.

As a summary, the establishment of organoids has been a milestone for the in vitro modeling of in vivo organ development, pathological studies, and drug screening albeit numerous difficulties remain to be resolved. The rapid and comprehensive progress in the organoid technologies shed light on the future breakthrough in overcoming the inaccessibility of human organs/systems via in vitro organoid-based platforms. However, being mindful that in vitro models cannot perfectly mimic in vivo counterparts will inspire investigators to make efforts to improve the technology and research strategies.

Conflicts of Interest

The authors declare that they have no conflict of interest.

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Research Article

Intestinal Organoids in Colitis Research: Focusing on Variability and Cryopreservation

Talke F. zur Bruegge,[1] Andrea Liese,[1] Sören Donath,[2,3] Stefan Kalies,[2,3] Maike Kosanke,[4] Oliver Dittrich-Breiholz,[4] Sandra Czech,[1] Verena N. Bauer,[1] André Bleich,[1] and Manuela Buettner[1]

[1]*Institute for Laboratory Animal Science, Hannover Medical School, Hannover, Germany*
[2]*Institute of Quantum Optics, Leibniz University Hannover, Hannover, Germany*
[3]*Lower Saxony Center for Biomedical Engineering, Implant Research and Development (NIFE), Hannover, Germany*
[4]*Research Core Unit Genomics, Hannover Medical School, 30625 Hannover, Germany*

Correspondence should be addressed to Manuela Buettner; buettner.manuela@mh-hannover.de

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In recent years, stem cell-derived organoids have become a cell culture standard that is widely used for studying various scientific issues that were previously investigated through animal experiments and using common tumor cell lines. After their initial hype, concerns regarding their standardization have been raised. Here, we aim to provide some insights into our experience in standardizing murine colonic epithelial organoids, which we use as a replacement method for research on inflammatory bowel disease. Considering good scientific practice, we examined various factors that might challenge the design and outcome of experiments using these organoids. First, to analyze the impact of antibiotics/antimycotics, we performed kinetic experiments using ZellShield® and measured the gene expression levels of the tight junction markers *Ocln*, *Zo-1*, and *Cldn4*, the proliferation marker *Ki67*, and the proinflammatory cytokine *Tnfα*. Because we found no differences between cultivations with and without ZellShield®, we then performed infection experiments using the probiotic *Escherichia coli* Nissle 1917 as an already established model setup to analyze the impact of technical, interexperimental, and biologic replicates. We demonstrate that interexperimental differences pose the greatest challenge for reproducibility and explain our strategies for addressing these differences. Additionally, we conducted infection experiments using freshly isolated and cryopreserved/thawed organoids and found that cryopreservation influenced the experimental outcome during early passages. Formerly cryopreserved colonoids exhibited a premature appearance and a higher proinflammatory response to bacterial stimulation. Therefore, we recommend analyzing the growth characteristics and reliability of cryopreserved organoids before to their use in experiments together with conducting several independent experiments under standardized conditions. Taken together, our findings demonstrate that organoid culture, if standardized, constitutes a good tool for reducing the need for animal experiments and might further improve our understanding of, for example, the role of epithelial cells in inflammatory bowel disease development.

1. Introduction

Over the past decade, stem cell-derived organoid culture is a well-known system that has evolved from an exciting new tool for investigating scientific issues to a standard cell culture and *in vitro* method. Organoids were initially introduced as a promising model for basic research on disease development and progression, toxicological drug testing, and regenerative medicine [1–5]. Much progress has been made, for example, generating organoid structures from many different origins and establishing various protocols for all types of applications. There was much hype on organoids when they were first introduced, but some skepticism regarding their standardization combined with experimental considerations has recently emerged [5–7]. However, since their introduction, the definition of organoids has been

agreed upon, and most scientists are currently aware of organoids [8] and their classification [9]. Specifically, organoids are 3D structures generated from pluripotent stem cells, such as iPSCs or ESCs, or from tissue-resident neonatal or adult stem or progenitor cells that are cultured in a tissue-like extracellular matrix (ECM). In the presence of niche and growth factors, these cells differentiate into all functionally relevant cell types and spontaneously self-assemble into 3D structures that can perform some of the donor organ's functions [1–3, 10].

In our research group, we mainly focus on intestinal epithelial organoids from the murine colon, hereafter also referred to as colonoids [9]. According to the 3R principles, we use these colonoids as a replacement tool for investigating the pathomechanism of inflammatory bowel disease (IBD) instead of performing *in vivo* studies. IBD is a multifactorial disease that depends on various factors, such as genetic predisposition, environmental factors, and alterations in the microbial gut flora [11]. Many *in vivo* models that exhibit genetic predisposition have been established, for example, the *Il10*-knockout mouse strain [12]. However, environmental and microbial factors are more difficult to display due to marked differences between the mouse model and the human situation. Furthermore, current *in vitro* models using, for example, Caco-2 cells often lack the physiological properties of a human tissue. Therefore, Dotti and Salas [6] reviewed the usage of *ex vivo* human intestinal organoids for research on IBD and judged them to be a suitable tool for analyzing disease mechanisms, although methods for their standardization are needed.

This recommendation is in accordance with the claims made by another research group [5], who states that the general reproducibility of organoid cultures is essential for their use, e.g., in developmental and drug testing studies. It is also important to consider the scalability and safety of organoids when attempting to use these systems in human regenerative medicine [5].

What we learned over the last few years in our organoid research is that the pace of experimental progress is rather slow due to the need to understand the fundamental basis of organoid formation and its requirements. As also stated by Huch et al. [5], we can only exhibit progress by "carefully laying the groundwork" for creating a useful product and facing the hype of organoid research with realistic expectations. As commented by Spence [7], organoids lack a commonly well-accepted standard regarding their use in experiments, and every laboratory using organoids has established its own standardizing methods.

This paper is aimed at collecting several strategies for standardizing 3D organoids to increase their value for the study of various scientific issues. According to good scientific practice, we want to note important aspects that should be considered in experimental designs to render 3D organoids a more predictable and reliable tool, for instance, regarding their technical, interexperimental, and biologic replicability. Additionally, we provide the first demonstration that the cryopreservation of intestinal epithelial organoids might influence the experimental outcome and should thus be analyzed in preliminary tests.

2. Materials and Methods

2.1. Animals. All experiments in this study were conducted in accordance with the European Directive 2010/63/EU [13] and German animal protection laws and were approved by the Local Institutional Animal Care (File: 2015/78). Male, 9- to 12-week-old C57BL/6J (wild-type) mice were obtained from the Central Animal Facility (Hannover Medical School, Hannover, Germany), where they were formerly housed in individually ventilated cage systems under standardized room and specific pathogen-free conditions according to the recommendations of the Federation of European Laboratory Animal Science Association [14]. Routine microbiological monitoring did not reveal any evidence of infection with common murine pathogens with the exception of [*Pasteurella*] *pneumotropica*, *Staphylococcus aureus*, *Klebsiella oxytoca*, and *Helicobacter* sp.

Prior to this study, we carefully timed and interweaved all of our experiments to ensure the use of the minimum number of animals for the maximum number of experiments. In total, 17 animals were sacrificed to obtain the 17 independent colonoid lines that were used in this study. All generated colonoid lines were cryopreserved and can be reused in further studies. For reduction reasons, most of the data of the freshly isolated colonoid group (see Section 2.9) were obtained from the analysis of technical and interexperimental replicability and biological variability (see Section 2.8); therefore, the data are repetitively used in the various experiments.

2.2. Preparation of Organoids. The isolation and plating of crypts were performed as already published by us in Brooks/zur Bruegge et al. [15] with the following alterations/specifications: After transfer into dissociation buffer (DPBS containing 54.9 mM sorbitol and 43.4 mM sucrose), the colonic tissue pieces were thoroughly mixed by hand until the suspension became turbid with detached crypts. The crypt suspension was filtered (70 μm pores) and centrifuged, and the pellet was resuspended in Matrigel® (Corning™, New York, NY, USA) and organoid growth medium (DMEM [high glucose, pyruvate, GlutaMAX™] [Thermo Fisher Scientific, Waltham, USA] with 50% L-WRN supernatant [ATCC® CRL3276™ in DMEM [high glucose, pyruvate, GlutaMAX™] plus 10% fetal calf serum [FCS]] supplemented with 10% FCS [total concentration], 1× B27 [Invitrogen, Carlsbad, CA, USA], 1× N_2 [Invitrogen, Carlsbad, CA, USA], 10 μM Y-27632 [Tocris, Bristol, UK], 50 ng/μL recombinant mouse epidermal growth factor [Sigma-Aldrich, St. Louis, MO, USA], and 1× ZellShield® [Biochrom, Berlin, Germany]) in a ratio of two-thirds to one-third, respectively. Under continuous mixing, 50 μL of the Matrigel®-and-crypt mixture was pipetted into wells of a 24-well plate in alternating diagonal rows. For a better nutrient distribution throughout the gel, the droplets were mechanically flattened with the pipette tip, polymerized at 37°C for 30 minutes and overlaid with 500 μL of organoid growth medium. Organoids were cultured with 5% CO_2 at 37°C, and the medium was changed every 3-4 days unless stated otherwise.

2.3. Passaging of Organoids. The organoids were passaged weekly unless stated otherwise. For each passage, the Matrigel® droplets were dissolved by thorough pipetting after the addition of ice-cold DPBS, and the organoids were split through a 27G 1/2″ cannula. The crypt suspension was centrifuged, and the pellet was resuspended in fresh Matrigel® and organoid growth medium and processed as described above (Section 2.2).

2.4. Cryopreservation and Thawing of Organoids. Freshly isolated organoids were grown for 1 week until passage 1 and then processed as described in Section 2.3, titled "Passaging of Organoids." Instead of Matrigel®, the pellet was resuspended in FCS with 10% DMSO and then frozen at -20°C in a Mr. Frosty™ (Fisher Scientific GmbH, Schwerte). After 24 h, the Mr. Frosty™ was transferred to -80°C, and 24 h later, the cryovials were transferred to liquid nitrogen until further use.

The cryopreserved organoids used for infection experiments were rapidly thawed at 37°C until the suspension became liquid. The organoids were then immediately transferred into ice-cold DPBS with 10% FCS, centrifuged, and plated as described in Section 2.2, titled "Preparation of Organoids."

2.5. Effects of ZellShield® on Organoid Kinetics. The organoids used to assess the effects of ZellShield® on organoid kinetics were isolated and cultivated as mentioned above (Sections 2.2 and 2.3) over 3 weeks in the presence of ZellShield® until passage 3. The organoids from passage 3 were then cultivated for 10 days in the presence of ZellShield®, and the medium was changed every 3 days, with the last change occurring one day prior to the experiment. On day 10, the old organoid growth medium was replaced by fresh organoid growth medium with or without ZellShield®, and the organoids were cultured for 1, 2, 4, 6, and 12 hours at 37°C in the presence of 5% CO_2. After incubation, the supernatant was removed and stored at -20°C. The plate was immediately placed on ice, the Matrigel® was dissolved, and the organoid structures were disrupted by thorough pipetting after the addition of ice-cold DPBS. The suspension was centrifuged, and the pellet was resuspended in RNA Quick-RNA™ Micro Prep Kit Lysis Buffer (Zymo Research, Irvine, CA, USA) and stored at -80°C until further processing for quantitative real-time PCR (qPCR) analysis. Two wells of each condition were pooled to obtain the supernatant and lysed samples. The whole experimental setup from isolation to sample collection was repeated in five independent experiments.

2.6. Cultivation of E. coli Nissle 1917. The *E. coli* Nissle 1917 strain (*Ec*N) for the infection experiments was cultivated as already published by us in Brooks/zur Bruegge et al. [15].

2.7. EcN Infection Experiments. The organoids used in the *Ec*N infection experiments were cultivated as described in Section 2.5, titled "Effects of ZellShield® on Organoid Kinetics"; again, organoids at passage 3 were used on day 10 for the infection. The old growth medium was exchanged with fresh medium without ZellShield® to obtain the control samples, and the infection samples were administered the bacterial suspension at 1 : 25 dilution in organoid growth medium without ZellShield®. The control and infection samples were incubated for 1 hour at 37°C in 5% CO_2. After incubation, all supernatants and lysed organoid samples were collected and stored using the protocol described in Section 2.5.

2.8. Technical and Interexperimental Replicates and Biological Variability. For the analysis of technical and interexperimental differences and biological variability, we performed five independent *Ec*N infection experiments as described above (Sections 2.6 and 2.7) on five different days using three different biological replicates (organoid lines) per experiment and three technical replicates (two wells pooled to prepare each sample) per organoid line. In total, we used 15 independent organoid lines for this experiment.

2.9. Freshly Isolated vs. Cryopreserved Organoids. For the comparison between freshly isolated and cryopreserved organoids, we performed seven independent *Ec*N infection experiments according to the experimental setup described above (Sections 2.6 and 2.7) using both types of organoids. The data from 15 freshly isolated organoid lines were obtained from the experiments described in Section 2.8, titled "Technical and Interexperimental Replicates and Biological Variability," and were repetitively used in this context. In addition, two additional freshly isolated organoid lines were generated as a comparison group for the last cryopreserved batches. The 12 cryopreserved organoid groups were thawed as described in Section 2.4, titled "Cryopreservation and Thawing of Organoids," at passage 1 one week after the corresponding freshly isolated organoid groups that were simultaneously infected; therefore, the same passage was used for both conditions.

2.10. RNA Isolation and cDNA Synthesis. To quantify the gene expression levels in the collected lysis samples, intracellular RNA was isolated using the RNA Quick-RNA™ Micro Prep Kit (Zymo Research, Irvine, CA, USA), and up to 1 μg of RNA was transcribed into cDNA using the QuantiTect® Reverse Transcription Kit (Qiagen®, Hilden, Germany) according to the manufacturer's instructions. Organoid samples for cDNA library generation and sequencing were lysed and processed according to the manufacturer's instructions using the RNeasy Mini Kit (Qiagen®, Hilden, Germany) with additional DNase digestion with the RNase-Free DNase Set (Qiagen®, Hilden, Germany) and stored at -80°C until further use.

2.11. Quantitative Real-Time PCR (qPCR). The qPCR-based quantification of the gene expression levels of the cDNA samples was performed using a TaqMan®-based singleplex assay with *Actb* (Mm00607939_s1) as the endogenous control gene, *Mki67* (Mm01278617) and *Slc5a1* (Mm00451210_m1) as the target, and the following TaqMan®-based multiplex assays: 4-plex 1 [*Actb* (Mm00607939_s1_qsy_ABY) as the endogenous control gene, *Cldn2* (Mm00516703_s1_VIC; data not shown), *Cldn7* (Mm00516817_m1_qsy_JUN; data not shown), and *Tnfα* (Mm00443258_m1_FAM)] and 4-plex 2 [*Cldn4* (Mm_00515514_s1_qsy_ABY), *Cldn8* (Mm00516972_s1_qsy_JUN;

data not shown), *Ocln* (Mm00500912_m1_FAM), and *Tjp1* (Mm01320638_m1_VIC)] (all from Thermo Fisher Scientific, Waltham, MA, USA). SYBR® Green-based QuantiTect Primer Assays (Qiagen®, Hilden, Germany) were used for *Actb* (Mm_Actb_1_SG) as the endogenous control gene, and *Chga1* (Mm_Chga_1_SG) and *Muc2* (Mm_Muc2_2_SG) as target genes. Each sample was either measured in duplicate or triplicate using a QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems™, Foster City, CA, USA). Relative quantification (RQ) was performed using the $2^{-\Delta\Delta CT}$ method [16].

2.12. Library Generation, Sequencing, and Raw Data Processing. Library Generation, Quality Control, and Quantification Were Performed as Described Previously [17]. 500 ng of total RNA per sample was utilized as input for mRNA enrichment procedure with "NEBNext® Poly(A) mRNA Magnetic Isolation Module" (E7490L; New England Biolabs) followed by stranded cDNA library generation using "NEBNext® Ultra II Directional RNA Library Prep Kit for Illumina" (E7760L; New England Biolabs). All steps were performed as recommended in user manual E7760 (Version 1.0_02-2017; NEB) except that all reactions were downscaled to 2/3 of initial volumes [17]. Furthermore, one additional purification step was introduced at the end of the standard procedure using 1× "Agencourt® AMPure® XP Beads" (#A63881; Beckman Coulter, Inc.) [17].

cDNA libraries were barcoded by dual indexing approach using "NEBNext Multiplex Oligos for Illumina–96 Unique Dual Index Primer Pairs" (6440S; New England Biolabs) [17]. All generated cDNA libraries were amplified with 7 cycles of final PCR.

Fragment length distribution of individual libraries was monitored using "Bioanalyzer High Sensitivity DNA Assay" (5067-4626; Agilent Technologies) [17]. Quantification of libraries was performed by use of the "Qubit® dsDNA HS Assay Kit" (Q32854; Thermo Fisher Scientific) [17].

2.12.1. Library Denaturation and Sequencing Run. Equal molar amounts of 12 individually barcoded libraries were pooled for a sequencing run. The library pools were denatured with NaOH and were finally diluted to 1.8 pM according to the *Denature and Dilute Libraries Guide* (Document # 15048776 v02; Illumina) [17]. 1.3 mL of denatured pool was loaded on an Illumina NextSeq 550 sequencer using a High Output Flow Cell kit for 1 × 76 bp single reads (20024906; Illumina) [17]. Sequencing was performed with the following settings: sequence reads 1 and 2 with 38 bases each and index reads 1 and 2 with 8 bases each.

2.12.2. BCL to FASTQ Conversion. BCL files were converted to FASTQ files using bcl2fastq Conversion Software version v2.20.0.422 (Illumina) [17].

2.12.3. Raw Data Processing and Quality Control. Raw data processing was conducted by use of nfcore/rnaseq (version 1.4.2) which is a bioinformatics best-practice analysis pipeline used for RNA sequencing data at the National Genomics Infrastructure at SciLifeLab, Stockholm, Sweden [17]. The pipeline uses Nextflow, a bioinformatics workflow tool. It preprocesses raw data from FASTQ inputs, aligns the reads, and performs extensive quality control on the results [17]. The genome reference and annotation data were taken from http://GENCODE.org (Mus musculus; GRCm38.p6; release M25).

2.12.4. Normalization and Differential Expression Analysis. Normalization and differential expression analysis were performed with DESeq2 (Galaxy Tool Version 2.11.40.6; DESeq2 version 1.22.1) with default settings except for "Output normalized counts table," "Turn off outliers replacement," "Turn off outliers filtering," and "Turn off independent filtering," and all of which were set to "True" [17]. The *Ec*N infection was selected as a primary factor, whereas the donor was used as a secondary factor in DESeq2 analyses (two-factor design). The results of the DESeq2 analysis are displayed in Supplementary Table 1. DESeq2 result table was loaded into Qlucore Omics Explorer (version 3.7) software using the Wizard function for visualization via heat map.

For gene set enrichment analysis, Enrichr gene set enrichment analysis web server was utilized [18]. Visualization for enrichment analysis was performed with the Appyters [19] programmatically run from the Enrichr results page with default settings for the Enrichr library KEGG 2019 Mouse.

2.13. Statistical Analysis. The statistical analyses were performed using GraphPad Prism 6® software (San Diego, CA, USA). The values are plotted either directly with the means and 95% confidence intervals (CIs) or standard deviations (SDs) or as the means with 95% CIs or SDs. Before calculating the means, all technical replicates were statistically tested using the Grubbs outlier test. All means from pooled groups were statistically tested via the ROUT outlier test. The following tests were performed for data with equal variances: the data from the analysis of the effects of ZellShield® on organoid kinetics were assessed by two-way analysis of variances followed by Tukey's multiple comparisons tests, the independently plotted data from experimental and biologic replicates were analyzed by a one-way analysis of variances followed by Tukey's multiple comparisons tests, and the pooled data from the biological replicates were analyzed using an unpaired *t*-test. In addition, the control and *Ec*N-infected samples of freshly isolated and cryopreserved organoids were subjected to the following pairwise comparisons by one-way analysis of variances followed by Sidak's multiple comparisons tests: fresh Ctrl vs. fresh *Ec*N, fresh Ctrl vs. thawed Ctrl, fresh *Ec*N vs. thawed *Ec*N, and thawed Ctrl vs. thawed *Ec*N. A P value of <0.05 was defined as significant (∗) for all experiments with the following grading: $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

3. Results

3.1. ZellShield® Does Not Affect Gene Expression in Colonoids. Due to practical reasons such as easier handling, the culturing of cell lines using antibiotics is a common practice. Because most conventional cell lines are derived from tumors and therefore do not properly recapitulate the

physiological state, the possible side effects of antibiotics can be neglected in most studies. For primary cells collected from a nonsterile environment such as the gut, it is important to avoid the overgrowth of bacteria or fungi. Thus, the use of antibiotics is crucial for achieving and maintaining a sterile environment. However, it is commonly known that the microbial flora shapes and maintains, for example, a strong intestinal barrier [20, 21]. Therefore, its removal due to antibiotic administration could alter the physiology of primary cells and their reaction to environmental stimuli, for example, when conducting infection experiments. Additionally, antibiotics could have a direct impact on gene expression levels; thus, omitting them might have an impact on the experimental outcome.

To analyze possible side effects of antibiotics on colonoids, we performed a kinetic experiment with controlled addition (+ ZS) and removal (Ø ZS) of ZellShield®, a defined purchasable mix of antibiotics/antimycotics, over the course of 12 hours (h) with a sample collection 1, 2, 4, 6, and 12 hours after media administration (Figure 1). Gene expression levels of the tight junction proteins *Cldn4* (claudin 4), *Ocln* (occludin), and *Zo-1* (zonula occludens-1; tight junction protein 1) and the cytokine *Tnfα* did not differ between the two conditions and were mostly stably expressed over time with a rather high standard deviation for *Cldn4*, *Ocln*, and *Tnfα*. The proliferation marker *Ki67* was also equally expressed in both conditions, but as expected, expression slowly decreased over time. This is consistent with our earlier findings [15] and represents the consumption of fresh media. In summary, ZellShield® seemed to have no effect on tight junction expression, proliferation, or induction of *Tnfα* in colonoids. However, to minimize effects related to medium changes and to acclimate organoids, fresh medium should be administered 12-16 hours prior to all experiments.

3.2. Experimental Data Are Predominantly Affected by Interexperimental Differences. To analyze the technical and interexperimental reliability, as well as the biological variability among organoids, we measured the acute effects of infection with *E. coli* Nissle 1917 (*Ec*N) on gene expression in colonoids. We thus performed five independent infection experiments, hereafter referred to as experimental replicates. For each experiment, we generated three different organoid lines (biological replicates) and prepared three technical replicates of each control and *Ec*N-infected sample from each organoid line. We wanted to analyze the reproducibility of our data and which type of replicate (technical, biological, and experimental) has the highest impact on the experimental outcome.

First, we examined the clustering of the technical replicates (Figure 2(a); representative results from the control samples of one organoid line from each experiment are shown). We noted four different clustering patterns: most technical replicates clustered closely together with a rather small SD and no visual and statistically significant outlier, as observed for, for example, *Cldn4* expression in organoid lines no. 2 and 8. Other technical replicates clustered evenly apart from one another with the mean laying around the middle value, as was observed for, e.g., *Tnfα* expression in organoid lines no. 2 and 8. Few replicate groups had visual outliers but no statistical relevance, as was detected for, for example, *Zo-1* and *Cldn4* expression in organoid line no. 9. Only two technical replicate groups had statistically relevant outliers (shown as black dots), namely, *Zo-1* and *Ki67* expression in organoid line 12, and these groups were later omitted from the mean calculation. Overall, the analysis of all technical samples revealed an acceptable statistical outlier frequency of at most 2.15% per gene (maximum of two outliers out of 93 individual values per gene). Therefore, our technical replicates were rather reliable and had only a low impact on the experimental outcome.

In the next step, we analyzed the clustering of biological replicates per experiment (Figure 2(b)). We observed a clustering pattern similar to the four different patterns found for the technical replicates, but in general, the biological replicates within an experiment clustered rather closely together. Statistically, only two relevant outliers (shown as red triangles) within a biological replicate group were detected for all the genes: *Tnfα* expression in organoid line 3 and *Ki67* expression in organoid line 4. This finding equates to an overall statistical outlier frequency of at most 3.33% per gene (max. 1 out of 30 mean values per gene). After pooling the data, including the two previously mentioned outliers (Figure 2(c)), no statistically significant outliers could be detected. Therefore, the sole biological variability had a rather low impact on our data.

In the final step, we further analyzed the effects of experimental replicates on variability (Figure 2(b)) and detected several significant interexperimental differences by the ANOVA: the *Ocln* gene expression levels differed significantly between two control groups (Ctrl 3 and 5, $P = 0.0239$). In addition, the *Cldn4* expression levels were significantly different between two *Ec*N-infected groups (*Ec*N 3 and 5, $P = 0.0054$). Additionally, after *Ec*N infection, the *Tnfα* gene expression level in one experiment was significantly higher than that in three other experiments [*Ec*N 4 and *Ec*N 1 ($P = 0.0089$), 2 ($P = 0.0201$), and 3 ($P = 0.0021$)]. Although several significant differences were detected between the experiments, it is important to mention that the increasing or decreasing trends in gene expression between the control and infected samples were mostly the same in the independent experiments. For example, the *Ki67* gene expression levels differed significantly between both control samples ($P = 0.0297$) and both *Ec*N-infected samples ($P = 0.0133$) of the same experiments (nos. 3 and 5). This trend was also visually observed for other expression patterns showing nonsignificant differences. However, experimental replicates had a higher impact on our data than technical and biological replicates.

Focusing again on the pooled data (Figure 2(c)) and the overall experimental outcome, we measured a significant increase in *Cldn4* gene expression after *Ec*N infection ($P = 0.0439$), but this finding could not be detected in the single experiments, presumably due to the rather low effect. In contrast, the significant increase in *Tnfα* expression after *Ec*N infection ($P < 0.0001$) was also observed in the independent experiments (Figure 2(b); the following significant differences are not shown in the graph: comparison of Ctrl and *Ec*N in Exps. 1 to 5: $P = 0.0073$, $P = 0.0026$, $P = 0.0098$

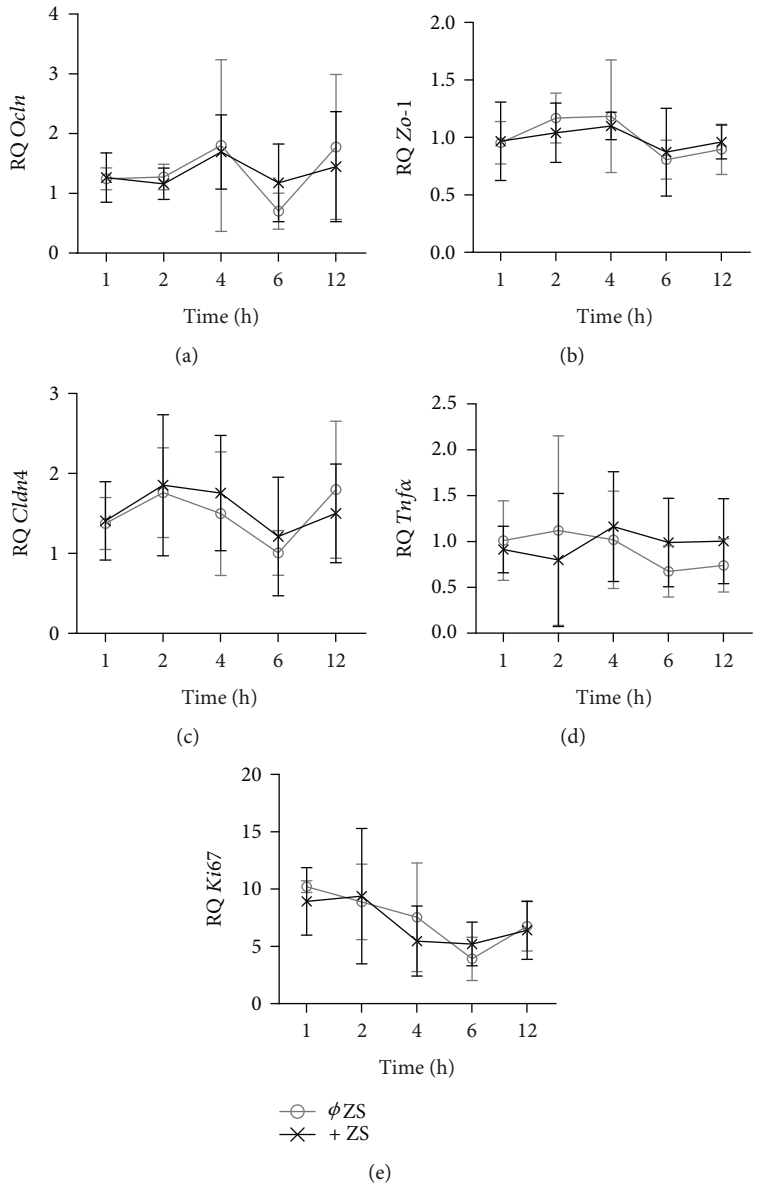

Figure 1: ZellShield® does not affect organoid kinetics. Comparison of the gene expression levels in colonoids over the course of 12 hours after the administration of fresh medium with (+ ZS) and without (Ø ZS) ZellShield® (each: $n = 5$). The graphs show the means with SDs. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$. (a) Relative *Ocln* expression, ANOVA ($F(9, 41) = 0.9771$; $P = 0.4728$). (b) Relative *Zo-1* expression, ANOVA ($F(9, 42) = 1.088$; $P = 0.3915$). (c) Relative *Cldn4* expression, ANOVA ($F(9, 42) = 0.9054$; $P = 0.5295$). (d) Relative *Tnfα* expression, ANOVA ($F(9, 42) = 0.4397$; $P = 0.9055$). (e) Relative *Ki67* expression, ANOVA ($F(9, 41) = 1.946$; $P = 0.0719$).

, $P < 0.0001$, and $P < 0.0001$, respectively), but these mostly exhibited a lower P value than that obtained for the pooled data. The gene expression levels of the tight junction proteins *Ocln* ($P = 0.4695$) and *Zo-1* ($P = 0.1021$) and the proliferation marker *Ki67* ($P = 0.6469$) did not differ between the control and *Ec*N-infected samples (Figure 2(c)). Taken together, these results show that *Ec*N has an impact on the gene expression levels of the tight junction protein *Cldn4* and on the induction of *Tnfα*.

3.3. Cryopreserved Organoids Show Attenuated Responses in EcN Infection Experiments. One of the major advantages of the organoid system is the ability to propagate organoids shortly after isolation and subsequently cryopreserve them until further use [6], similarly to regular nonprimary cell lines. However, it is commonly known that the freezing and thawing of cells is an invasive treatment that can alter not only the cell viability but also other parameters within a cell, such as gene expression patterns. To analyze the possible effects of cryopreservation on primary organoid cell culture and on the outcome of infection experiments, we first compared the morphology of freshly isolated colonoids with cryopreserved and subsequently thawed colonoids (hereafter referred to as cryopreserved colonoids, Figures 3(a) and 3(b)); then, we performed infection experiments on both colonoid types in passage 3. We compared the acute effects of *Ec*N infection

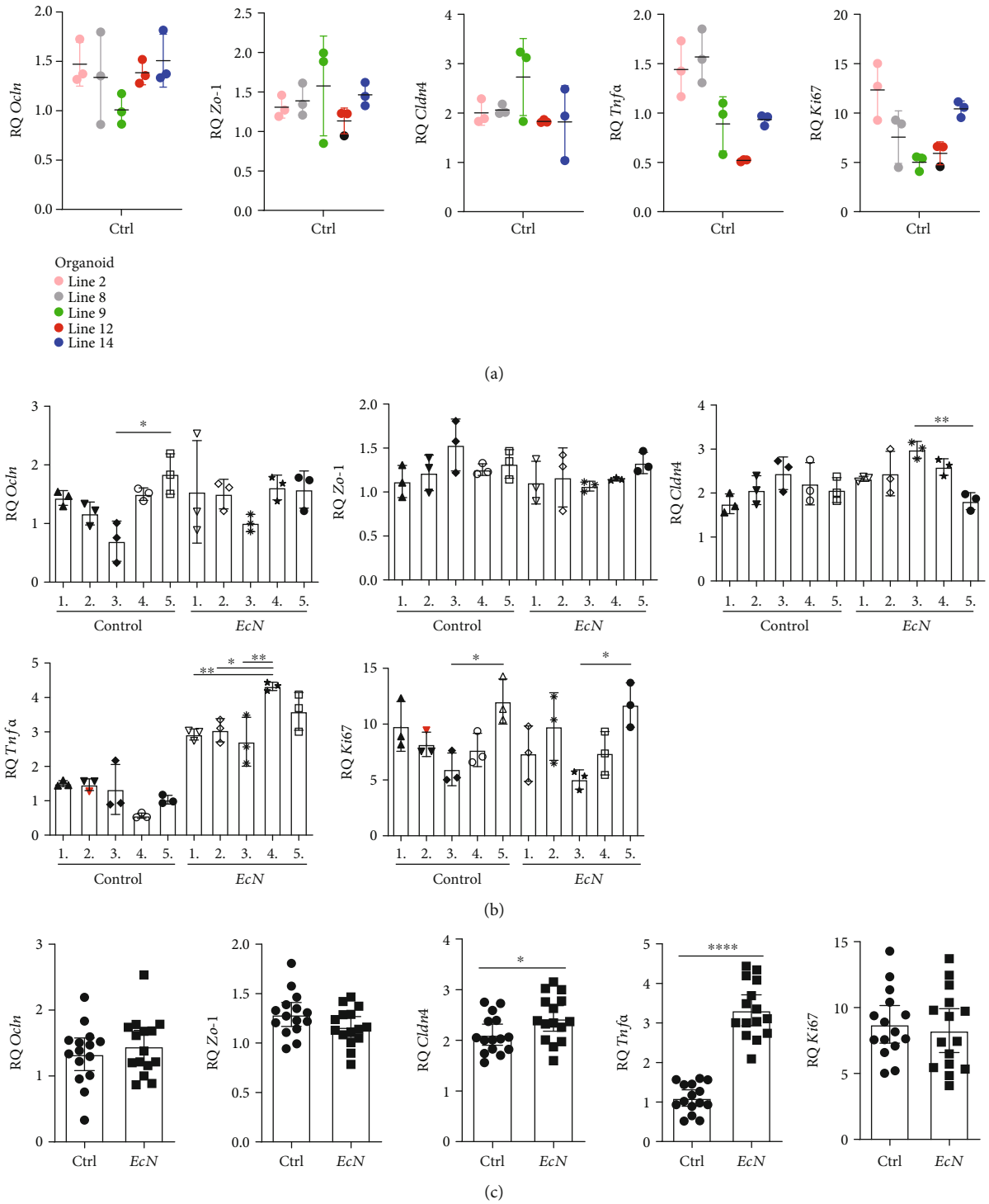

Figure 2: Experimental data are predominantly affected by interexperimental differences. The technical and experimental reproducibility and the biological variability in colonoids were compared using the EcN infection model. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$. (a) Technical replicates. The graphs plot individual values of five representative organoid lines with the technical replicate mean and SD values; the outliers, as identified using the Grubbs outlier test, are shown as black dots. (b) Experimental and biological replicates. The graphs plot five independent experiments with the technical replicate means from three different organoid lines (biological replicates) per experiment plus the overall mean and SD per experiment; the outliers, as identified using the Grubbs outlier test, are shown as red triangles; ANOVA: Ocln ($F(9, 20) = 2.609$, $P = 0.0355$), Zo-1 ($F(9, 20) = 1.538$, $P = 0.2019$), Cldn4 ($F(9, 20) = 4.227$, $P = 0.0035$), Tnfα ($F(9, 20) = 29.69$, $P < 0.0001$), and Ki67 ($F(9, 20) = 4.150$, $P = 0.0039$). (c) Pooled data. The graphs plot the technical replicate means of all biological replicates ($n = 15$) from all independent experiments plus the overall mean and 95% CI.

on gene expression levels and different signaling pathways (Figures 3(c) and 4–6). For a direct comparison within the same biological replicates, freshly isolated colonoids were also used for cryopreservation, thawed, and then infected together with new freshly isolated colonoids of the same passage number. During passage 1, more and bigger colonosphere structures were observed in the cryopreserved culture compared to freshly isolated organoids (Figure 3(a)). No differences were detectable in passages 2 and 3. Immunohistological staining for CD326, a marker for epithelial cells, showed a positive signal in the outer cell layer of colonospheres and organoids (Figure 3(b)). High amounts of KI67-positive cells were found throughout the whole epithelium of all colonospheres, and positive cells in mature colonoids were located at the base and sides of the intestinal crypts. Colonospheres as well as organoids were positive for the intracellular TJ protein ZO-1 (Figure 3(b)). In addition, epithelial cell subtypes such as enterocytes (*Slc5a1*), enteroendocrine cells (*Chga1*), and goblet cells (*Muc2*) were analyzed using qPCR before and after *Ec*N infection. All analyzed genes did not differ between freshly isolated and cryopreserved colonoids (Figure 3(c)). After *Ec*N infection, *Ki67* expression was significantly higher in the cryopreserved organoids ($P = 0.0425$) and tended to be higher in the control cryopreserved organoids than in freshly isolated colonoids.

Furthermore, we performed RNA sequencing and pathway analysis from freshly isolated and cryopreserved colonoids after *Ec*N infection. Although donor and interexperimental-specific differences were detectable, overall, all genes which were significantly (adjusted P value < 0.01; Supplementary Table 2) differentially expressed in *Ec*N samples in fresh colonoids compared to control counterparts displayed a comparable expression pattern in thawed organoids (Figure 4). The change in gene expression levels between *Ec*N and control samples was attenuated in cryopreserved colonoids compared to fresh ones, though. While in fresh colonoids, the significantly differentially expressed genes (DEGs) amounted up to 290; in cryopreserved colonoids, only 140 significantly DEGs were detected between *Ec*N and control samples (Supplementary Table 3). Notably, almost all of those 140 significantly DEGs were also found among the 290 genes in the group of fresh organoids without indication for any additional effect of the thawing process on *Ec*N treatment outcome.

Correspondingly, both fresh and cryopreserved colonoids experienced gene upregulation in, overall, the same pathways or biological processes in response to *Ec*N infection (Figure 5). Upregulated genes were especially associated with the TNF, IL-17, MAPK, or NF-kappa B signaling pathway (Figure 5(a)). Although the same signaling pathways were activated in fresh and cryopreserved organoids after *Ec*N infection, the association with each gene set, except the TNF and IL-17 signaling pathway (Figure 5(a)), was more significant for the fresh colonoids due to the higher number of significantly upregulated genes (Figure 5(b)).

In addition, the relative gene expression of the tight junction genes *Ocln*, *Zo-1*, and *Cldn4* before or after infection did not significantly differ between the two types of colonoids (Figure 6). However, the *Cldn4* expression levels were significantly elevated in fresh organoids after *Ec*N infection ($P = 0.0182$), whereas no significant differences were detected by ANOVA in the cryopreserved organoids ($P = 0.1144$). But a direct comparison using an unpaired t-test showed a significant difference between the control and *Ec*N-infected samples of cryopreserved colonoids ($P = 0.0124$). In addition, *Tnfα* expression was significantly upregulated in both types of colonoids (both $P < 0.0001$) in response to bacterial stimulation. However, after *Ec*N infection, significantly higher expression was detected in the cryopreserved organoids than in the fresh colonoids ($P = 0.0110$). Together, these results indicate that strong effects can be easily observed in cryopreserved colonoids, whereas smaller effects might remain undetected.

4. Discussion

As often stated in various articles, comments, and reviews, standardization techniques have been needed in the field of organoid research for a longer period [5–7, 22]. Because every laboratory uses its own methods for conducting experiments and uses different tissue/cell sources, among other variations, the recreation of results and their transferability to other labs are extremely difficult. According to our early personal experience with organoid culture, even the reproducibility of our results proved to be challenging, and we therefore developed several methods for standardizing our colonoids. Here, we provide some insights into these techniques and aim to answer several questions we encountered during our colonoid research over the last few years.

As stated previously, the commonly used cell lines and colonoids are usually cultured in the presence of antibiotics and/or antimycotics, such as ZellShield®. A culture without these agents would lead to severe infection and overgrowth of the endogenous microbiota and fungi that are naturally present in the donor tissue and cannot be mechanically removed during crypt isolation. Another option is the use of a tissue derived from germ-free mice, but germ-free animals often pose other challenges and do not recapitulate the physiological state of the gut. For example, germ-free mice have a weakened intestinal barrier, reduced metabolic rates, and an enlarged cecum because their body has to cope with the lack of digestive microbiota [23]. In addition, biopsy samples for the preparation of human intestinal epithelial organoids cannot be obtained from a germ-free individual; therefore, antibiotic administration is necessary. However, it is known that antibiotic treatment can alter the gene expression levels of epithelial cells and immune cells [24–27], and the sudden lack of antibiotics might be responsible for any effects detected in these cells. Therefore, we analyzed the impact of ZellShield® removal on the gene expression levels of the tight junction proteins *Ocln*, *Zo-1*, and *Cldn4* and the proliferation marker *Ki67* and the induction of the proinflammatory cytokine *Tnfα* in colonoids over the course of 12 hours. We did not detect any differences between culture with and without ZellShield® and concluded that ZellShield® has no effect on the expression of the analyzed genes in colonoids. However, this study and our previously published work [15] revealed that the administration of fresh medium has a direct impact on colonoid gene

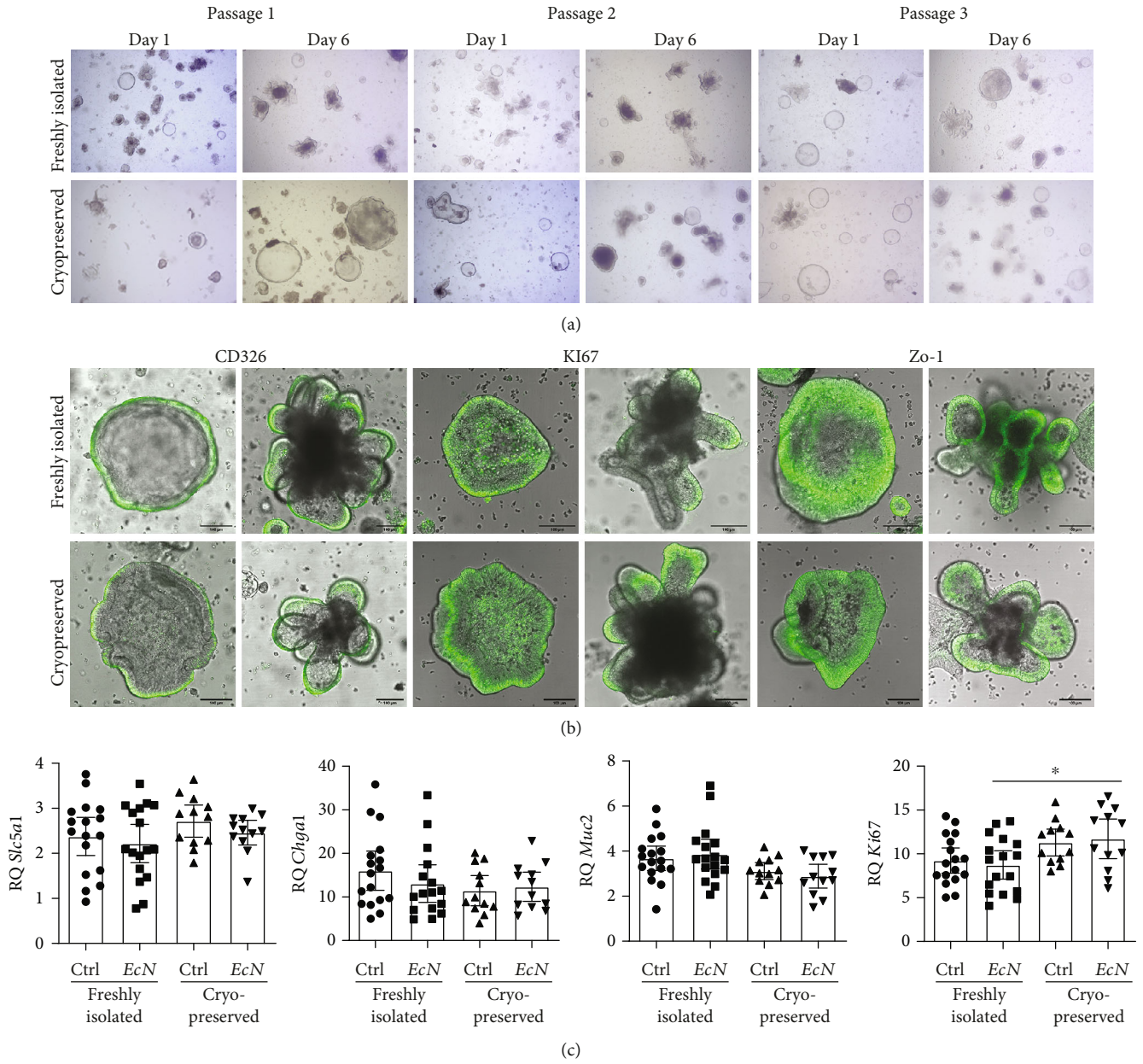

Figure 3: Characterization of freshly isolated and cryopreserved colonoids during growth and after *Ec*N infection. (a) Representative light phase-contrast images. (b) Immunofluorescent staining for epithelial cell adhesion molecule (CD326), KI67, and tight junction protein 1 (ZO-1). (c) The gene expression levels in freshly isolated colonoids ($n = 17$) and cryopreserved and thawed organoids ($n = 12$) of the same passage were compared before and after infection with *Ec*N. The graphs plot the pooled technical replicate means of biological replicates plus the overall mean and 95% CI. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$. Relative *Slc5a1* expression (ANOVA: $F(3, 54) = 1.179$; $P = 0.3264$). Relative *Chga1* expression (ANOVA: $F(3, 53) = 1.104$; $P = 0.3555$). Relative *Muc2* expression (ANOVA: $F(3, 54) = 2.932$; $P = 0.0416$). Relative *Ki67* expression (ANOVA: $F(3, 54) = 3.435$; $P = 0.0231$).

expression. It has been shown that standardized and improved culture conditions [28, 29] together with well-timed media administration will result in optimized experimental settings [15]. Therefore, we recommend the administration of fresh medium 12-16 hours before performing any experiments with new medium to minimize the effects related to medium changes. For optimal results, specific analyses for each case should be performed.

As mentioned previously, at the beginning of our work with intestinal organoids a few years ago, we experienced a lack of reproducibility in our experimental data. We considered factors that might influence the outcome of experiments with organoids, and according to good scientific practice, a well-planned study design is an important factor for obtaining reproducible data. Therefore, we were interested in the reliability of the experimental data obtained from technical and experimental replicates of colonoids and the extent to which biological variability might influence these data. The last factor, biological variability, is also important because the organoid system is aimed at replacing

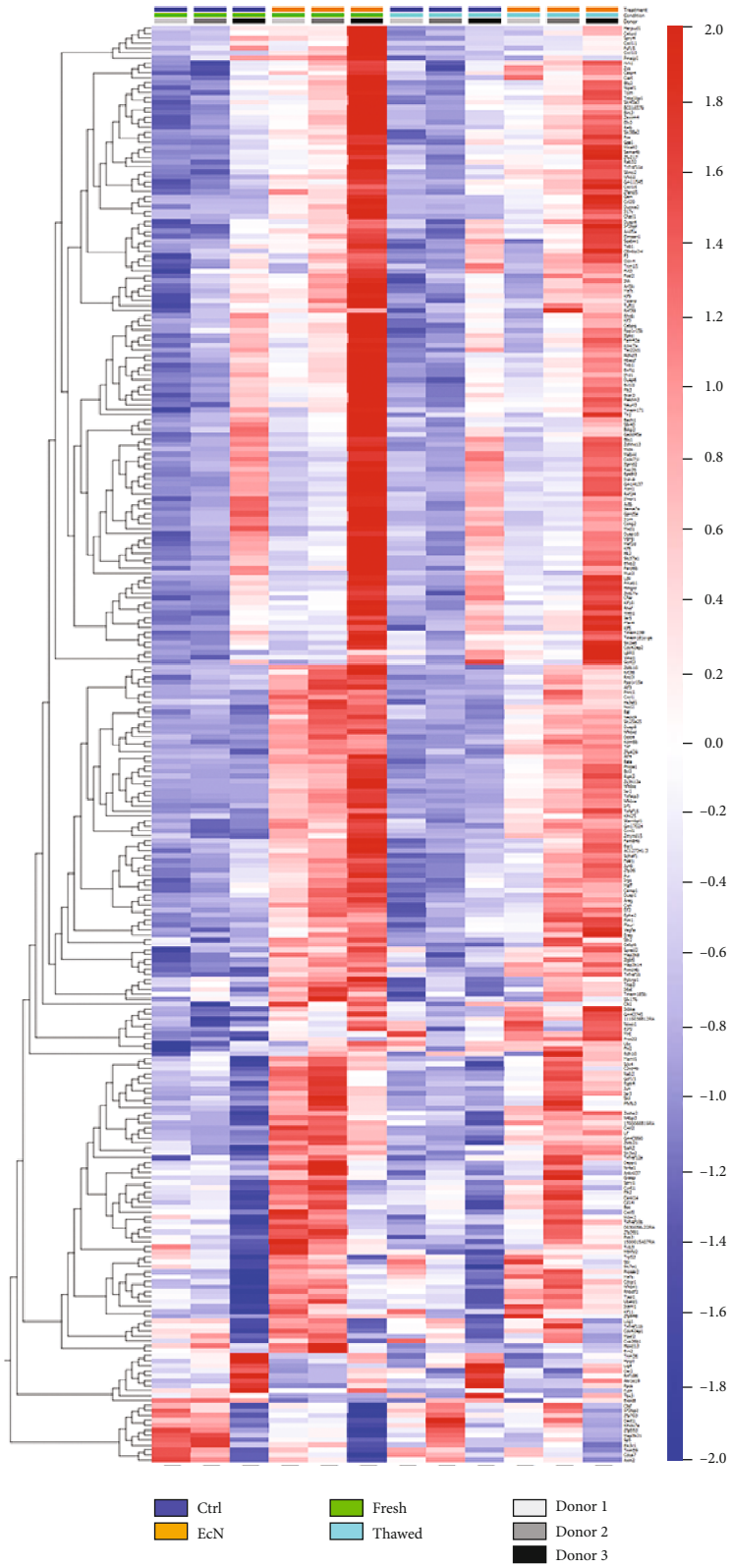

Figure 4: Fresh and cryopreserved organoids exhibit a similar gene expression profile in response to *Ec*N injection. Heat map displaying significantly (adjusted P value < 0.01) differentially expressed genes detected between *Ec*N and control samples in fresh colonoids. Besides the normalized and log transformed expression levels of the samples originating fresh organoids, the heat map includes in an analogous manner the samples of thawed colonoids.

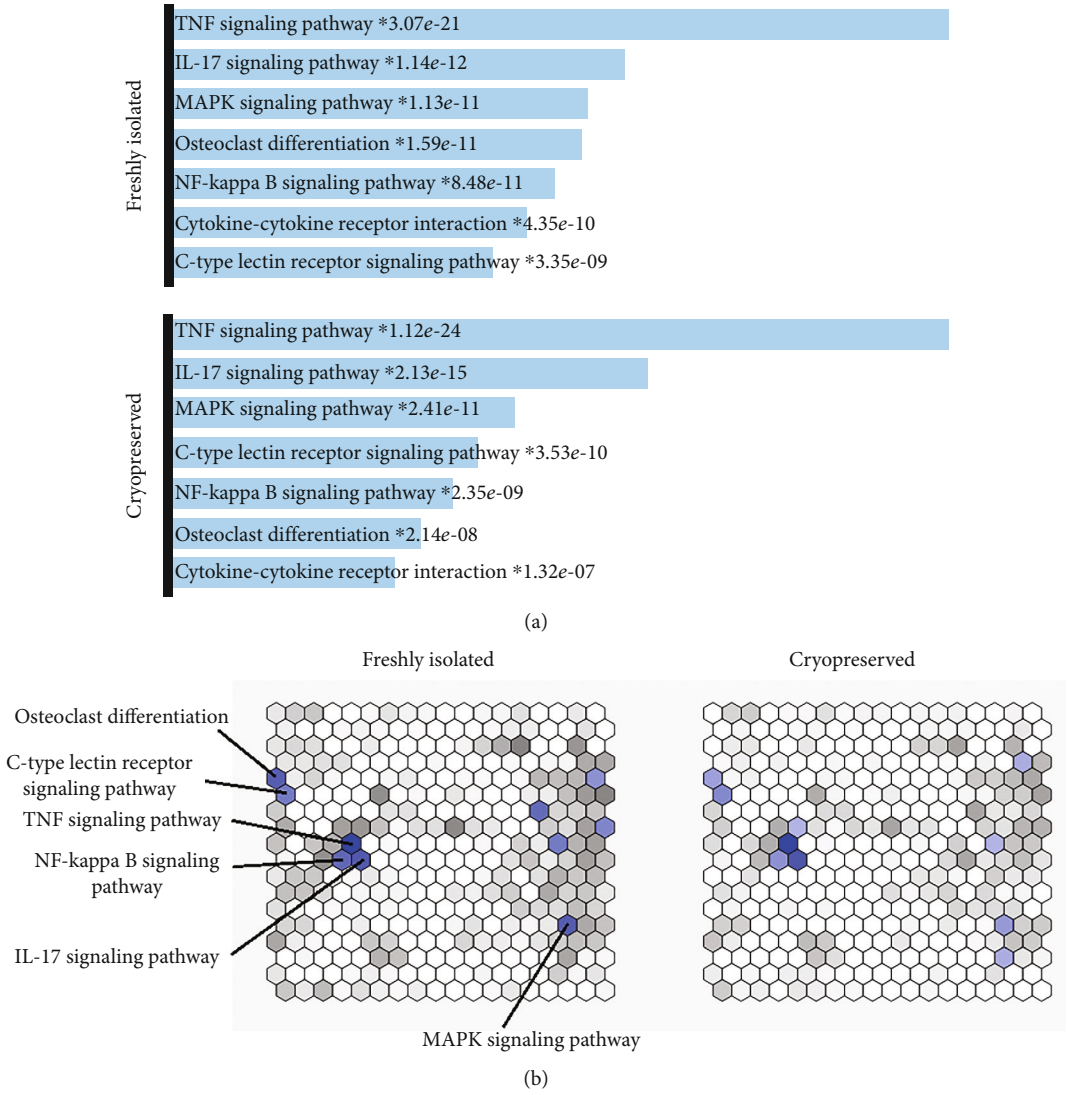

Figure 5: Gene set enrichment analysis. Significantly (adjusted P value < 0.01) upregulated genes in *Ec*N compared to control samples originating from fresh and cryopreserved colonoids, respectively, were examined for their similarity to gene sets of the KEGG 2019 Mouse library via Enrichr gene set enrichment analysis web server. (a) Top enriched terms in the KEGG 2019 Mouse library, with P values. Asterisk symbolizes the term has an adjusted P value < 0.05. (b) Each hexagon represents one gene set from the KEGG 2019 Mouse library. The brighter the blue color is, the more similar and, therefore, significant the specific gene set. Hexagons that are grouped together represent similar gene sets.

animal experiments and reducing the number of animals used according to the 3Rs. As a model setup for this study, we used an infection experiment with the probiotic bacterium *E. coli* Nissle 1917 that we previously established in our colonoids [15] and measured the gene expression levels of the abovementioned markers. Our observations revealed that both technical and biological replicates were rather reliable with few statistical outliers and thus had a rather low impact on our data. In contrast, experimental replicates exhibited more interexperimental differences and therefore had a higher impact on the produced data. These findings are consistent with those obtained by Pamies et al. [22], who state that standardizing organoids is highly demanding due to their complexity, which "can be associated with variability between individual […] experiments, thus affecting reproducibility of […] quality and functionality and hence any downstream readouts." Overall, we conclude from our analyses that it is more important to conduct an experiment several times instead of adding many technical and biological replicates to only one or two experiments. The exact quantity of these parameters also depends on the expected outcome and statistical power, which should be measured in preliminary tests. In our previously published work [15], we standardized our data from infected/stimulated samples to the corresponding controls to further account for interexperimental differences. The rather low impact of biological variability on gene expression levels indicates that reducing the numbers of animals used for the generation of organoids of animal origin is possible. However, Voelkl et al. [30] note that it is also important to introduce some heterogenization

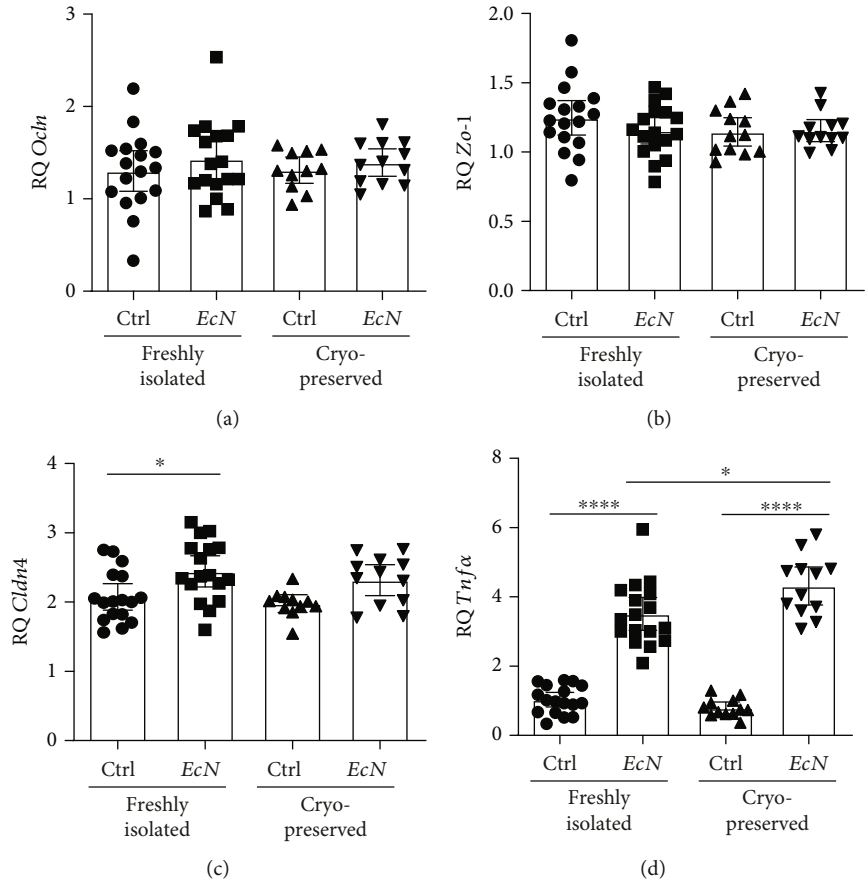

Figure 6: Cryopreservation affects gene expression levels in colonoids after *Ec*N infection. The gene expression levels in freshly isolated colonoids ($n = 17$) and cryopreserved and thawed organoids ($n = 12$) of the same passage after infection with *Ec*N were compared. The graphs plot the pooled technical replicate means of biological replicates plus the overall mean and 95% CI. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$. (a) Relative *Ocln* expression (ANOVA: $F(3, 53) = 0.4720$; $P = 0.7031$). (b) Relative *Zo-1* expression (ANOVA: $F(3, 54) = 1.010$; $P = 0.3953$). (c) Relative *Cldn4* expression (ANOVA: $F(3, 53) = 4.962$; $P = 0.0041$). (d) Relative *Tnfα* expression (ANOVA: $F(3, 54) = 91.30$; $P < 0.0001$).

to achieve increased reliability and to avoid idiosyncratic results, for example, from only using mice of the same age or the same genetic/microbial background. Regarding the observed effects obtained after pooling our data, the increases in the gene expression levels of the tight junction protein *Cldn4* and the proinflammatory cytokine *Tnfα* are consistent with our prior experimental results and might suggest a positive probiotic effect of *Ec*N on the epithelium, which might involve enhancing the barrier through the upregulation of tight junction components and the recruitment of immune cells via cytokine induction [15]. In tubular cells, *Tnfα* increases the gene expression and surface levels of *Cldn4* and thereby contributes to an increase in the transepithelial resistance [31]. Other in vitro studies with IECs have also shown that the response to *Ec*N stimulation and other probiotic bacteria is transiently proinflammatory [32, 33]. Therefore, our research group suggested that the upregulation of cytokines such as *Tnfα* might be part of the probiotic effect of *Ec*N [34]. Furthermore, Yan et al. [35, 36] reported that *Tnfα* is responsible for the activation of both pro- and anti-inflammatory signaling pathways and that their balance is crucial in IBD.

Again, to reduce the use of experimental animals and for the storage of patient-derived organoid cultures, using cryopreserved organoids is a major advantage of the whole organoid system. However, Pamies et al. [22] note that the cryopreservation of organoids (among others) is more complex than that of standard cell culture; hence, the maintenance of their functionality has to be ensured. To the best of our knowledge, the experimental reliability of cryopreserved murine organoids has not yet been investigated, and in our early experience with cryopreserved and then thawed organoids, we noticed that these showed a different growth pattern during the first passage compared with freshly isolated organoids. Formerly cryopreserved colonoid cultures appear to have a higher quantity of premature spheroid structures and exhibit delayed development. Therefore, we wanted to analyze their experimental behavior compared with that of freshly isolated colonoids in early passages. For this purpose, we also used *Ec*N infection as our experimental setup. To heed the European directive 2010/63/EU [13] to reduce animal numbers, we cryopreserved our freshly isolated colonoids used in our other experiments and thawed most volumes for comparison. In addition, most

of the data from the freshly isolated organoids were obtained from the experiments with "different replicates," which were conducted simultaneously. Hence, the same colonoids were used for both states, which also increase the comparability of the results. Further comparison of the cryopreserved and freshly isolated organoids showed no differences in cell subtype composition but a higher expression of *Ki67* in the cryopreserved colonoids, which hints that these are found at a presumably more premature state than fresh colonoids at the same passage. For additional analysis, we performed RNA sequencing analysis and revealed more DEGs in *Ec*N-infected freshly isolated colonoids compared to cryopreserved colonoids. However, cryopreserved organoids displayed a comparable expression pattern. As previously observed, the gene expression levels of the tight junction marker *Cldn4* and the cytokine *Tnfα* were significantly increased in the *Ec*N-infected samples of both types, although lower upregulation of *Cldn4* expression was observed in the cryopreserved colonoids. This different cell status might influence the response to bacterial challenge and might also be responsible for the significantly higher expression of the cytokine *Tnfα* observed in the cryopreserved compared with the freshly isolated colonoids. For example, during the tumor progression of gastroenteropancreatic neuroendocrine neoplasms, the expression of *Tnfα* is positively correlated with high proliferation rates, as indicated by *Ki67* expression [37]. Taken together, these results indicate that strong effects, such as the increase in *Tnfα* expression, can be easily detected in cryopreserved and possibly more premature colonoids, whereas smaller effects, such as the upregulation of *Cldn4*, might be more easily detected in freshly isolated, more mature colonoids. Therefore, the developmental characteristics of organoids should be tested before to their use in experiments to analyze which passage is optimal for experimental usage. Whether cryopreserved organoids also show different behaviors at older passages remains to be analyzed, but another study using bovine colonoids showed that formerly in-plate *in situ* cryopreserved colonoids showed similar growth rates to unfrozen colonoids of the same passage and found no significant increase in cytotoxic sensitivity to staurosporine after *in situ* freeze-thawing [38]. Other studies regarding aging in intestinal epithelial organoids describe organoid culture as an aging system similar to the *in vivo* state [39–42]. Therefore, it is likely that cryopreserved murine colonoids also mature over time and can be reliably used for experiments, which would enable a further reduction in animal numbers according to the 3Rs. For this purpose, another possibility might be the cryopreservation of whole tissue samples using the DMSO slow-freeze technique for later organoid generation, as was previously described for tumor-derived organoids by Walsh et al. [43]. These researchers observed similar *Ki67* expression and a matching drug response in organoids generated from a fresh and DMSO frozen tumor tissue a few days after generation.

In general, studies involving organoids should be carefully designed such that the lowest number of animals is used for the highest number of experiments. Of course, this poses a challenge related to the planning and interweaving of all experiments being conducted, but the same stipulation has to be considered in official animal experiments.

5. Conclusions

In conclusion, cultivation with or without ZellShield® had no impact on the analyzed genes of interest. Regarding good scientific practice, we showed that the experimental outcome is predominantly influenced by interexperimental differences and that the technical and biological variabilities are rather low. In addition, the cryopreservation of organoids might also influence the experimental outcome due to a possibly premature character of organoids at early passages and their higher proinflammatory response to bacterial stimulation. Therefore, testing the growth characteristics of organoids prior to their use in experiments would be recommended and will aid further standardization of organoid culture.

Data Availability

All protocols used or data generated in this study are available from the corresponding author on reasonable request.

Conflicts of Interest

The authors declare no conflicts of interest.

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Supplementary Materials

Table 1: DESeq2 result table. Table 2: Significantly different gene expression from *Ec*N samples in fresh colonoids compared to control counterparts. Table 3: Significantly differentially expressed genes between *Ec*N and control samples. *(Supplementary Materials)*

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Review Article

Brain Organoids: Studying Human Brain Development and Diseases in a Dish

Jie Xu[1] **and Zhexing Wen**[2,3,4]

[1]*The Graduate Program in Genetics and Molecular Biology, Laney Graduate School, Emory University, GA 30322, USA*
[2]*Department of Psychiatry and Behavioral Sciences, Emory University School of Medicine, Atlanta, GA 30322, USA*
[3]*Department of Cell Biology, Emory University School of Medicine, Atlanta, GA 30322, USA*
[4]*Department of Neurology, Emory University School of Medicine, Atlanta, GA 30322, USA*

Correspondence should be addressed to Zhexing Wen; zhexing.wen@emory.edu

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With the rapid development of stem cell technology, the advent of three-dimensional (3D) cultured brain organoids has opened a new avenue for studying human neurodevelopment and neurological disorders. Brain organoids are stem-cell-derived 3D suspension cultures that self-assemble into an organized structure with cell types and cytoarchitectures recapitulating the developing brain. In recent years, brain organoids have been utilized in various aspects, ranging from basic biology studies, to disease modeling, and high-throughput screening of pharmaceutical compounds. In this review, we overview the establishment and development of brain organoid technology, its recent progress, and translational applications, as well as existing limitations and future directions.

1. Introduction

Being the control center of the nervous system in humans, the brain is one of the most complex and advanced organs in the body, and thus, it has never been easy to study the biological basis of brain development and brain disorders. The current knowledge of the human brain is mostly based on postmortem brain specimens, mainly due to the difficulties in accessing human brain tissues. As a result, animal models, including nonhuman primates, have been widely used to study the development and function of the brain for many decades. However, the human brain differs from those of other species not only in size, shape, and structure but also in cellular and molecular composition and developmental trajectory [1–5]. Hence, a model system that can better recapitulate human brain development is urgently needed to deepen our understanding in human-specific developmental processes and molecular mechanisms.

The advent of stem cell technology has opened a new avenue to study human brain development *in vitro*, providing new platforms for modeling neurological disorders, especially those involves developmental processes that are unique to human [6–8]. For the last decade, human stem cells, including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), have been widely used in the differentiation of monolayer neural cells to investigate the cellular and molecular mechanisms of neurodevelopment and neurological disorders. While monolayer (two-dimensional) cell culture has provided a system that can efficiently produce relatively homogeneous population of a cell type, they still cannot recapitulate many characteristic features of the human brain, such as self-organizing properties and interactive dynamics [9, 10].

These limitations inspired the innovation of a more sophisticated model system and thus led to the invention of brain organoids. Brain organoids are stem-cell-derived 3D suspension cultures that are capable of self-assembling into an organized structure with features resembling the developing brain, such as ventricle formation, cortical layer organization, and neuronal migration [11–18]. Transcriptomic and

epigenomic analysis also revealed that brain organoids recapitulate many features of early-to-mid and mid human fetal brain [19]. Additionally, whereas monolayer cultures can only be maintained for a short period of time, long-term culturing of brain organoids promotes further maturation and thus provides opportunities for investigating late-stage developmental events such as gliogenesis, neuronal maturation, and neuronal network formation. For example, high-depth bulk and single-cell RNA-sequencing confirmed the presence of astrocyte-lineage cells in human cortical spheroids and that these astrocytes resemble primary human fetal astrocytes [18]. Moreover, cerebral organoids cultured for eight months exhibited spontaneously active neurons and neuronal networks and generated photosensitive cells that can respond to light stimulation [20]. Most recently, a comprehensive assessment on the maturation of human cortical organoids reported attainment of early postnatal features when cultured for 250~300 days *in vitro*, which was in a timeline paralleling *in vivo* development. These features included switches in the histone deacetylase complex and NMDA receptor isoform, as well as the emergence of superficial layer neurons and astrocytes at later stages [21].

With the application of diverse advanced technologies such as genome editing, single-cell sequencing, biomaterials, and bioengineering, progress has been made in brain organoids to better recapitulate features of the human brain, including supplementation of brain-blood barrier, vasculature, and microglia. Here, we summarize some of the recent innovations on brain organoid techniques and review the use of human brain organoids on the investigation of neurological and neurodevelopmental disorders as well as potential treatments (Table 1). At the end, we also discuss the limitations of organoid models and highlight potential improvements that would allow brain organoids to progress further in the future.

2. Main Text

2.1. Current Methodologies of Generating Three-Dimensional Brain Organoids. In general, protocols for induction of brain organoids from stem cells can be classified into two main categories: unguided methods that make use of the spontaneous morphogenesis and intrinsic signaling potential of human pluripotent stem cell (hPSC) aggregates to generate brain organoids that contain a variety of cell lineage identities [14, 22, 23], as well as guided methods that induce regional cell fate specification by applying patterning factors to the culture and lead to the production of brain region-specific organoids [11, 13, 24, 25]. Unguided brain organoids are advantageous in that they have the capacity to develop into various kinds of cell lineages, including dorsal forebrain, ventral forebrain, midbrain, hindbrain, hippocampus, retina, choroid plexus, and even nonneural lineages [14, 20, 22]. Single-cell transcriptomic analyses revealed the presence of neural progenitors, excitatory neurons, inhibitory neurons, astrocytes, oligodendrocyte precursor cells, and photosensitive cells in unguided cerebral organoids, confirming the heterogeneous cellular population of these organoids [20, 26–29]. However, as every coin has two sides, the stochastic nature of hPSC spontaneous differentiation also leads to problems such as unpredictable proportion and arrangement of each cell lineage in the unguided cerebral organoids. Although the variety of cell lineages present in the unguided brain organoids has provided a unique opportunity for examining interactions between different brain regions, the high variability across batches and cell lines has made systematic and quantitative studies difficult and challenging and thus prompts interest in generating brain region-specific organoids through guided differentiation.

The principle of guided differentiation is to utilize small molecules and growth factors to promote a certain cell lineage, forming cells and structures representative of a specific brain region. Typically, neural lineages are promoted by the inhibition of the BMP/TGF-β signaling pathway; with subsequent application of relevant patterning factors (i.e., WNT3A, SHH, BMP7, FGF8, FGF2, and insulin) can the brain organoids be further directed to a discrete brain region, such as cerebral cortex, optic cup, midbrain, hippocampus, thalamus, hypothalamus, cerebellum, ganglionic eminences, and choroid plexus [11–13, 15, 17, 30–38]. Importantly, brain region-specific organoids have been shown to have less variation across batches and cell lines, which makes experiments more reproducible and quantitative analyses more reliable and easier [18].

Nevertheless, the choice between unguided and guided methods should be dictated by the scientific questions of interest. Unguided brain organoids may be more ideal for questions related to spontaneous differentiation and self-organization properties during brain development, but at the price of having high variability and heterogeneity across samples. Guided brain region-specific organoids, in contrast, show less variability and heterogeneity and are thus best suited for questions related to cell fate specification, differentiation programs, and developmental trajectory within a specific brain region.

2.2. Recent Advances of Brain Organoid Techniques. The tremendous promise of brain organoids in modeling human neurodevelopmental processes *in vitro* has inspired scientists to continuously innovate and improve the current methods. Recent advances include fusion of different brain region-specific organoids to model interactions between brain regions, incorporation of important cellular and structural components into brain organoids to better recapitulate features of the human brain, and other technical advances that benefit the development of brain organoids (Figure 1). We will review these major advances in this section.

2.2.1. Fusion of Brain Region-Specific Organoids. Interregional interactions are critical processes in the developing brain. Although unguided brain organoids contain a variety of neural structures resembling interacting brain regions, they are less efficient to utilize in scientific experiments due to having high variability and heterogeneity among individuals. To improve the current methodology, brain region-specific organoids are generated separately as desired and fused together via coculture to form "assembloids," by which developmental processes such as cellular interactions

Table 1: Selected studies investigating neurological disorders/deficits using human brain organoids.

Disease	Studies	Organoid type	Methods of generation	outcomes
Primary microcephaly	Lancaster et al., 2013 [14]	Cerebral organoids	Patient iPSC-derived; *CDK5RAP2* mutation	Fewer progenitor cells, premature neuronal differentiation; *CDK5RAP2* overexpression rescued the mutant phenotypes
	Li et al., 2017 [70]	Cerebral organoids	Patient iPSC-derived; *ASPM* mutation	Reduced organoid size, fewer progenitor cells in VZ and oSVZ, poor lamination, reduced neuronal calcium activity
	Gabriel et al., 2016 [75]	Cerebral organoids	Seckel patient iPSC-derived; *CPAP* mutation	Delayed cilia disassembly led to premature differentiation of NPCs and reduced progenitor pools
	Zhang et al., 2019 [134]	Cerebral organoids	hPSC-derived; CRISPR/Cas9-mediated homozygous knockout of *WDR62*	Delayed cilia disassembly and retarded cell cycle progression led to reduced proliferation and premature differentiation of NPCs
Autism spectrum disorder (ASD)	Mariani et al., 2015 [15]	Cortical organoids	Idiopathic ASD patient iPSC-derived	Altered transcriptomic profiles, particularly *FOXG1* upregulation; accelerated cell cycles; increased GABAergic neuron production, can be rescued by RNAi-mediated *FOXG1* knockdown
	Wang et al., 2017 [76]	Cerebral organoids	hiPSC-derived, CRISPR/Cas9-mediated heterozygous mutation of *CHD8* (*CHD8*$^{+/-}$)	Upregulation of genes involved in neurogenesis, neuronal differentiation, forebrain development, Wnt/β-catenin signaling, and axonal guidance
Tuberous sclerosis complex (TSC)	Blair et al., 2018 [81]	Cortical spheroids	CRISPR/Cas9-mediated homozygous knockout of *TSC1* or *TSC2* in hESCs	mTORC1 hyperactivation, reduced neurogenesis, increased gliogenesis; dysplastic cells in *TSC2*$^{-/-}$ cortical spheroids can be rescued by early and continuous rapamycin treatments
Neonatal hypoxia-ischemia injury	Boisvert et al., 2019 [82]	Cerebral organoids	hESC-derived; 72-hour under hypoxic environment	Inhibition of dorsal-related genes such as FOXG1, CTIP2, and TBR1; could be alleviated by minocycline
	Pasca et al., 2019 [84]	Cortical spheroids	hiPSC-derived; 48-hour under hypoxic environment	Reduction of TBR2^{+} intermediate progenitors led to cell cycle damage and premature neural differentiation; rescued by ISRIB treatments
ZIKV-associated microcephaly	Qian et al., 2016 [17]	Cortical organoids	hiPSC-derived; MR766 and FSS13025 ZIKV strain infected	Reduced organoid size, reduced neuronal layer thickness, expanded ventricular lumen, increased cell death
	Dang et al., 2016 [87]	Cerebral organoids	hESC-derived; MR766 ZIKV strain infected	Reduced organoid size, TLR3 upregulation and TLR3-mediated transcriptomic alterations; direct inhibition of TLR3 reduced phenotypes
	Watanabe et al., 2017 [88]	Cortical organoids	hPSC-derived; PRVABC59 ZIKV strain infected	Activated innate immune responses led to increased progenitor apoptosis and reduced organoid size; duramycin or ivermectin rescued the teratogenic effects of ZIKV infection
SARS-CoV-2-associated neurological deficits	Jacob et al., 2020a [98]	Cortical, hippocampal, hypothalamic, midbrain, and ChP organoids	hiPSC-derived; SARS-CoV-2 USA-WA1/2020 infected	Particular tropism for ChP epithelial cells, caused increased cell death, transcriptional dysregulation, disrupted ChP epithelial integrity and barrier function
	Pellegrini et al., 2020 [100]	Cerebral and ChP organoids	hPSC-derived; SARS-CoV-2 spike pseudovirus and live virus infected	Particular tropism for ChP epithelial cells of cerebral organoids; infected cells expressing ACE2 and lipoproteins; ChP epithelial integrity and barrier function were disrupted
Alzheimer's disease (AD)	Gonzalez et al., 2018 [102]	Cerebral organoids	Familial AD or DS patient iPSC-derived	β-Amyloid (Aβ) aggregation, formation of neurofibrillary tangle-like structures, hyperphosphorylated tau, increased cell apoptosis
	Lin et al., 2018 [109]	cerebral organoids	CRISPR/Cas9-generated isogenic iPSC lines homozygous for APOE4 alleles	Increased Aβ accumulation and tau phosphorylation

Table 1: Continued.

Disease	Studies	Organoid type	Methods of generation	outcomes
Parkinson's disease (PD)	Kim et al., 2019a [34]	Midbrain organoids	CRISPR/Cas9-generated isogenic iPSC lines harboring LRRK2 G2019S mutation	Shortened neurite length and decreased marker expression of mDAN; increased aggregation and abnormal clearance of α-synuclein; inhibition of upregulated TXNIP ameliorated mutant phenotypes
	Wulansari et al., 2021 [114]	Midbrain organoids	CRISPR/Cas9-mediated homozygous knockout of DNAJC6 in hESCs	mDAN degeneration, α-synuclein aggregation, increased neuronal firing frequencies, mitochondrial and lysosomal defects
Huntington's disease (HD)	Conforti et al., 2018 [133]	Cerebral organoids	Patient iPSC-derived	Defective progenitor identity acquisition, abnormal neuronal specification, and disrupted cellular organization
	Zhang et al., 2019 [134]	Cerebral organoids	Patient iPSC-derived and isogenic HD hESC-derived	Impaired cell cycle, disrupted neuroepithelial structures, and premature neurogenesis
Glioblastoma	Linkous et al., 2019 [139]	Cerebral organoid glioma (GLICO)	Patient-derived glioma stem cells cocultured with hESC-derived cerebral organoids	Rapid and deep invasion of glioblastoma cells into cerebral organoids; invasive tumor phenotypes in hybrid organoids
	Jacob et al., 2020b [143]	Glioblastoma organoids	Patient-derived	Recapitulated histological, cellular, and transcriptomic features of glioblastoma; aggressive infiltration after transplantation

hPSC: human pluripotent stem cell, including hiPSC and hESC; hiPSC: human-induced pluripotent stem cell; hESC: human embryonic stem cell; VZ: ventricular zone; oSVZ: outer subventricular zone; NPC: neural progenitor cells; ChP: choroid plexus; DS: Down syndrome; mDAN: midbrain dopaminergic neuron.

between distinct regions, synaptic formation, and establishment of early circuits can be investigated [39–42]. For example, the fusion of ventral and dorsal forebrain organoids revealed a unidirectional cell migration pattern; ventral-derived inhibitory neurons and interneurons were both observed to migrate in a saltatory pattern, with a single or branched process leading towards the dorsal side as previously reported in animal models [39–41]. These interneurons, after migrated into the dorsal side, exhibited increased branching complexity, showed changes in gene expression profiles, and connected and formed microcircuits with dorsal-derived excitatory neurons [40]. Similarly, corticothalamic interactions that are critical for sensory-motor processing were modeled by fusing cortical and thalamic organoids together [42]; corticostriatal circuits that regulate motivated behaviors and movements were also modeled by assembling human striatal spheroids with cortical organoids [43]. Notably, corticostriatal assembloids from patients with 22q13.3 deletion exhibited disease-associated defects in calcium activity [43], indicating the possibilities of using patient-derived assembloids in the investigation of disease-related interregional connectivity.

Most recently, a three-part system resembling the corticospinal-motor circuit was established by assembling human cortical spheroids, hindbrain/cervical spinal cord spheroids, and skeletal muscle spheroids together. Results have shown that stimulation of cortical spheroids triggered robust contraction of muscle spheroids, and these assembloids were able to stay intact both morphologically and functionally for up to 10 weeks postfusion [44], suggesting the possibilities of modeling more complex circuits with multipart assembloids. Despite the promising results found in these studies, further investigations are needed to examine whether assembloids actually model the endogenous regional interactions and, if so, what stage of development they are modeling.

2.2.2. Incorporation of Glial Cells. Glial cells have fundamental roles in the regulation and support of the nervous system. Despite having astrocytes and oligodendrocyte progenitor cells developed in cortical organoids after long-term culturing [17, 18, 20, 40], mature oligodendrocytes have not been observed in typical cortical organoids [18, 45]. As oligodendrocytes are essential for many developmental processes, such as myelination, axonal maintenance, and nutrition and metabolic support of neurons, it is important to establish a system where they can be generated and functioning. By exposing cortical spheroids to certain differentiation inducers and accelerating such process with promyelinating drugs, oligodendrocyte-like cells are generated in "oligocortical spheroids" with features consistent to those of functionally mature oligodendrocytes [46]. Later on, a protocol that promotes the development of so-called human oligodendrocyte spheroids, which contains oligodendrocytes, astrocytes, and neurons, was established and thus provided a system to investigate oligodendrocyte development, myelination, and interactions with other cell types [47].

Another major subtype of glial cells is microglia, which act as the immune cells of the nervous system and regulate its health by responding to inflammation, phagocytosing infectious microorganisms, and pruning redundant synapses. However, despite being innately developed within unguided and self-organized cerebral organoids [48], microglia are

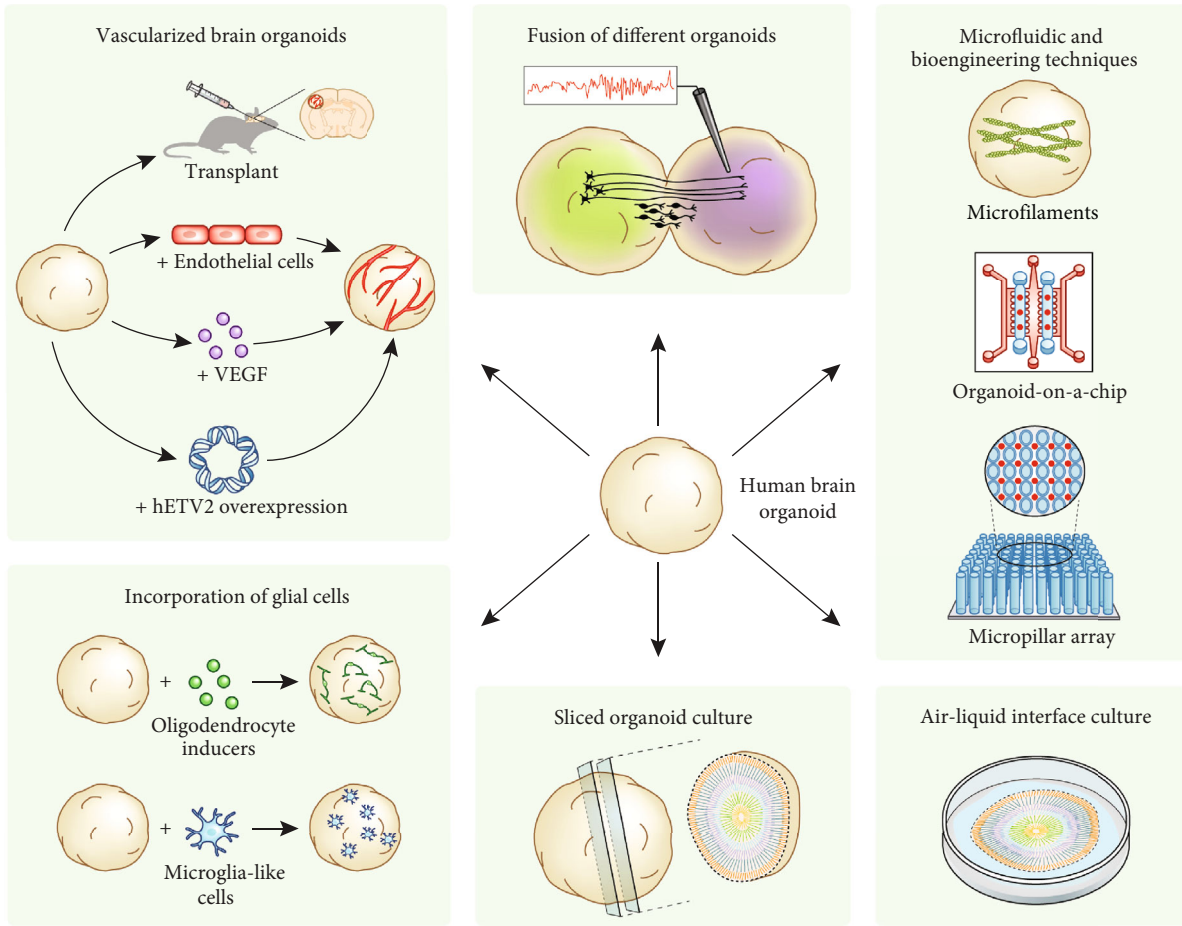

Figure 1: Recent advances of brain organoid techniques. (1) Different region-specific brain organoids can be fused together to generate so-called "assembloids" for the investigation of interregional interactions. (2) The lack of oligodendrocytes and microglia in cortical organoids has inspired the incorporation of these cell types into brain organoids. Strategies include exposure to oligodendrocyte inducers and coculturing with microglia-like cells. (3) The addition of vasculature in brain organoids is beneficial for oxygen and nutrient delivery under long-term culturing and hence the development of vascularized brain organoids. Strategies include transplantation of brain organoids into the mouse brain, coculturing with endothelial cells, exposure to vascular endothelial growth factor (VEGF), and overexpression of human ETS variant 2 (ETV2) in brain organoids. (4) Air-liquid interface culture technique has been shown to benefit neuronal survival and axonal growth. (5) Sliced organoid culture technique is able to overcome the diffusion limit in conventional brain organoid culture, leading to more expanded cortical plate and distinct layering of neurons. (6) Microfluidic and bioengineering techniques help improve the repeatability and uniformity of brain organoid culture, providing possibilities for generating organoids with simple procedure, high reproducibility, and low cost.

completely absent from guided cortical organoids as they originate from nonneural lineage. Dysregulation of microglia has been shown to affect normal brain function and contribute to neurodegenerative disorders such as Alzheimer's and Parkinson's disease [49–51], and hence the importance of establishing microglia-containing brain organoids. Attempts have been made by coculturing microglia-like cells with neuron aggregates or brain region-specific organoids [52–55]. Notably, microglia migrated into the organoid would cluster near an injured site and change morphology to that of activated microglia upon injury of the central nervous systems [52]. Moreover, differential cellular phenotypes were observed between the coculture of microglia-like cells with dorsal organoids and with ventral organoids, including differences in migration ability, intracellular Ca^{2+} signaling, and the response to proinflammatory stimuli [55]. Changes of gene expression in microglia-like cells before and after coculturing were detected by transcriptome analysis [53–55], prompting interests in studying how the presence of microglia in brain region-specific organoids will in return affect their development and functions.

2.2.3. Incorporation of Structural Components. Due to being derived from nonneural lineage, functional vasculature is absent in brain organoids, resulting in the insufficient delivery of oxygen and nutrient into organoids under long-term culturing and hence the increased apoptosis and cell death in the inner zone that forms a necrotic core [56–58]. Functional vasculature is critical for the differentiation and maturation of neuronal/glial progenitor cells [59], and thus, several approaches have been established in attempts to induce vascularization of brain organoids. Coculturing of

cerebral organoids at early developmental stage with endothelial cells allowed robust vascularization of the organoid after 3-5 weeks *in vitro* or 2 weeks *in vivo* after transplanted into immunodeficient mice, in which human $CD31^+$ blood vessels were found inside and in-between rosettes within the center of the transplanted organoid [60]. Other approaches, including induction of endothelial cell differentiation in cerebral organoids by vascular endothelial growth factor (VEGF) treatment [61] or by overexpressing human ETS variant 2 (ETV2) [62], as well as coculture of hPSCs with human umbilical vein endothelial cells [63], have also successfully generated a functional vascular-like system in brain organoids without affecting neurogenesis. More importantly, vascularized organoids acquired many characteristics of blood-brain barrier, including expression of tight junctions, molecular transporters, and other genes related to blood vessel morphogenesis, and supported the formation of blood vessels *in vivo* [61–63], providing a potential platform for studying blood-brain barrier and drug discovery.

2.2.4. Other Technical Advances. Additional advances mainly focus on the improvement of organoid culture system, either by alternative culture techniques that allow better recapitulation of neurogenesis or by state-of-art bioengineering technologies that increase the repeatability and uniformity of brain organoid cultures. For example, air-liquid interface culture techniques were established to improve neuronal survival and axonal growth, resulting in active neuronal networks and circuit formation with functional neuronal output [64]. Later on, a sliced neocortical organoid system was established, which overcame the diffusion limit in typical brain organoids and prevented cell death over long-term culturing. Sustained neurogenesis, which led to an expanded cortical plate, was observed by this system, forming distinct upper and deep cortical layers for neurons and astrocytes similar to the neocortex in the third trimester [65].

Additionally, the application of state-of-art microfluidic and bioengineering techniques has greatly improved the repeatability and uniformity of brain organoid culture. For example, poly (lactide-co-glycolide) copolymer (PLGA) fiber microfilaments were engineered to be used as a floating scaffold to generate elongated embryoid bodies, which then self-organized into cerebral organoids, with more-consistent formation of enlarged ventricular structures and neuroepithelium [66]. Moreover, microchip culture systems have been developed and utilized to generate brain organoids in confined compartments for the investigation of surface wrinkling, a biological process that is significant for the formation of gyrus and sulcus formation in the cortical plate. In this study, two opposing forces, the cytoskeletal contraction at the organoid core and the nuclear expansion during cell cycle at the organoid perimeter, were identified contributing to the formation of surface wrinkling [67]. More recently, benefited from the rapid development of microfluidic devices and the establishment of air-liquid interface culture techniques, a one-stop microfluidic platform has been developed to generate and culture cerebral organoids for investigating the effect of prenatal cannabis exposure on early brain development [68]. This platform is advantageous in that it greatly simplifies the experimental procedure and improves productivity. Hopefully with the continuous advances and improvement of culture techniques and bioengineering technology, brain organoid can soon become a sophisticated model system that not only recapitulates human brain development but also has the characteristics of fast generation, high reproducibility, and low cost.

2.3. Disease Modeling Using Brain Organoids. Brain organoids, owing to having 3D structures mimicking key features of the developing brain, are particularly suitable for translational research. Patient iPSC-derived brain organoids, for instance, contain genetic abnormalities that lead to the disease and are therefore capable of recapitulating the disease pathology as well as phenotypes in a dish. On the other hand, isogenic brain organoids generated via gene-editing techniques can help reveal the necessity and essentiality of a specific gene mutation to the disease. As a result, brain organoids have been extensively explored for the modeling of various neurological disorders, including neurodevelopmental disorders, neurodegenerative disorders, infectious diseases, and brain cancers. We will summarize and discuss some of these studies in this section (Table 1).

2.3.1. Modeling Neurodevelopmental Disorders

(1) Primary Microcephaly. Primary microcephaly, also known as autosomal recessive primary microcephaly (MCPH), is a condition where abnormalities occur at the early developmental stage of the human brain, resulting in reduced head circumference and most likely intellectual disability and seizures [69]. Well-known genetic causes of primary microcephaly are mainly genes involved in the assembly of centrosomes and cilium, such as *CDK5RAP2*, *ASPM*, *CPAP*, and *WDR62* [14, 45, 70–72]. However, rodent models of primary microcephaly did not exhibit a significantly reduced brain size as observed in human [73, 74], and thus prompting interest in developing human-specific models of this disease.

The first microcephalic cerebral organoids were derived from iPSCs of a microcephaly patient, harboring heterozygous truncation mutations in CDK5 regulatory subunit-associated protein 2 (CDK5RAP2), a component of the pericentriolar material (PCM) in centrosomes that regulates the organization of spindle microtubules [14]. The mutant organoids were significantly smaller in size and exhibited reduced number of progenitor cells as well as premature neuronal differentiation compared to the controls. RNAi-mediated knockdown of *CDK5RAP2* in the control organoids recapitulated the mutant phenotypes, while overexpression of this gene in the mutants rescue the phenotypes [14]. Later on, patient iPSC-derived cerebral organoids harboring mutations in the abnormal spindle-like microcephaly-associated (*ASPM*) gene were generated [70]. ASPM is a mitotic spindle protein; mutations in the *ASPM* gene are the most common cause for primary microcephaly. These mutant organoids exhibited significantly reduced overall size, fewer progenitor cells in both ventricular zone and outer subventricular zone, poor lamination, and a reduction in neurons with calcium activity [70]. Centrosomal-P4.1-associated protein (CPAP) is a centriole wall protein

required for the assembly and recruitment of PCM proteins to the centrosome; mutations in the *CPAP* gene can cause Seckel syndrome and microcephaly. Brain organoids generated from the iPSCs of a Seckel syndrome patient were significantly smaller in size [75]; NPCs in these mutant organoids had delayed cilia disassembly that caused a retardation in cell cycle progression, leading to premature differentiation of NPCs into early neurons and thus an overall reduction in the progenitor pools [75]. Similarly, *WDR62* ablated iPSC-derived brain organoids showed delayed cilia disassembly and retarded cell cycle progression, resulting in reduced proliferation and premature differentiation of NPCs [71]. It turns out that WDR62 interacts with CEP170, promoting CEP170 to locate in the matrix of primary cilia; CEP170 then recruits the microtubule depolymerization factor KIF2A to disassemble cilium [71].

(2) Autism Spectrum Disorder. Autism spectrum disorder (ASD) is a developmental condition related to neurodevelopment that affects a person's perception and interaction with other people, characterized by difficulties in communication and social-emotional reciprocity, restricted interests, and repetitive behavior. The utilization of brain organoids has deepened our understanding on the cellular and molecular mechanisms of ASD pathophysiology. Cortical organoids generated from the iPSCs of severe idiopathic ASD patients exhibited upregulation of genes involved in cell proliferation, neuronal differentiation, and synaptic assembly, as well as cellular alterations including accelerated cell cycles and increased number of GABAergic neurons [15]. *FOXG1* was one of the most upregulated genes in ASD organoids; RNAi-mediated knockdown of *FOXG1* was able to rescue the overproduction of GABAergic neurons, suggesting that the overexpression of *FOXG1* may initiate a shift towards the GABAergic lineage, which results in an imbalance between excitatory and inhibitory neurons and eventually leads to ASD [15]. In addition to *FOXG1*, an exome-sequencing study has identified *CHD8* (chromodomain helicase DNA-binding protein 8) as one of the most commonly mutated genes in ASD. Combined with the CRISPR/Cas9 gene-editing technique, cerebral organoids harboring a heterozygote mutation of *CHD8* ($CHD8^{+/-}$) were generated [76]. Differentially expressed genes (DEGs) between heterozygote mutant organoids and isogenic controls were identified by RNA-sequencing; pathway analysis revealed an upregulation of genes involved in neurogenesis, neuronal differentiation, forebrain development, Wnt/β-catenin signaling, and axonal guidance [76]. This study, again, highlights the possibility that the imbalance between excitation and inhibition in the brain is a pathogenic cause of ASD.

(3) Tuberous Sclerosis Complex. Tuberous sclerosis complex (TSC) is an autosomal dominant genetic disorder characterized by the growth of benign tumors in multiple organ systems including the brain, kidneys, lungs, and skin. Among these manifestations, neurological abnormalities attract the most attention due to being the most complicated and therapeutically challenging conditions in TSC. In addition to brain lesions such as cortical tubers (focal regions of disorganized and dysmorphic neurons and glia), subependymal nodules, and subependymal giant cell astrocytomas, neurological deficits such as epilepsy, ASD, and intellectual disability are often seen in TSC patients [77–79]. Studies have shown that mutations in the *TSC1* or *TSC2* gene are the causes of TSC as they lead to *TSC1*/*TSC2* deficiency in organs and hyperactivation of the mTOR signaling pathway, which plays an important role in regulating cell growth and proliferation [79, 80]. So far, the molecular mechanisms underlying TSC are still unclear. A recent study using CRISPR/Cas9-mutated *TSC1* and *TSC2* cortical spheroids revealed that homozygous knockout of *TSC1* or *TSC2* disrupted the developmental suppression of mTORC1 signaling, resulting in reduced neurogenesis, increased gliogenesis, and dysmorphia of neurons and glia similar to those observed in patients' cortical tubers [81]. Moreover, it has been found that biallelic inactivation of *TSC2* was necessary and sufficient to cause the formation of dysplastic cells in cortical spheroids. Therapeutically, it has been shown that treatments with rapamycin since either early stage (day 12-110) or later stage (day 80-110) of development strongly reduced mTORC1 signaling and reversed cellular hypertrophy in *TSC2*-deficient spheroids. However, only early treatment with rapamycin could rescue neuronal differentiation defects in *TSC2*-deficient spheroids, and continuous treatments were required to sustain these effects, highlighting the importance of timing and duration of pharmacological treatments [81].

2.3.2. Modeling Congenital/Infectious Diseases

(1) Neonatal Hypoxia-Ischemia Injury. Neonatal hypoxic-ischemia (HI) injury, synonymous with hypoxic-ischemic encephalopathy (HIE) that occurs at 36 gestational weeks or later, is the most common cause of death and disability in neonates. Even though early interventions and improvements in care have led to an increase in survival rate after hypoxic insult, many survivors still suffer from life-long neurodevelopmental deficits such as cerebral palsy, seizures, epilepsy, and cognitive impairment [82, 83]. Recently, in order to better examine the effects of hypoxia on neurodevelopment, cerebral organoids of neonatal HI were generated and cultured at different oxygen concentrations [82]. Hypoxic environment had an inhibition effect on dorsal-related genes such as *FOXG1*, *CTIP2*, and *TBR1* but had no effect or minimal effect on more ventral genes such as *ENG1*, *DLX2*, and *NKX2.1*. Notably, the inhibition of dorsal genes under hypoxic environment could be alleviated by the application of minocycline, demonstrating the therapeutic potential of this small molecule [82]. Another study using hiPSC-derived 3D-cultured cortical spheroid revealed a reduction of $TBR2^+$ intermediate progenitors after 48-hour cultivation under hypoxic environment [84]. This cell-specific defect was related to changes in the unfolded protein response (UPR) pathway in $TBR2^+$ progenitors, resulting in cell cycle damage and premature neural differentiation in these cells. Treatments with the UPR modulator ISRIB were able to rescue these phenotypes observed after the hypoxic insult [84].

(2) ZIKV Infection. In addition to the well-known genetic causes mentioned in the previous section, external factors such as viral infection and environmental cues can also lead to microcephaly, which is termed acquired microcephaly. Zika virus (ZIKV) is a member of the flavivirus family. Zika virus (ZIKV) infection is the most studied condition as its outbreak in South America cooccurred with an increased incidence of microcephalic neonates, arousing suspicion in a causal relationship between the two. Due to the inaccessibility of live infected human fetal tissues and the variability of postmortem tissues, brain organoids have been widely used to model ZIKV infection and investigate the cellular mechanisms underlying it. For example, hiPSC-derived forebrain organoids exposed to ZIKV revealed specific tropism of ZIKV towards NPCs over intermediate progenitor cells or immature neurons in the organoids [17]. Infected NPCs provided material and machinery for virus production, leading to the amplification of ZIKV and the propagation of infected cells over time [17, 85]. Transient exposure (i.e., one day) of early-stage forebrain organoids to ZIKV was sufficient to cause microcephalic-like phenotypes, including thinning of the neuronal layer, decrease in overall size, and dilation of the ventricular lumen, which was in agreement with the clinical finding that ZIKV infection during the first trimester is the most dangerous [17]. Mechanistically, it has been shown that suppression of NPC proliferation and increased cell death in ZIKV-infected forebrain organoids were responsible for the decrease in organoid size [17]. Remarkably, these effects of ZIKV infection are not a general feature of viruses in the flavivirus family as exposure of cerebral organoids to dengue virus 2 (DENV2), another member in the flavivirus family that causes dengue fever, did not attenuate NPC growth [85]. Meanwhile, different strains of ZIKV were tested to see if there is intrinsic difference in the pathogenicity of virus. Interestingly, $ZIKV^{B}$, a more recent clinical isolate from Brazil, appeared to have stronger deleterious effects in cerebral organoids than the original African strain $ZIKV^{M}$, showing more severe NPC depletion and neuronal layer disruption [86]. However, it is worth noting that passage history is important for the pathogenicity of virus and thus should be taken into consideration when drawing conclusions.

Other studies focused on the molecular mechanisms of ZIKV infection have revealed several biological pathways affected by the virus. For example, transcriptome analysis of human cerebral organoids infected with ZIKV exhibited upregulation of toll-like receptor 3 (TLR3), an innate immune receptor [87]. Further analysis revealed a TLR3-mediated downregulation of neurogenesis and upregulation of proapoptotic pathways in the infected organoids. Interestingly, a direct competitive TLR3 inhibitor rescued ZIKV-mediated apoptosis and partially rescued the reduced size of infected organoids [87]. Later on, another study also revealed activated innate immune responses in ZIKV-infected cortical organoids, which could explain the increased progenitor apoptosis and restricted growth of infected organoids [88]. Interestingly, administration of either duramycin or ivermectin to infected organoids dramatically reduced the teratogenic effects of ZIKV infection on cortical development, highlighting the potential therapeutic role of these drugs in anti-ZIKV infection [88]. Translational studies have also been performed to search for potential therapeutic agents that could alleviate ZIKV-mediated phenotypes. A high-content screening in hiPSC-derived NPCs identified hippeastrine hydrobromide (HH) and amodiaquine dihydrochloride dihydrate (AQ) as drug candidates to inhibit ZIKV infection [89]. It has been shown that HH rescued ZIKV-mediated growth and differentiation defects in NPCs and was even capable to suppress viral propagation in adult mice with active ZIKV infection [89]. Additionally, a recent study revealed an abundant production of virus-induced small interfering RNAs (siRNAs) in NPCs [90]. Ablation of key components in RNAi machinery significantly enhanced ZIKV replication in infected cells, and thus prompting interest in testing the effects of RNAi enhancers on these cells. Remarkably, enoxacin, an RNAi enhancer, completely prevents ZIKV infection and rescued ZIKV-mediated microcephalic-like phenotypes in infected organoids [90], bringing RNAi into the discussion of potential therapeutic targets.

(3) SARS-CoV-2 Infection. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection has caused the COVID-19 global pandemic since 2019, resulting in more than 216 million infected people and over 4.5 million deaths worldwide as of August 2021 (https://covid19.who.int). Even though the infection primarily affects the respiratory system, neurological complications have been reported in a significant number of patients, including headache, dizziness, cerebrovascular injury, encephalitis, hypogeusia, and hyposmia, as well as neuropsychiatric symptoms such as confusion and new-onset psychosis [91–94]. Although a few cases reported the presence of viral RNA in the brain and cerebrospinal fluid (CSF) of infected patients [93, 95–97], it is hard to draw conclusions on the prevalence of central nervous system infection based on these sporadic reports. Therefore, it remains unclear whether the neurological symptoms in COVID-19 are caused by direct neural infection or by some more indirect mechanisms. Due to the difficulties in accessing human brain tissue, brain organoids were utilized to investigate this question. By exposing hiPSC-derived monolayer cortical neurons, astrocytes, and microglia, as well as 3D-cultured cortical, hippocampal, hypothalamic, and midbrain organoids to SARS-CoV-2, the viral tropism in various cell types was revealed [98]. It has been shown that SARS-CoV-2 had limited tropism for neurons and astrocytes under clinically relevant conditions but rather had a particularly high rate of infection in choroid plexus (ChP) epithelial cells, a cell type present in some of the hippocampal organoids tested in this study [98, 99]. Indeed, this finding was confirmed in further examinations using choroid plexus organoids (CPOs), from which a productive infection of SARS-CoV-2 in ChP epithelial cells was revealed [98, 100]. This high susceptibility of CPOs to SARS-CoV-2 may be explained by the finding that ACE2 and TMPRSS2, the key cell entry receptors for SARS-CoV-2, were highly expressed in the ChP *in vivo* and *in vitro*

[98, 100, 101]. The infection of SARS-CoV-2 in CPOs caused an increase in both cell-autonomous and non-cell-autonomous cell death, transcriptional dysregulation, and disruption of ChP epithelial integrity and barrier function [98, 100]. In fact, recent clinical data reported leakage of blood proteins into CSF in more than 40% of patients tested [97], which was in support of this finding as the disruption of ChP integrity would be expected to lead to leakage in the blood-CSF barrier (B-CSF-B). Subsequently, a breakdown of the B-CSF-B would allow abnormal entry of immune cells and cytokines, which could lead to harmful neuroinflammation and neural tissue injury. Taken together, so far, it has been proposed that the neurological symptoms in COVID-19 patients are more likely to be consequences of indirect effects of viral infection. However, this proposal requires further verifications by animal models and post-mortem ChP from infected patients, as current clinical data did not reveal high prevalence of SARS-CoV-2 in the circulating bloodstream [96], raising questions on the pathway(s) of viral entry.

On the other hand, the remarkable variability in terms of symptom severity among infected individuals has prompted interest in investigating the potential molecular mechanism(s) underlying it. A recent study reported the host gene FURIN as a mediator for SARS-CoV-2 infection and a common variant rs4702 that is located in the 3′UTR of this gene being an influencer of SARS-CoV-2 infection *in vitro*. Moreover, CRISPR/Cas9-mediated allelic conversion (from AA to GG) at rs4702 decreased the neuronal and alveolar expression of FURIN and led to reduced SARS-CoV-2 infection [99], which was in agreement with the idea that host genome is associated with SARS-CoV-2 infection and might dictate the severity of clinical outcomes.

2.3.3. Modeling Neurodegenerative Disorders

(1) Alzheimer's Disease. Alzheimer's disease (AD) is the most common neurodegenerative disease and is characterized by progressive decline in memory, thinking, language, behavior, and other cognitive abilities. On a cellular level, AD is characterized by the extracellular deposition of β-amyloid plaques as well as intracellular formation of neurofibrillary tangles that are composed of aggregated hyperphosphorylated tau (pTau). Even though brain organoids are thought to recapitulate embryonic brain development, which seems far from neurodegeneration, several studies have reported successful establishment of brain organoids harboring AD-like pathologies. For example, an early study revealed that 3D-differentiated neuronal cells overexpressing APP or PSEN1 gene variants from familial AD (fAD) patients exhibited robust deposition of β-amyloid plaques and aggregates of pTau, recapitulating the two pathological hallmarks of AD. Similarly, AD-like pathologies were observed in fAD patient iPSC-derived brain organoids, including β-amyloid (Aβ) aggregation, hyperphosphorylated tau, and endosome abnormalities. These pathologies were excluded from various control lines and occurred at consistent incidence among several fAD lines that carried different mutations [102, 103]. Moreover, treatments with β- and γ-secretase inhibitors were able to significantly reduce amyloid and tau pathology in AD-like brain organoids [103, 104], suggesting the potential of utilizing these organoids as platforms for preclinical drug discovery in AD.

Other studies focus on the investigation of sporadic AD (sAD). *APOE4* is the E4 allele of *APOE* and is the earliest identified and most significantly associated genetic risk factor for sAD, leading to increased AD risk relative to the *APOE3* allele [105–108]. Isogenic *APOE4* brain organoids, which were generated by switching the *APOE3* allele in healthy individual iPSCs to *APOE4* allele via CRISPR/Cas9 gene-editing technique, showed an increased Aβ accumulation and pTau compared to *APOE3* organoids. Conversely, switching *APOE4* in sAD patient iPSCs to *APOE3* was sufficient to alleviate most of the AD-related phenotypes in brain organoids, supporting the central role of *APOE4* in sAD pathology [109].

(2) Parkinson's Disease. Parkinson's disease (PD), being the second most common neurodegenerative disease after AD, is a chronic and progressive nervous system disorder affecting movement. Symptoms commonly include tremors, slowness in movement, muscle stiffness, and difficulties with speech, balance, and coordination. On a cellular level, PD is characterized by the loss of dopaminergic neurons in the substantia nigra of the midbrain as well as the development of neuronal Lewy bodies (α-synuclein) [110, 111]. The current cellular and animal models have some limitations in recapitulating pathological hallmarks of PD [112], leading to the development of midbrain organoids (MOs) as a better alternative for modeling PD *in vitro* [113, 114]. Previous studies have shown that missense mutations in the leucine-rich repeat kinase 2 (*LRRK2*) gene locus, particularly *LRRK2* G2019S mutation, are common causes of late-onset familial and sporadic PD [115, 116], prompting interest in studying the pathogenic mechanisms of LRRK2-associated PD. In a recent study, isogenic MOs harboring a *LRRK2* G2019S mutation were generated from CRISPR/Cas9-edited iPSCs. These organoids exhibited several PD-like phenotypes, including shortened neurite length in dopaminergic neurons (mDANs), decreased expression of mDAN-specific marker (e.g., TH, AADC, and DAT), and increased aggregation and abnormal clearance of α-synuclein. Notably, analysis of differentially expressed genes revealed an upregulation of TXNIP, a thiol-oxidoreductase, in the *LRRK2*-G2019S mutant organoids specifically; inhibition of this gene was able to ameliorate the mutant phenotypes induced by *LRRK2*-G2019S mutation, indicating the possibility of TXNIP in mediating disease phenotypes of patients with *LRRK2*-associated PD [113]. In line with these findings, another study using MOs derived from PD patients who carried the *LRRK2* G2019S mutation also demonstrated a decrease in the number and complexity of mDANs compared to the control organoids [117]. Moreover, FOXA2-positive progenitor cells were found to be significantly increased in these patient-derived organoids, suggesting a neurodevelopmental defect is likely associated with the

LRRK2 G2019S mutation. Importantly, it has been shown that introduction of the LRRK2-G2019S mutation within a healthy background was sufficient to cause deleterious effects on the complexity of mDANs, consistent with the findings in Kim et al. (2019), and yet, correction of the LRRK2-G2019S mutation within a PD patient background was not able to rescue the mutant phenotypes [117], supporting the hypothesis that genetic background of PD patients may influence the *LRRK2*-induced mDAN degeneration [118].

Additionally, as homozygous loss-of-function mutations in DNAJC6 were previously identified in familial juvenile/early-onset PD [119–121], MOs harboring CRISPR/-Cas9-mediated DNAJC6 mutations were generated and utilized for investigating the roles of DNAJC6 in PD pathogenesis [114]. These mutant organoids exhibited key PD pathologic features, including mDAN degeneration, α-synuclein aggregation, increased neuronal firing frequencies, and mitochondrial and lysosomal defects. DNAJC6 ablation also led to impairment of WNT-LMX1A regulation, which is critical for early ventral midbrain (VM) patterning and mDAN development, and thus resulted in VM patterning defects and vulnerable mDANs in mutant MOs [114]. Moreover, MOs derived from idiopathic PD patients were also utilized for investigating the pathophysiology of this disease subtype [122]. Changes in the expression of LIM homeobox transcription factor alpha (early) and tyrosine hydroxylase (late) markers were observed in patient-derived MOs; several crucial genes associated with idiopathic PD, e.g., TH, PTX3, LMX1A, and FOXA2, were also identified in this study [122].

(3) Huntington's Disease. Huntington's disease (HD) is an autosomal dominant genetic disorder characterized by motor impairments such as chorea, dystonia, and incoordination, cognitive decline such as forgetfulness, impaired judgement, and learning difficulties, and psychiatric problems such as insomnia and depression. The cause of HD has been shown to be a polymorphic CAG repeat expansion in the *huntingtin* (*HTT*) gene located on chromosome 4 that leads to abnormal degeneration of neurons within the striatum and cortex [123, 124] through several biological mechanisms including altered gene expression profile, disrupted mitochondrial and metabolic function, direct toxicity of the mutant protein, and aberrated ATP levels. Also, the length of CAG repeats in the *HTT* gene has been found to be crucial for disease onset and severity: fewer than 36 repeats are normal; 36–39 repeats are abnormal but might result in HD with reduced penetrance; more than 40 repeats result in adult-onset HD; and more than 60 repeats generally result in Juvenile Onset HD (JHD) [125, 126]. As JHD progresses significantly faster than adult-onset HD, researchers have brought up the possibility that mutant HTT may lead to neurodevelopmental deficits in addition to neurodegenerative manifestations in HD. Indeed, many studies have examined the role of HTT in brain development in both rodent models and monolayer cell cultures [127–132], and yet, the impact of mutant HTT on neurodevelopment, especially early neurogenesis and cortical layer formation, was less clear, most likely due to the difficulties of accessing human embryonic brain tissues with HD. To address this question, one group took advantage of patient iPSC-derived cerebral organoids to investigate early neurodevelopmental processes in HD [133]. They found that CAG repeat expansion caused significant defects in early telencephalic induction and progenitor identity acquisition, leading to abnormal neuronal specification and disrupted cellular organization. They also observed severer phenotypes in the organoids with larger repeat expansion than those with shorter expansion, which were in line with the clinical representation that the longer the CAG repeats are, the earlier and severer the symptoms tend to manifest [133]. A later study using cerebral organoids derived from patient iPSCs and a panel of TALEN-mediated isogenic HD hESCs reported similar results, as HD organoids showed impaired cell cycle regulatory processes and reduced symmetric division of apical progenitors that eventually led to disrupted neuroepithelial structures and premature neurogenesis in these organoids [134].

2.3.4. Modeling Brain Cancer. Glioblastoma (GBM) is the most lethal and devastating type of glioma, accounting for 54% of all gliomas [135]. Current treatments are very limited and mainly focus on slowing the progression of the cancer and reducing signs and symptoms, as the rapid development and invasion of GBM often make surgical resection improbable. The prognosis of GBM is dismal, with a median survival time of approximately 15 months and a 5-year survival rate of less than 5% [136]. In order to study the formation and progression of GBM *in vitro*, several strategies have been taken. For example, unguided cerebral organoids were utilized, in which oncogenes and/or tumor suppressors were manipulated using CRISPR/Cas9- and/or transposon-mediated approaches to induce mutagenesis and tumorigenesis [137, 138]. Many features of GBM cells were observed in transformed organoids, including capability of expansion and invasion (both *in vivo* and *in vitro*), cellular markers, and gene expression profiles [137, 138]. Another strategy was taken by coculturing either patient-derived glioma stem cells (GSCs) [139] or glioblastoma spheroid [140] with human cerebral organoids. Both studies revealed a rapid and deep invasion of glioblastoma cells into the host tissue, forming hybrid organoids that exhibited an invasive tumor phenotype [139, 140]. Such GBM hybrid organoids would provide a scalable and easily manipulable system for the investigation of tumorigenesis and progression, as well as for the screening of anticancer drugs [141, 142]. More recently, a different method was established, generating glioblastoma organoids (GBOs) directly from resected tumor tissue without additional manipulation [143]. These GBOs recapitulated inter- and intratumoral heterogeneity as well as many key features of glioblastoma, including histological features, cell type diversity, transcriptomic signatures, mutation profiles, and aggressive infiltration after transplantation. This method allows for rapid generation of patient-specific glioblastoma organoids, which can be utilized for testing personalized therapies, treatments, and drugs [143].

3. Conclusions and Discussion

With less than a decade of development, brain organoid technology has revolutionized our toolbox for investigating cellular and molecular mechanisms of neurodevelopment and neural disorders. In this review, we summarized many recent advanced techniques in the field of brain organoids, such as the development of assembloids, incorporation of cellular and structural components, and other optimized culture systems. We also discussed some of the translational applications of brain organoids, including disease modeling and screening or testing potential pharmaceutical compounds. Attracted by the unique advantages of brain organoids, more and more researchers devoted themselves into this field and established many more disease models for the investigation of disease mechanisms. For example, most recently, MECP2 knockout neurospheres and cortical organoids were generated for modeling Rett syndrome [144]; Down syndrome cerebral organoid models were established from patient-derived iPSCs [145]; and iPSC-derived brain organoids infected by a "clinical-like" human cytomegalovirus (HCMV) strain were utilized for studying HCMV-induced microcephaly [146]. Furthermore, such disease-modeling organoids also provide a platform for drug screening [88, 89] and act as a subject in the investigation of potential organoid transplantation therapy for neurological disorders [147–149].

Despite the numerous promising results researchers have obtained from brain organoid models, there are still limitations in the current system. Firstly, as NPCs with high metabolic demands are often located in the inner zone of brain organoids, continuous apoptosis and cell death caused by the insufficient delivery of oxygen and nutrients to the inner zone have greatly hampered the neurogenesis and further maturation of brain organoids, leading to the incompetence of modeling late-stage events such as distinct cortical layering, cortical expansion, and cortical folding. Improvements can be achieved by overcoming the diffusion limit in long-term organoid cultures. For example, the use of spinning bioreactors or orbital shakers as well as elevated oxygen concentration in the incubator has been shown to be beneficial in some ways [13, 17, 66]. Alternative culture methods such as an air-liquid interface culture system [64] and sliced neocortical organoid system [65] have also contributed to the development of a better organoid model. Additionally, there is no doubt that the incorporation of vasculature into brain organoids would largely improve the delivery of oxygen and nutrients. Methods involving building or providing a vascular system in brain organoids, such as constructing vascular-like networks with perfusion via bioengineering or grafting organoids into animal brains to allow invasion of the host vasculature, are therefore being actively studied and developed [150].

Secondly, a recent study has revealed that brain organoids generated from current methods did not resemble their cortical progenitor counterparts at the earliest developmental stages, despite having increased fidelity of cell types after the radial glia and neuronal populations emerged [151]. Specifically, a mesenchymal-like population marked by ALX1 and LUM expression was identified in samples at or before Carnegie stages (CS) 16 but was not detected in cortical organoids until week 7, highlighting the importance of continuing optimizing brain organoid protocols for the investigation of developmental processes prior to neurogenesis [151].

Another major limitation of the current organoid model is that the maturation process takes too long and is therefore costly and labor-intensive. Future improvements in terms of speeding up this process would not only benefit the generation of brain organoids but also create a more "aged" model for studying age-dependent neurodegenerative disorders. Moreover, the use of bioengineering technology such as microfluidics, biomaterial, and bioprinting may further improve the efficiency of generating organoids with low variability, high reproducibility, and low cost.

Lastly, the introduction of assembloid has opened a new avenue for the investigation of interregional connections and activities using different guided brain region-specific organoids. Future directions include establishing more sophisticated assembloid systems that compose more brain regions as well as incorporating nonneural lineages such as microglia, endothelial cells, hematopoietic cells, and meninges into the assembloid to better mimic the *in vivo* condition. The ultimate goal is to assemble a whole brain-like structure that comprehensively models human brain development and function.

Taken together, brain organoid technology, although still being at its primary stage, has become an invaluable tool for studying neurodevelopment and neural disorders. While new methods and improvements are being made to generate more advanced organoid systems, it is important to keep in mind that no model is perfect. Thus, we should always choose a model system based on the biological question of interest and be cautious when drawing conclusions. Only when interpreted comprehensively and complementarily with other models can we gain new insight into the biological basis of human brain development.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Review Article

Organoid Models for Salivary Gland Biology and Regenerative Medicine

Chen Zhao, Cuida Meng, Na Cui, Jichao Sha, Liwei Sun, and Dongdong Zhu

Department of Otolaryngology Head and Neck Surgery, China-Japan Union Hospital of Jilin University, Changchun, China

Correspondence should be addressed to Dongdong Zhu; zhudd@jlu.edu.cn

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The salivary gland is composed of an elegant epithelial network that secrets saliva and maintains oral homeostasis. While cell lines and animal models furthered our understanding of salivary gland biology, they cannot replicate key aspects of the human salivary gland tissue, particularly the complex architecture and microenvironmental features that dictate salivary gland function. Organoid cultures provide an alternative system to recapitulate salivary gland tissue in vitro, and salivary gland organoids have been generated from pluripotent stem cells and adult stem/progenitor cells. In this review, we describe salivary gland organoids, the advances and limitations, and the promising potential for regenerative medicine.

1. Introduction

Three major salivary glands (the parotid, submandibular, and sublingual glands) and numerous minor ones located in the upper aerodigestive tract produce saliva by a wide range of environmental and biological stimuli. Like most exocrine glands, salivary glands undergo their morphogenesis during the embryonic period when the branched ductal structures originate from an initial epithelial placode and grow into the mesenchyme [1]. The branched ductal structure comprises acinar, ductal, and myoepithelial cells. Acinar cells are responsible for protein and fluid secretion upon parasympathetic neuron stimulation, while ductal cells form a tubular conduit for saliva transportation and slight modification of ionic composition. Acini are wrapped by contractile myoepithelial cells inside the basement membrane embedded in the stroma containing immune cells, vasculature, and nerves [2]. The epithelial-mesenchymal crosstalk guides the morphogenesis of the salivary glands [3]. Any impairment to the architecture and/or function of the salivary glands may result in hyposalivation, manifested as xerostomia or "dry mouth syndrome" [4, 5]. The situation can be caused by systemic diseases including, but not limited to, Sjögren's syndrome, uncontrolled diabetes mellitus, and granulomatous diseases, however, more frequently by radiotherapy for head and neck cancer (HNC) [6, 7]. Patients with xerostomia suffer from swallowing and speaking difficulties, as well as oral and dental infections, each of them is life-disrupting. However, current therapeutic options mainly rely on artificial substitutes and systemic sialogogues, but they provide only temporary relief, not long-term benefits.

Three-dimensional architecture is the cornerstone of morphogenesis, and functional differentiation has been accepted during the last several decades, with the simultaneous advent of in vitro 3D culture technologies, and they enabled the generation of "organoids." Organoids can be established from adult stem cells (ASCs) and pluripotent stem cells (PSCs), including induced pluripotent stem cells (iPSCs) and embryonic stem cells (ESCs) [8]. When placed into a hydrogel with an appropriate exogenous factor cocktail, stem cells develop into several cell types through cell sorting and lineage commitment that mimic the process in vivo. Since their remarkable ability reflects the properties of organs structurally and functionally, organoids are utilized to model organ development and diseases, drug discovery, and personalized therapy; meanwhile, they also shed light on regenerative medicine [9]. Increasing studies indicate that they are potential sources for regeneration of new salivary gland units. In this review, we will summarize the current literature concerning salivary gland organoid, the development

of technology, the emerging roles in understanding salivary gland morphogenesis, and their great potential use in regenerative medicine. Furthermore, challenges to salivary gland organoid research and future directions are discussed.

2. Salivary Gland Development, Homeostasis, and Regeneration

To produce sufficient saliva within a limited space of the craniofacial complex, the salivary glands need to maximize the surface area to volume ratio during morphogenesis. This is realized by the programmed formation of interconnected and branched secretory acinus and ductal structures, which are highly similar in rodents and human. Most of our knowledge of salivary gland development come from ex vivo cultures of mouse embryonic submandibular glands [10, 11]. At mouse embryonic day (E) 11.5, the oral epithelium thickens and invaginates into the condensed mesenchyme, which is the beginning of salivary gland formation. Branching morphogenesis occurs during E12.5-14.5, and single epithelial bud sequentially undergoes several branching cycles, including bud enlargement, cleft formation, and terminal bud expansion. At E13, axons elongate along the epithelial cells and envelop the newly formed terminal buds, which finally differentiate into secretory acini. At E14.5, $KRT19^+$ duct progenitor cells begin to proliferate and lead to duct extension; meanwhile, they condense at the midline and microlumen fuse to form a contiguous lumen [12]. Tubulogenesis ends at E18.5, and in parallel, proacinar cells mature with the hallmark of mucin protein production. This complex morphological transformation is rigorously regulated by multiple epithelial-mesenchymal crosstalks via growth factors (i.e., FGF, EGF, EDA, BMP, Wnt, and Hedgehog) and neurotransmitter- (i.e., acetylcholine and vasoactive intestinal peptide-) mediated signaling pathways [13]. However, the exact functions of each signaling molecule are difficult to define because epithelial arborization is an integrated process including cell duplication, branch point generation, and finally branch elongation.

Studies focusing on salivary gland cell proliferation and differentiation utilizing bromodeoxyuridine and ^{3}H-thymidine labeling have accumulated evidences that stem cells play an important role in maintaining salivary gland homeostasis [14, 15]. These putative stem cells are mainly distributed to the excretory and intercalated ducts and maintain the morphological and molecular characteristics of undifferentiated stem cells [16]. Researchers have identified these putative stem/progenitor cells relied on the expression of c-Kit (CD117), keratin 5 (K5), keratin 14 (K14), Ascl3, CD24, CD29, and CD49f, based on molecular markers identified in other tissues and lineage tracing assays [17–21]. The label retaining cell (LRC) assay demonstrated that LRC, which are considered to be slow-cycling stem cells, colocalize with several stem cell markers, without obvious overlapping with each other, indicated that stem/progenitor cells of salivary glands are heterogeneous [22]. Their stemness of self-renewal and differentiation into acinar, ductal, and myoepithelial cells has been verified in vitro; however, the differentiation capacity in vivo needs to be further identified, especially in irradiated glands [17, 18, 21, 23]. Radiation hampers the replacement capacity of primitive stem cells by classical mitotic cell death, preventing their supplement to damaged secretory epithelia, a process generally irreversible [15]. However, after injuries such as main excretory duct ligation, the remaining intact duct is the source of stem cells leading to regeneration and substitution of excretory cells after ligation removed [24]. A recent fate-mapping experiment [25] revealed that differentiated acinar cells still self-duplicate, with relatively less contribution of stem/progenitor cells maintaining homeostasis [26]. The roles of distinct putative stem/progenitor cells as well as differentiated cells contribute to homeostasis remain to be clarified with the consideration of conditions.

3. Salivary Gland Organoids

3.1. Establishment of Salivary Gland Organoid Models. Salivary gland organoids are commonly established from fragments of mouse or human salivary glands dissociated utilizing mechanical and/or enzymatic digestion and then embedded in reconstructed ECM-like material, traditionally animal tissue derived protein extracts, such as Matrigel, fibrin gel, and collagen gel [27, 28] (Figure 1). Dispersed salivary gland cells develop and self-assembly into acini and/or ductal-like structures that express subsets of critical lineage markers. While organoids derived from rodent salivary gland are popular because our knowledge of salivary gland development mainly depends on rodent cells ex vivo cultures, human organoids derived from biopsies and resected salivary glands of preirridiation head and neck cancer surgical patients provide increasing information of human development biology. Importantly, distinct salivary gland stem/progenitor cells demonstrate different organoid formation abilities [17, 21, 29, 30]. Abundant studies provide proof of concept that fully functional regeneration of the salivary glands that can be achieved by reciprocal epithelial and mesenchymal interactions reproducing mimicking that during embryogenesis [31–33]. However, relatively less information of specific niche factors that promote the differentiation and formation of salivary gland organoids from pluripotent stem cells is far from clear, and several groups have succeeded in taking the first step [28, 33].

3.1.1. Salivary Gland Organoids Generated from Pluripotent Stem Cells. PSCs can undergo differentiation into various cell lineages when induced by signals positioning and patterning the way during embryogenesis. Organoids generated from PSCs were first developed for brain by Lancaster and Knoblich, after acquisition neuroectoderm from embryoid bodies (EBs) ,and they generated them into organoids in spinning bioreators [34]. The protocol is pattern growth factors independent and thus gives PSCs the most freedom to self-organize. Since then, organoids generated from the endoderm, mesoderm, and ectoderm-derived PSCs were reported, including intestine, stomach, liver, pancreas, lung, and kidneys [35–40]. Aiming to replicate salivary gland development in vitro, Ogawa team has succeeded in differentiating mouse embryonic salivary gland epithelial cells into

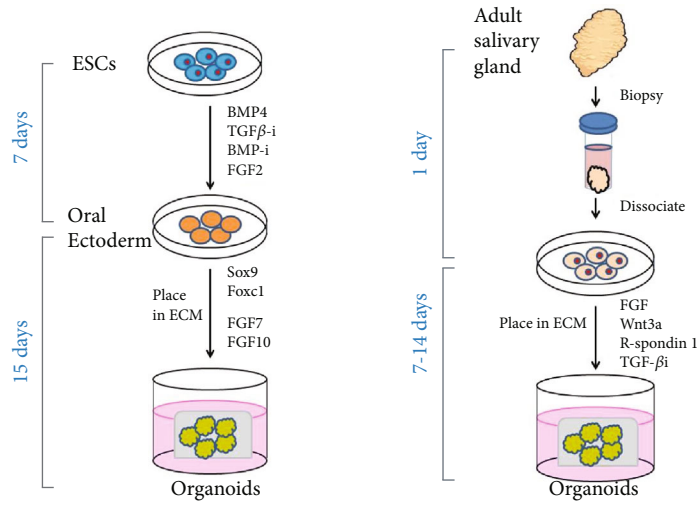

Figure 1: Salivary gland organoids can be derived from distinct origins by modulating niche factors during in vitro culture. (a) Salivary gland organoids derived from ESCs by a step-wise method that recapitulates the signaling pathways during salivary gland development. ESCs are first inducted towards oral ectoderm fate by exposure to BMP4, TGFβ-i, BMP-i, and FGF2. These oral ectoderm aggregates with the forced expression of Sox9 and Foxc1 develop into branching structures following induction of FGF7 and FGF10. (b) Salivary gland organoids can also be generated from adult stem cells isolated from biopsies. Dissociated cells can be placed in ECM with cytokines important for organogenesis, regeneration, and development including FGF, Wnt3a, R-spondin 1, and TGFβ-i. ESCs: embryonic stem cells; BMP4: bone morphogenetic protein 4; TGFβ-i: transforming growth factor beta inhibitor; BMP-i: bone morphogenetic protein inhibitor; FGF: fibroblast growth factor; ECM: extracellular matrix; Sox9: sex-determining region Y (SRY) box 9; Foxc1: forkhead box C1; Wnt3a: wingless-type MMTV integration site family member 3a; R-spondin1: roof plate-specific spondin 1.

functionally mature gland germs with embryonic mesenchymal cells [41]. The organ germ underwent sequential branching morphogenesis, stalk elongation, and cleft formation after 3 days culture. However, this early approach was performed in 2D culture without 3D information for salivary gland development in vitro. The first attempt to differentiate PSCs into 3D salivary gland tissue that recapitulated embryonic salivary gland features was the establishment of organ rudiment cultures by Tanaka and colleagues that introduced a step-wise method [28]. Sox9 and Foxc1 are identified as essential organ-inductive transcriptional factors inducing oral epithelium (OE) thickening during initial stage of salivary gland development. Thus, after mouse submandibular ESCs' derived EBs are inducted into OE with cytokines (i.e., BMP4, SB-431542, LDN-193189, and FGF2), the forced expression of Sox9 and Foxc1 induce primitive OE to develop into branching structures, namely, salivary gland rudiment, composed of $AQP5^+$ acinar-like cells, $CK18^+$ ductal-like cells, and α-SMA^+ myoepithelial-like cells after 15 days culture (Figure 2). On orthotopical transplantation into parotid gland-defective mice, the rudiment developed into tissue exhibiting mature salivary gland features. While groundbreaking, ESCs' inaccessibility for the human tissues is a concern that cannot be ignored, hampering the translational application of the model.

3.1.2. Salivary Gland Organoids Generated from Adult Stem/Progenitor Cells. Early studies by Aileen and colleagues, in which fragments of rat submandibular gland were cultured in three-dimensional collagen gel matrix, led to maintained topological organization of the parent tissue; however, the outgrowth of these cultures was accompanied by central necrosis which led to a short surviving period [42]. Self-renewing salivary gland organoids during long-term culture were reported by Lombaert and colleagues in 2008. When cultured in rat tail collagen, isolated cells from rodent submandibular glands formed salispheres expressing stem cell markers including c-Kit, Sca-1, and Musashi; moreover, these salispheres were able to proliferate and differentiate towards acinar and ductal cells both in vitro and in vivo [17]. Enrichment of c-Kit^+ cells in primary and secondary salispheres suggests that this three-dimensional sphere is a feasible way to concentrate salivary gland adult stem cells. Feng [29] reported the striking similarities of primitive human salivary gland stem cells to form organoids that differentiate into acinar and ductal lineages in collagen type I. The expansion of adult salivary gland organoids was enabled by culture conditon optimization. In addition to the cytokines such as FGF, Wnt3a, and R-spondin 1, which had been described to be important for organogenesis, regeneration and development of salivary glands [43, 44] and Alk (also known as TGF-β/Smad) signaling inhibitors were supplemented in the culture condition to suppress squamoid differentiation. This protocol was adapted to generate organoids from healthy human salivary glands and to recapitulate inflammatory diseases such as sialadenitis [45].

It is well known that in contrast to pluripotent stem cells, adult stem cells are difficult to proliferate and expand. Since salivary gland stem cell organoids shed light on autologous transplantation to restore irradiated salivary gland function, getting potent enough and sufficient stem cells for organoid establishment is the first problem to be

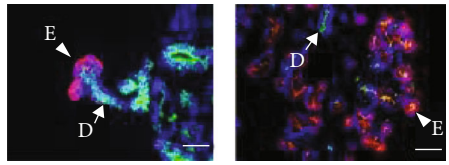

Figure 2: Immunofluorescence images of salivary gland organoid derived from mouse PCSs (a) and mouse embryonic salivary gland E18 (b). The ductal marker K18 (green) and the acinar cell marker AQP5 (red) were shown. Scale bars, 50 μm. Arrows indicate ducts (d). Arrowheads indicate an epithelial bud (e) (immunofluorescense images taken from Tanaka et al. [28]).

addressed. Nanduri [46] reported an enhanced regenerative potential of cells derived from murine salispheres by selection of $CD24^{hi}CD29^{hi}$ subset, and an exciting 4-fold increased number of selected cells was generated after seven passages compared to expansion from unselected population. Similarly, using multiple surface markers, Xiao [47] identified $Lin^{-}CD24^{+}c\text{-}Kit^{+}Sca1^{+}$ a highly enriched population of adult salivary gland stem cells, and in vivo serial transplantation studies demonstrated self-renewal and multipotency of their progenies for at least 6 months after initial isolation.

3.2. Salivary Gland Organoids for Development and Morphogenesis. Branching morphogenesis is the key developmental process for salivary glands and other glandular organs including kidney, mammary gland, and lungs [48]. One hallmark of organoid models that the composition completely separated from the adjacent ECM ensures their efficiency of attempting to study the physical and chemical properties' roles of ECM in morphogenesis. In fact, organoids have been successfully used to study salivary gland epithelial branching morphogenesis. Using embryonic submandibular gland single epithelial cells cocultured in Matrigel with bone marrow-derived mesenchymal cells (MSCs), Farahat [32] demonstrated that MSCs induced a self-assembly organoid with branching morphology, and the process was sensitive to the initial cell ratio and total number, but growth factors are independent. Another study identified laminin-111 and FGF2, but not EGF, as niche factors that driven epithelial progenitor cell development into terminal buds displaying the robust AQP5 expression [27]. Organoids have also been applied to investigate salivary stem/progenitor cells as a branching driver. For instance, Coppes and colleagues have expanded single adult stem cells into an organoid with distinct lobular or ductal/lobular structures in a short-term culture manner [46]. In their follow-up study, robust Wnt signaling activation by the addition of R-spondin and Wnt3A guaranteed a long-term expansion of organoids comprising all the differentiated cell types [49]. These various organoid models investigated both human and rodent salivary gland branching morphogenesis, although recapitulated some, but not all aspects of that observed in vivo.

To realize the primary function of the salivary gland to produce saliva and then deliver to the oral cavity, multiple elements remain to be recapitulated in salivary gland organoid models. While α-amylase and AQP5 expression by acinar cells has been induced in several organoid models, some loss the expression during a quite short maturity [50]. Until recently, a model with neural cells and neuotrophic factor, neurturin input into a fetal mesenchyme containing laminin-111 extracellular matrix supporting an innervated branching epithelium was reported by Vining and colleagues [51], and it is noteworthy that abundant basal progenitors remained close proximity to nerves; moreover, the proacinar cells exhibited a prolonged maturing period mimicking that in vivo spatiotemporally. The mechanism of bidirectional interaction between nerve and epithelial progenitor cells was verified by this coculture organoid model, the highlight of researches on branching morphogenesis, and acinar cell function maintenance [23, 52].

As saliva secretion from acini depends both on membrane transport of acinar cells and actomyosin-mediated contraction of myoepithelial cells, salivary gland organoids can also be used to identify $Ca2^{+}$-dependent mechanisms that drive myoepithelial cell contractility. A functional model was developed using a bottom-up approach, when isolated human salivary myoepithelial cells were added into adult stem/progenitor cell derived spheroids in HA hydrogel, and they self-assembled around the spheroids; more importantly, the newly formed spheroid retained responding ability when stimulated by neurotransmitters [53]. Compared to acinar and myoepithelial cells, there are relatively less information of niche factors that induce ductal cell differentiation in organoids, and thus far, no elongated network with branching morphology of ductal cells has been obtained.

3.3. Salivary Gland Organoids for Regenerative Medicine. Aiming to replace (or aid to regenerate) the functions of injured or diseased tissues, regenerative medicine has gathered the endeavor of engineering scientists and physicians during the last thirty years. Salivary gland organoids containing stem/progenitor cells, acini, and ductal-like structures hold promise for providing a radical solution for xerostomia, and they have shown their capability of restoring the function of irradiation-damaged glands. The first evidence was provided by Tanaka and colleagues, after orthotopically transplanted into parotid gland-defective mice, the ESC-derived salivary rudiment connected to surrounding tissues, developed into mature phenotype, and secreted saliva by gustatory stimulation [28]. Up to date, several groups have demonstrated ASC-derived organoids' efficiency in rescuing functional loss of postirradiation glands in murine models [46, 54–57]. In these models, the traditionally used ECM-like materials extracted from animal tissues are proven to be conducive to multiple cell behaviors such as adhesion, migration, assembly, and differentiation; however, the first obstacle for translation into the clinical settings is their potential tumorigenicity and immunogenicity. Several biocompatible and/or biodegradable scaffolds and matrices have been generated to solve the problem, including inregion ones such as hyaluronic acid (HA), alginate, chitosan, silk, and synthetic ones such as poly-lactic acid, poly-lactic-*co*-glycolic acid, poly-glycolic acid, and polyethylene glycol (reviewed in [58]). An ideal material

needs suitable stiffness and porosity, resembling the native extracellular matrix to support cell behavior during organogenesis. Besides these scaffold-based culture models, the striking advances in microwell culture and bioprinting platform allow salivary gland organoids formed faster and more uniformly [59–62]. Hurdles ahead concern the way organoids get connected with the existing gland, including excretory ducts, blood vessels, and nerves to ensure their long-term functional maintenance.

4. Conclusions and Perspectives

Salivary gland organoids allow us to recapitulate exocrine epithelial cells functionally and structurally in vitro by harnessing salivary gland cells' potential. PSC-derived salivary gland organoids containing multiple salivary gland cell lineages can be a hopeful model for salivary gland development and morphogenesis studies. To engineer more faithfully recapitulating models, continued characterization of salivary gland tissue is the cornerstone. Recent advances in the single cell transcriptome revealed the molecular identity and cellular diversity of both epithelial and mesenchymal cells of adult and embryonic mouse salivary glands [63, 64]. Although difficult to collect samples, such analyses of human would be necessary for organoid engineers. As a potential strategy for regenerative medicine, ASC-derived salivary gland organoids are facing challenges that require combined approaches of stem cell biology and bioengineering. Current salivary gland organoids are lack of vascular cells; although, microvascular endothelial cells can be cocultured with salivary gland cells [65], but insufficient to form a functional network to guarantee nutrient supply as organoids expand. Microfluidic systems and biomaterials can be incorporated in future work, and organoid transplantation into an existing vascularized bed of host animals to allow the vasculature to grow into would be a promising attempt [56]. In conclusion, salivary gland organoids provide an unprecedented manner to study salivary gland development, biology, and morphogenesis. Bioengineering holds the promise to establish salivary gland organoids more physiologically relevant and more amenable to biomedical applications.

Data Availability

The data used to support the findings of this study are included within the article. Previously reported histoimmunochemistry figure data were used to support this study and are available at DOI 10.1038/s41467-018-06469-7. This prior study is cited at relevant place within the text as reference [28].

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Development of a Human Intestinal Organoid Model for *In Vitro* Studies on Gut Inflammation and Fibrosis

Leonidas Kandilogiannakis,[1] **Eirini Filidou**,[1] **Ioannis Drygiannakis**,[2] **Gesthimani Tarapatzi**,[1] **Stylianos Didaskalou**,[3] **Maria Koffa**,[3] **Konstantinos Arvanitidis**,[1] **Giorgos Bamias**,[4] **Vassilis Valatas**,[1,2] **Vasilis Paspaliaris**,[5] **and George Kolios**[1]

[1]*Laboratory of Pharmacology, Faculty of Medicine, Democritus University of Thrace, Alexandroupolis, Greece*
[2]*Gastroenterology and Hepatology Research Laboratory, Medical School, University of Crete, Heraklion, Greece*
[3]*Laboratory of Molecular Cell Biology, Department of Molecular Biology and Genetics, Democritus University of Thrace, Alexandroupolis, Greece*
[4]*GI-Unit, Third Department of Internal Medicine, National & Kapodistrian University of Athens, Sotiria Hospital, Athens, Greece*
[5]*Tithon Biotech Inc., San Diego, USA*

Correspondence should be addressed to George Kolios; gkolios@med.duth.gr

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Inflammatory Bowel Diseases (IBDs) are characterized by chronic intestinal inflammation and fibrosis, the latter being the predominant denominator for long-term complications. Epithelial and mesenchymal 2D cultures are highly utilized *in vitro* models for the preclinical evaluation of anti-inflammatory and antifibrotic therapies. More recently, human intestinal organoids (HIOs), a new 3D *in vitro* model derived from pluripotent stem cells, have the advantage to closely resemble the architecture of the intestinal mucosa. However, the appropriate timing for the study of inflammatory and fibrotic responses, during HIO development, has not been adequately investigated. We developed HIOs from the human embryonic stem cell line, H1, and examined the expression of mesenchymal markers during their maturation process. We also investigated the effect of inflammatory stimuli on the expression of fibrotic and immunological mediators. Serial evaluation of the expression of mesenchymal and extracellular matrix (ECM) markers revealed that HIOs have an adequately developed mesenchymal component, which gradually declines through culture passages. Specifically, CD90, collagen type I, collagen type III, and fibronectin were highly expressed in early passages but gradually diminished in late passages. The proinflammatory cytokines IL-1α and TNF-α induced the mRNA expression of fibronectin, collagen types I and III, tissue factor (TF), and alpha-smooth muscle actin (α-SMA) primarily in early passages. Similarly, HIOs elicited strong mRNA and protein mesenchymal (CXCL10) and epithelial (CXCL1, CCL2, CXCL8, and CCL20) chemokine responses in early but not late passages. In contrast, the epithelial tight junction components, CLDN1 and JAMA, responded to inflammatory stimulation independently of the culture passage. Our findings indicate that this HIO model contains a functional mesenchymal component, during early passages, and underline the significance of the mesenchymal cells' fitness in inflammatory and fibrotic responses. Therefore, we propose that this model is suitable for the study of epithelial-mesenchymal interactions in early passages when the mesenchymal component is active.

1. Introduction

Inflammatory Bowel Diseases (IBDs), a group of diseases that includes Crohn's disease and ulcerative colitis, are characterized by chronic intestinal inflammation of unknown etiology [1]. Mucosal and systemic immunology has been the mainstream of IBD research for many decades resulting in the successful development of many biologics for the treatment of this debilitating group of diseases. However, epithelial and stromal biology has been largely overlooked. Recent studies

have indicated that the study of the intestinal epithelium and mesenchyme may provide keys in deciphering the heterogeneity that characterizes patient phenotypes and their responses to biologics [2–4]. Furthermore, due to the fibrotic complications that eventually develop in more difficult to treat patients and the lack of therapeutic approaches to reverse postinflammatory fibrosis, the biology of the mucosal stroma has recently been brought into focus [4–6].

Significant progress has recently been achieved in understanding intestinal stromal cell biology by using 2D culture systems of primary mesenchymal cells isolated from human diseased and normal guts and intestinal organoids. During embryonic development and homeostasis, stromal cells have been shown to control epithelial proliferation and restitution through the production of activators and inhibitors of the Wnt signaling pathway [7]. During IBD-related chronic inflammation, we among others have shown that the intestinal stroma is not an innocent bystander, as previously thought [8–10]. Mesenchymal cells exhibit a variety of cytokine receptors and orchestrate extracellular matrix (ECM) production, accumulation, and eventually fibrosis in response to various inflammatory stimuli [8–10].

The development of human intestinal organoids (HIOs) in 2011 has revolutionized mucosal research as a novel *in vitro* system that enabled to study epithelial and mesenchymal cells as an interacting unit [11]. HIOs are 3D formations developed by pluripotent stem cells, through a process that simulates organogenesis. They have a similar architecture with the intestinal tissue, where the lumen is surrounded by epithelial cells forming villi and crypts, which are further supported by an outer layer of mesenchymal cells. Therefore, HIOs are able to approach intestinal inflammation and fibrosis in a more spherical way than classic 2D *in vitro* models, as they consist of many different interacting epithelial and mesenchymal cell types. HIOs still lack vascular, neurological, or immune structures, in comparison to animal models of IBD, but do provide a more analytical tool to separately study mesenchymal and epithelial biology from immune responses [12].

Despite their growing use for the study of monogenic diseases, intestinal organoids have rarely been used to model polygenic multifactorial diseases such as IBD. Recent studies have shown that as organoids are formed and later cultured, they continue to mature and change throughout their culture, mimicking the process of embryonic to fetal and adult development [13–15]. Therefore, knowing the appropriate time during their culture period to study inflammatory and fibrotic responses that mimic closely the IBD cascade is vital for these to be used as an effective *in vitro* disease model.

In this study, we successfully developed and characterized HIOs from the human embryonic stem cell line, H1. We examined the expression of fibrotic and mesenchymal factors during their maturation process, as well as the effect of the proinflammatory cytokines, IL-1α and TNF-α, on the expression of fibrotic and inflammatory mediators in HIOs during different stages of their maturation period.

2. Materials and Methods

2.1. H1 Cells. H1 cells are human pluripotent embryonic stem cells, originally derived and isolated from a male human blastocyst in 1998 [16]. They were purchased from WiCell (Madison, Wisconsin, USA) and set to culture according to WiCell Feeder Independent Pluripotent Stem Cell Protocols. Briefly, H1 cells were seeded onto Matrigel-coated 6-well plates (Matrigel™; Corning, New York, USA), which contained the mTeSR™1 medium (StemCell Technologies, Vancouver, Canada), and cultured in 5% CO_2 at 37°C. H1 were fed daily and passaged every 5 days in a ratio of 1 : 6 using Dispase (MilliporeSigma, Burlington, Massachusetts, USA). Before passaging, H1 cells were first observed for any signs of differentiation, which can be visible when observed under a microscope, as differentiated cells significantly differ in morphology from undifferentiated embryonic stem cell colonies. According to the manufacturer's instructions, when the differentiation rate was above 5%, we removed the differentiated cells with a micropipette tip. H1 cells were maintained in culture and were regularly screened for the expression of pluripotent embryonic markers using immunofluorescence.

2.2. Development and Culture of HIOs. HIOs were developed from H1 embryonic stem cells using the STEMdiff™ Intestinal Organoid Kit (StemCell Technologies, Vancouver, Canada), according to the manufacturer's instructions. Briefly, H1 cells were seeded onto Matrigel-coated 24-well plates and cultured in the mTeSR™1 medium (StemCell Technologies, Vancouver, Canada) until they reached the appropriate confluency. H1 cells were then cultured in the Endoderm Basal medium containing Activin A and fed daily until day 3, when the Definitive Endoderm (DE) was created. DE was subsequently cultured in the Endoderm Basal medium containing Wnt3A and fibroblast growth factor 4 (FGF4) for another 5-6 days, until Mid-/Hindgut (MH) spheroids were released into the supernatant. MH spheroids were then collected, counted, seeded into domes made of Matrigel (Corning, New York, USA), and cultured in the Intestinal Organoid Basal (IOB) medium containing epidermal growth factor (EGF) and Noggin, until HIOs were finally formed. HIOs were continuously cultured in the EGF- and Noggin-supplemented IOB medium, fed every 3-4 days, and passaged every 10 days at a ratio of 1 : 3. HIOs and their intermediate developmental stages were characterized using immunofluorescence.

HIOs were cultured up to passage 13, and their intestinal structure proved stable until that passage (Supplementary Figure 1A). In addition, we semiquantitatively calculated the percentage of the organoid growth rate by measuring the diameter of three random organoids on day 0 and day 10 for each passage, which provides a semiquantitative estimate of their growth rate. As shown in Supplementary Figure 1B, the diameter increases by $50.8 \pm 14.7\%$ from day 0 to day 10 in passage 1, by $76.8 \pm 11.4\%$ in passage 6, and by $106 \pm 5.8\%$ in passage 13, suggesting that HIOs continue to mature their luminal structures even in late passages. In addition, we also performed double staining for the expression of Ki67, a well-known proliferation marker, and

EpCam, an epithelial marker, in late-passage organoids and we found Ki67-positive expression in epithelial cells. This finding suggests that even in late passages, organoids continue to grow, and this growth is mainly attributed to the active proliferation state of epithelial cells. Supplementary Figure 1C is showing a late-passage organoid expressing Ki67 in its epithelial cells.

HIOs were cultured, and prior to cytokine stimulation, they were left with no growth factors for 15 h. Next, HIOs were stimulated with 5 ng/ml IL-1α and 50 ng/ml TNF-α for 12 h, and at the end of this incubation period, HIOs were collected for RNA extraction and mRNA expression analyses through qRT-PCR. In addition, we semiquantitatively calculated the percentage of organoid growth change by measuring the diameter of three random organoids in each time period and for each condition (control and 2C), in passages 2, 6, and 12.

2.3. Characterization of H1 Cells and HIO Development Using Immunofluorescence. H1 cells and HIO development were characterized using immunofluorescence, as previously described [9]. Briefly, samples were first fixed in 4% ice-cold paraformaldehyde (PFA; Sigma-Aldrich, St. Louis, Missouri, USA) for 40 minutes, then washed in phosphate-buffered saline (PBS; Sigma-Aldrich, St. Louis, Missouri, USA), and treated with 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, Missouri, USA) for 15 minutes, in order to achieve membrane permeability. Samples were then treated with the blocking solution containing 5% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, Missouri, USA) for 1 hour and later incubated overnight at 4°C with primary antibodies in 0.5% BSA (Sigma-Aldrich, St. Louis, Missouri, USA). The next day, samples were washed and incubated with secondary fluorochrome-conjugated antibodies in 0.5% BSA (Sigma-Aldrich, St. Louis, Missouri, USA) for 2 hours. Finally, nuclei were stained either with DAPI (Sigma-Aldrich, St. Louis, Missouri, USA) and observed under a fluorescent microscope (Leica DM2000; Leica Microsystems GmbH, Germany) or with DRAQ5 (Novus Biologicals, Abingdon, UK) and observed in 3 dimensions under a light sheet fluorescent microscope (UltraMicroscope II; LaVision BioTec, Bielefeld, Germany).

In addition, we semiquantitatively calculated the percentage of vimentin-positive areas in passages 1, 5, and 10. In each passage, we measured the vimentin-positive area and compared it with the total organoid area, providing us with a semiquantitative estimate of the vimentin-positive area.

2.4. Light Sheet Microscope Setup and Imaging. The UltraMicroscope II (Bioimaging Facility, Department of Molecular Biology and Genetics, Democritus University of Thrace) is equipped with an Andor Neo 5.5 sCMOS camera (Andor Technology, Belfast, UK), with a pixel pitch of 6.5 μm, a Nikon 16x (0.8 NA) water immersion objective, and a zoom body of 1.8x magnification, for a total of 28.8x magnification. The illumination is achieved by three intersecting light sheets coming from the right side, achieving a uniform illumination across the sample and reducing shadows and stripe artifacts. The detection axis is perpendicular and above the illumination path. The illumination NA was set to 0.156 creating a light sheet with a thickness of $2w_0 = 4.53\,\mu$m (as reported from the software; InSpector Pro). Excitation and detection were performed using a 488 nm, 561 nm, or 640 nm laser and 525/50 nm, 620/60 nm, and 680/30 nm filters, respectively. z-stacks were acquired with a 1 or 2 μm step.

Fixed and stained HIOs were enclosed in the top surface of 1% low-melting agarose (in PBS) cubes and were immersed inside the imaging cuvette filled with distilled water. This technique ensures that the HIO structure remains undamaged and unpressurized, and therefore, the images taken depict their actual form. Image analysis, 3D rendering, and slice selection were performed in ImageJ (National Institutes of Health, USA).

2.5. Total RNA Extraction and Purification. Total RNA from HIOs was extracted and purified from genomic traces using the NucleoSpin RNA Plus XS kit (MACHEREY-NAGEL, Düren, Germany) according to the manufacturer's instructions. Briefly, HIOs were first lysed and homogenized, and DNA was removed by passing the lysate through the DNA removal columns. The purified lysate was then loaded onto the RNA extraction columns and washed 3 times, and finally, total RNA was eluted using RNase-free H_2O. The concentration and purity of total RNA were measured using a Q5000 UV-Vis spectrophotometer (Quawell, San Jose, California, USA).

2.6. cDNA Synthesis and Quantitative Real-Time PCR. cDNA synthesis was performed using the PrimeScript RT Reagent Kit (Perfect Real Time) (TaKaRa, Kusatsu, Shiga, Japan) according to the manufacturer's instructions. In brief, 250 ng of total RNA was mixed with the 5X PrimeScript Buffer, reverse transcriptase, oligo dT primers, random hexamers, and RNase-free H_2O and incubated at 37°C for 15 minutes. Reverse transcriptase was then inactivated by heat treatment. The gene-specific mRNA expression was quantified by quantitative real-time- (qRT-) PCR using the KAPA SYBR FAST qPCR Kit (Kapa Biosystems Ltd., Boston, MA, USA), as previously described [9]. Briefly, 25 ng of cDNA was mixed with the gene-specific primers, described in Table 1, and the KAPA SYBR FAST qPCR Master Mix, and a two-step amplification protocol was performed for almost all studied genes, except for tissue factor (TF), for which the annealing temperature was set at 52°C, and a three-step protocol was performed. All amplification reactions took place at a SaCycler-96 Real Time PCR system (Sacace Biotechnologies, Como, Italy), and the gene expression of each studied gene was normalized against GAPDH gene expression in the same sample using the $2^{-\Delta\Delta Ct}$ method. Regarding the results of the mesenchymal marker and ECM component expression through serial passages, passage 1 expression levels were set as a reference point and expression levels in later passages were compared to that.

2.7. Enzyme-Linked Immunosorbent Assay (ELISA). Human DuoSet® ELISAs (R&D Systems, Minneapolis, Minnesota, USA) were used to estimate the protein concentrations of CCL2, CXCL10, CXCL1, CXCL8, CXCL10, and CXCL11

Table 1: Gene-specific primers used in real-time PCR.

Gene	Forward	Reverse	Reference
GAPDH	GACATCAAGAAGGTGGTGAA	TGTCATACCAGGAAATGAGC	[9]
Collagen type I	CCCTGGAAAGAATGGAGATGAT	ACTGAAACCTCTGTGTCCCTTCA	
Collagen type III	GCTCTGCTTCATCCCACTATTA	TGCGAGTCCTCCTACTGCTAC	
Fibronectin	CCAGTCCACAGCTATTCCTG	ACAACCACGGATGAGCTG	
α-SMA	AATGCAGAAGGAGATCACGG	TCCTGTTTGCTGATCCACATC	
TF	TTCAGTGTTCAAGCAGTGATTCC	ATGATGACCACAAATACCACAGC	
CD90	CGCTCTCCTGCTAACAGTCTT	CAGGCTGAACTCGTACTGGA	[41]
CCL2	AGGAAGATCTCAGTGCAGAGG	AGTCTTCGGAGTTTGGGTTTG	[42]
CCL20	GCTGCTTTGATGTCAGTGC	GCAGTCAAAGTTGCTTGCTTC	[43]
CXCL1	GCCCAAACCGAAGTCATAGCC	ATCCGCCAGCCTCTATCACA	[44]
CXCL8	TGGGTGCAGAGGGTTGTG	CAGACTAGGGTTGCCAGATTTA	[42]
CXCL10	CCTGCTTCAAATATTTCCCT	CCTTCCTGTATGTGTTTGGA	
CXCL11	GACGCTGTCTTTGCATAGGC	GGATTTAGGCATCGTTGTCCTTT	[45]
CLDN1	CGATGCTTTCTGTGGCTAA	AGTGGCTGACTTTCCTTGT	[46]
OCLN	CCTATAAATCCACGCCGGTTC	TCAAAGTTACCACCGCTGCTG	
ZO1	AACAGCCCTACCCATCTCG	CGTGGAAAGTACCCTCGTT	
JAMA	CGAGAGGAAACTGTTGTGCC	AACGAGTCTGGTGGTGTCTC	[47]

chemokines in HIO supernatants, according to the manufacturer's instructions. Briefly, flat 96-well plates were coated overnight with a capture antibody for each chemokine, and the following day, plates were incubated with the recommended blocking buffer for 2 h. Next, duplicates of each supernatant and known concentrations of chemokine samples were added in wells and incubated for 2 h, and then, a biotinylated detection antibody for each chemokine was added for another 2 h. Streptavidin-horseradish peroxidase was then added for 20 min, and the following addition of tetramethylbenzidine with H_2O_2 produced different optical densities (OD) of color which were measured at 450 nm on a microplate reader (DIAReader ELX800; DIALAB, Wr. Neudorf, Austria). The chemokine concentration was calculated using a linear standard curve according to the manufacturer's instructions.

2.8. Statistics. Results are presented as means with the standard error of the mean (SEM). Comparison of values among sample groups was performed with ordinary one-way ANOVA. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Development and Characterization of HIOs. HIOs were developed from the embryonic stem cell line H1, as described in Materials and Methods. Prior to protocol initiation, the H1 pluripotent stem cell line was screened for embryonic stem cell marker expression, and it was found positive for Nanog, SOX2, and OCT4 (Supplementary Figure 1A). All major developmental stages of HIOs were assessed by relevant markers. The Definitive Endoderm (DE) was found positive for SOX17 and FOXA2, two transcription factors required for the development of the definitive gut endoderm and the intestinal tissue [17], respectively (Supplementary Figure 1B). Mid-/Hindgut (MH) spheroids were expressing CDX2, an intestinal epithelial marker [18], and vimentin and E-cadherin, mesenchymal and epithelial markers [6, 19], respectively (Supplementary Figure 1C), suggesting that the HIO formation were almost complete.

After 9 days, HIOs were formed (Figure 1(a)) and were morphologically studied by immunofluorescence in order to confirm the presence of intestinal-specific cellular components. Developed organoids, as seen in Figures 1(b) and 1(c), included both the mesenchymal and epithelial cells, as indicated by positive immunoreactivity to Desmin and E-cadherin, respectively. The epithelium of HIOs consisted of intestinal CDX2-expressing epithelial cells (Figure 1(d)), forming a compact epithelial barrier, as they intensively expressed the cell adhesion molecules E-cadherin and EpCam (Figures 1(c) and 1(d)), which was further supported by abundant cytokeratin expression (Figure 1(d)). In addition, HIOs contained various types of epithelial cells, such as goblet (stained positive for MUC2, Figure 1(e)) and enteroendocrine cells (stained positive for Chromogranin A, Figure 1(e)), and formed villi as shown by their positivity for Villin (Figure 1(e)). Finally, SOX9 and KLF5 apparent staining revealed the concomitant presence of intestinal epithelial stem cell niches, possibly supporting the renewal of specialized epithelial cell subtypes.

3.2. The Mesenchymal Component Is Gradually Reduced upon Continuous Passaging. Previous studies have shown that organoids continue to mature and change throughout their culture. Since the presence of mesenchymal lineage cells is an essential difference in the cell components of embryonic stem cell-derived and adult stem cell-derived organoids, we decided to evaluate the persistence and functional fitness of mesenchymal cells during continuous passaging.

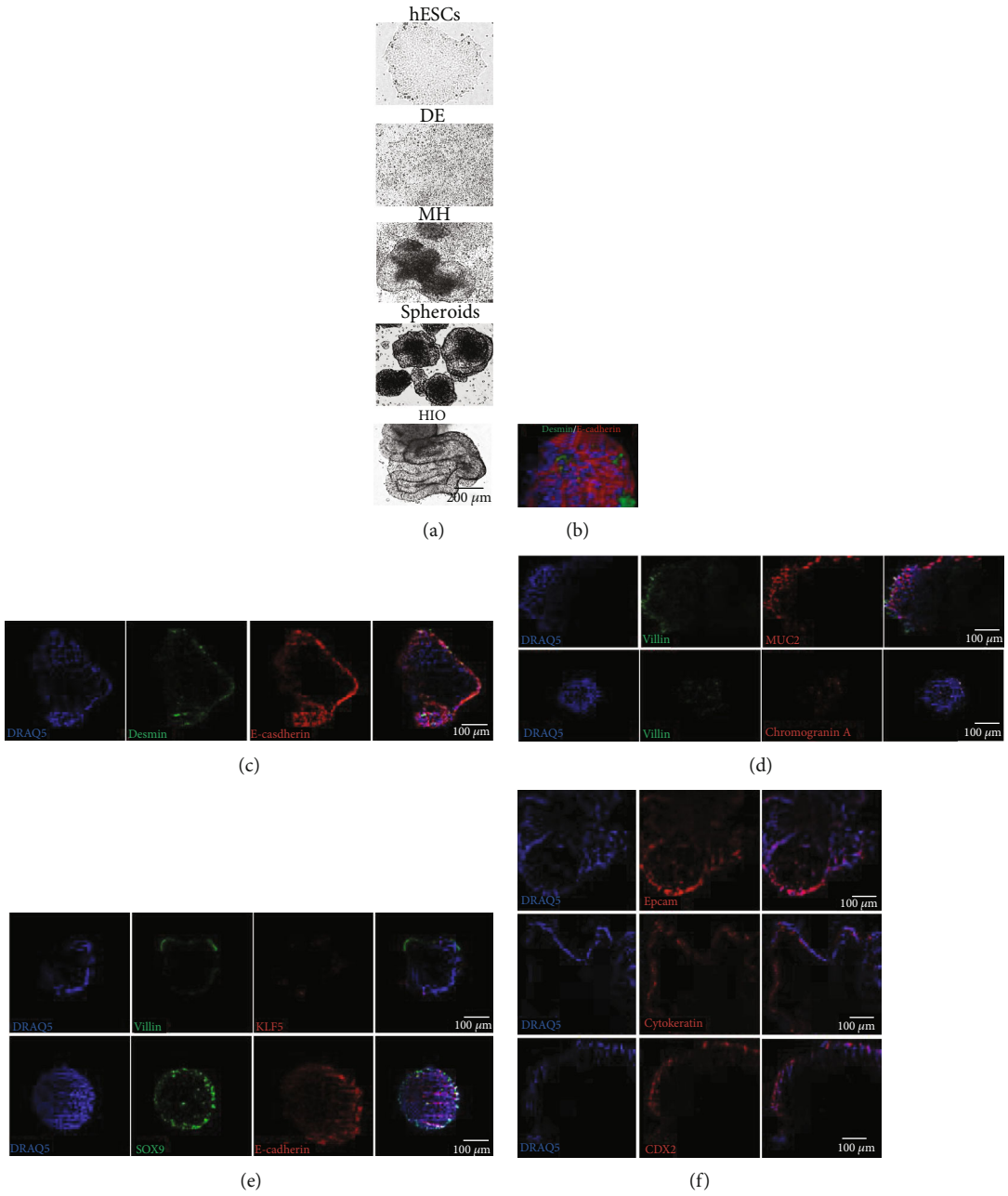

Figure 1: Development and characterization of HIOs. (a) Developmental stages of HIO formation. (b, c) HIOs stained against Desmin and E-cadherin, indicating fibroblast and epithelial cell populations, respectively. (d) HIOs stained positive for EpCam, cytokeratin, and CDX2, indicating intestinal epithelial cells. (e) MUC2-positive goblet cells and Chromogranin A-positive endocrine epithelial cells found in HIOs, surrounded by Villin-expressing epithelial cells. (f) HIOs stained positive for either KLF5 or SOX9, indicating the existence of intestinal epithelial stem cell niches that support the already-differentiated E-cadherin- and Villin-expressing epithelial cells. Representative 40x snapshots are shown in (a) and 28.8x immunofluorescence images in (b–f). (b–f) Images were obtained using a light sheet microscope. (c–f) Images are selected z-slices from the HIO total volume. (b) A 3D volume of an organoid.

We therefore studied changes in the expression of vimentin and E-cadherin, two characteristic markers for mesenchymal and epithelial cells, respectively. Once fully developed, HIOs were maintained in culture and passaged every 10 days. At the end of each passage and prior to subculturing, a portion of HIOs was collected and stained using immunofluorescence. As seen in Figure 2, vimentin expression was affluent during the early passages but was later decreased. Indeed, semiquantitative calculation of the vimentin staining area in each passage revealed $68 \pm 5.8\%$ positivity in passage 1, $36.6 \pm 7.9\%$ in passage 5, and $14.6 \pm 3.2\%$ in passage 10 (Figure 2(b)), suggesting that the mesenchymal component was gradually reduced towards late passages. We further examined the mRNA expression of various fibrotic and

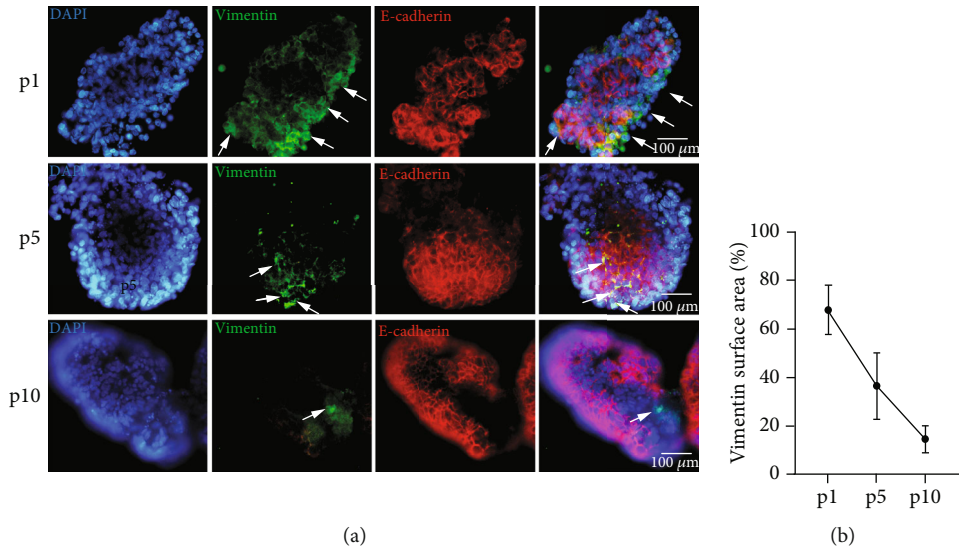

Figure 2: Mesenchymal evolution along HIO passaging. HIOs stained for the epithelial marker, E-cadherin, and the mesenchymal marker, vimentin, in three different passages (a). Vimentin-positive staining area is shown to be reduced through subsequent passages, suggesting that the mesenchymal component is gradually decreased (b). Representative 40x immunofluorescence snapshots are shown.

mesenchymal factors, as organoids progressed through the passages. The mRNA levels of CD90, fibronectin, and collagen types I and III were gradually reduced after passage 2 (CD90: 0.08-fold, ±0.01, and $p < 0.0001$; fibronectin: 0.029-fold, ±0.003, and $p < 0.0001$; collagen type I: 0.0082-fold, ±0.0009, and $p < 0.0001$; and collagen type III: 0.0042-fold, ±0.0002, and $p < 0.0001$, Figures 3(a)–3(d)), with the exception of α-SMA, which showed more stable expression pattern during passages (Figure 3(e)).

3.3. The Effect of Proinflammatory Cytokines on the Expression of Fibrotic Mediators. We proceeded to study mesenchymal responses of HIOs to inflammatory stimuli in order to evaluate their suitability for modeling postinflammatory intestinal fibrosis. The effect of IL-1α and TNF-α on the expression of mesenchymal activation markers, ECM components, and profibrotic mediators was evaluated in passages 2, 4, 6, 8, 10, and 12. Prior to the experiments with IL-1α and TNF-α, we examined the expression of their receptors, IL1R1, IL1R2, and TNFRSF1A, and found that HIOs had a basal expression of all the receptors in all passages (data not shown). In order to exclude the possibility that IL-1α and TNF-α stimulation could affect the HIO structure and growth rate, we semiquantitatively calculated the percentage of organoid growth in passages 2, 6, and 12. Supplementary Figure 3 depicts the controls or 2C-treated HIOs before and after all incubation periods, along with the percentage of diameter changes during these incubations, in three representative passages (2, 6, and 12). As shown in Supplementary Figure 3B, D, and E, the percentages of diameter changes are negligible among the passages and conditions. In passage 2, the diameter changes in controls are 2.2 ± 1.4% at 12 h, 5.5 ± 0.4% at 24 h, and 8.2 ± 1.7% at 48 h and in 2C 1.8 ± 0.8% at 12 h, 1.8 ± 0.5% at 24 h, and 8.1 ± 1.7% at 48 h. In passage 6, the diameter changes in controls are 4.8 ± 1.1% at 12 h, 3.1 ± 1.1% at 24 h, and 4.5 ± 0.6% at 48 h and in 2C 1.7 ± 0.8% at 12 h, 6.4 ± 1.3% at 24 h, and 3.3 ± 0.6% at 48 h. In passage 12, the diameter changes in controls are 4.2 ± 1.9% at 12 h, 2.5 ± 1.3% at 24 h, and 5 ± 0.9% at 48 h and in 2C 3.3 ± 0.7% at 12 h, 3.6 ± 0.6% at 24 h, and 6.3 ± 1.1% at 48 h.

We observed a differential response of HIOs to the inflammatory stimuli depending on the passage. Specifically, IL-1α and TNF-α induced a statistically significant upregulation of ECM components such as collagen types I and III and fibronectin in early passages with maximum responses observed in passage 4 (fibronectin: 2.69-fold, ±0.87, and $p < 0.0001$; collagen type I: 1.52-fold, ±0.17, and $p < 0.001$; and collagen type III: 3.39-fold, ±0.32, and $p < 0.0001$, Figures 4(a)–4(c)). Likewise, maximum responses of the profibrotic mediator TF and the mesenchymal activation marker α-SMA also occurred in early passages and specifically in passage 4 (TF: 5.04-fold, ±0.59, and $p < 0.0001$; α-SMA: 1.95-fold, ±0.12, and $p < 0.0001$, Figures 4(d) and 4(e)). Interestingly, fibrotic mesenchymal responses to proinflammatory cytokines were gradually reduced in later passages and eventually diminished in passage 12 (Figure 4).

3.4. The Effect of Proinflammatory Cytokines on the Expression of Mesenchymal and Epithelial Inflammatory Responses. We next proceeded in investigating the effect of the proinflammatory cytokines, IL-1α and TNF-α, on the mesenchymal and epithelial inflammatory responses of HIOs.

Similar to fibrotic mesenchymal responses, the chemokine responses of the HIO mesenchyme to inflammatory stimuli were strong during early passages but diminished in later passages. Specifically, IL-1α and TNF-α stimulation induced a statistically significant upregulation of CXCL10 and CXCL11 mRNA levels in passage 2 (CXCL10: 235.3-fold,

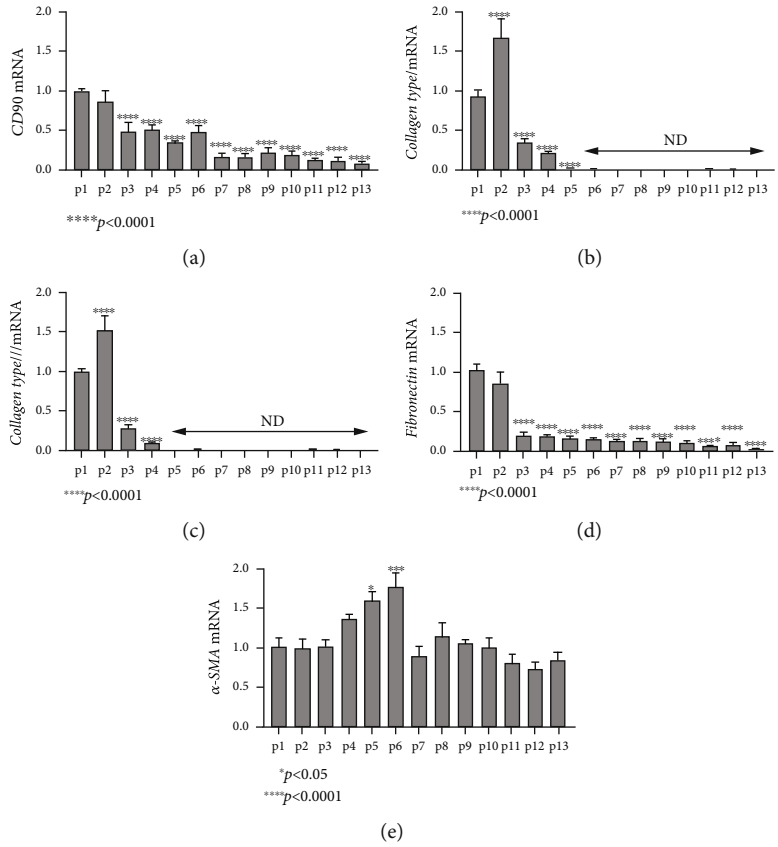

Figure 3: Expression of mesenchymal markers and ECM components during passaging. The mRNA levels of CD90, collagen types I and III, and fibronectin were gradually reduced after passage 2 (a–d), with the exception of α-SMA, which showed a tendency to increase during passages 4-6, but later decreased to basal levels (e). ND: nondetectable. All experiments were performed in triplicate. The gene expression of each studied gene was normalized against GAPDH gene expression in the same sample using the $2^{-\Delta\Delta Ct}$ method. Passage 1 expression levels were set as a reference point, and expression levels in later passages were compared to that. Data are presented as the mean ± standard error of the mean (SEM).

±20.73, and $p < 0.0001$; CXCL11: 14.53-fold, ±1.28, and $p < 0.0001$) but had no effect later on (Figures 5(a) and 5(b)).

As for the epithelial inflammatory responses of HIOs, we chose to study the effect of IL-1α and TNF-α on the expression of chemokines that are mainly produced by epithelial cells and on tight junctions, which characterize the epithelial component. Again, the effect of IL-1α and TNF-α was different depending on the passage.

Regarding the chemokine expression, in passage 2, IL-1α and TNF-α stimulation led to a statistically significant upregulation of all studied chemokines (CXCL1: 22.30-fold, ±1.30, and $p < 0.0001$; CXCL8: 13.30-fold, ±1.76, and $p < 0.001$; CCL2: 52.29-fold, ±2.59, and $p < 0.0001$; and CCL20: 23.85-fold, ±2.43, and $p < 0.0001$, Figures 5(c)–5(f)). In passage 4, the effect of IL-1α and TNF-α was even more intense for CXCL8, as it was even greater upregulated (51.98-fold, ±3.40, and $p < 0.0001$, Figure 5(d)), remained the same for CCL2 (48.91-fold, ±5.28, and $p < 0.0001$, Figure 5(e)), and was weaker for CXCL1 and CCL20, as their mRNA expression, although upregulated when compared to unstimulated organoids, was lower than passage 2 (CXCL1: 3.89-fold, ±0.22, and $p < 0.0001$; CCL20: 8.12-fold, ±0.87, and $p < 0.0001$, Figures 5(c) and 5(e)). In passage 6, only CXCL8 remained upregulated in response to proinflammatory cytokines, although its expression was significantly lower than that of passage 4 (5.76-fold, ±0.33, and $p < 0.05$, Figure 5(d)). As for passages 8, 10, and 12, none of the studied chemokines was increased in response to IL-1α and TNF-α.

The same pattern was observed in the protein level for chemokines CXCL10, CXCL1, CXCL8, CCL2, and CCL20 (Figure 6). Specifically, in passage 2, stimulation with IL-1α and TNF-α for 24 and 48 hours greatly upregulated CXCL10 (24 h: 1779 ± 234 pg/ml; 48 h: 3134 ± 305.3 pg/ml; and $p < 0.0001$; Figure 6(a)), CXCL1 (24 h: 5214 ± 113.9 pg/ml; 48 h: 10618 ± 296.2 pg/ml; and $p < 0.0001$; Figure 6(b)), CXCL8 (24 h: 312.5 ± 10.89 pg/ml; 48 h: 571.7 ± 33.9 pg/ml; and $p < 0.0001$; Figure 6(c)), CCL2 (24 h: 3458 ± 237.2 pg/ml; 48 h: 3965 ± 15.54 pg/ml; and $p < 0.0001$; Figure 6(d)), and CCL20 (24 h: 1870 ± 107.7 pg/ml; 48 h: 6669 ± 361.7 pg/ml; and $p < 0.0001$; Figure 6(e)). In passage 4, IL-1α and TNF-α stimulation also upregulated, but in a less extent, the chemokines CXCL1 (24 h: 356.3 ± 26.1 pg/ml; 48 h: 686.9 ± 30.21 pg/ml; and $p < 0.0001$; Figure 6(b)), CXCL8 (24 h: 103.4 ± 2.1 pg/ml; 48 h: 125 ± 11.3 pg/ml; and $p < 0.0001$; Figure 6(c)), CCL2 (24 h: 1848 ± 135.9 pg/ml; 48 h: 2396 ± 261.4 pg/ml; and $p < 0.0001$; Figure 6(d)), and CCL20 (24 h:

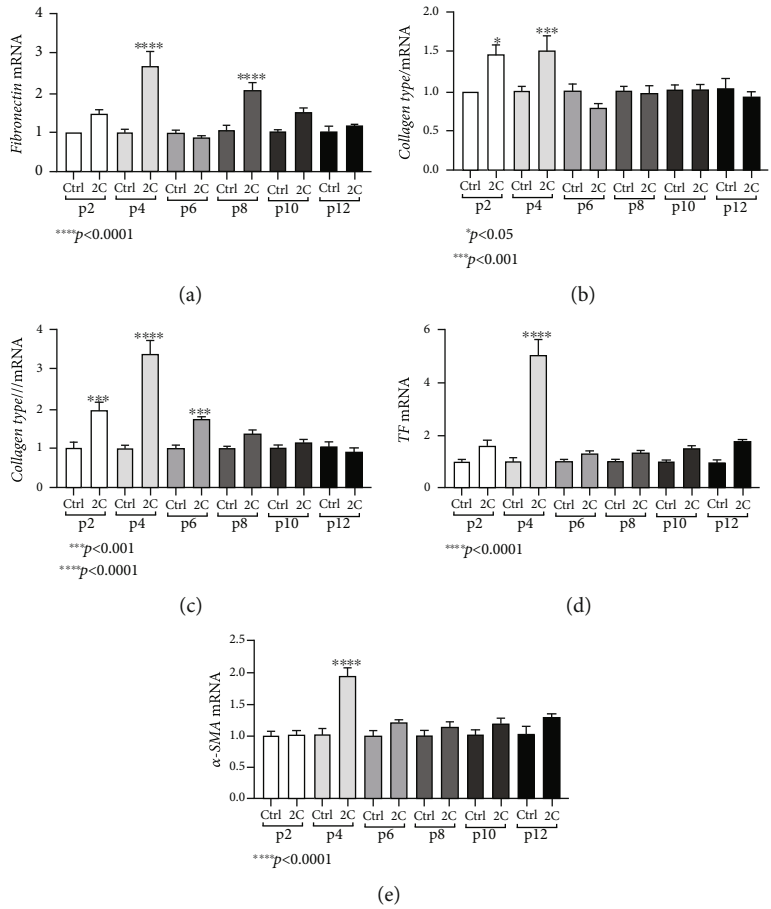

Figure 4: HIOs exhibit fibrotic responses to inflammatory cytokines. IL-1α and TNF-α (2C) induced the mRNA expression of α-SMA in passage 4 (a), fibronectin in passages 4 and 8 (b), TF in passage 4 (c), collagen type I in passages 2 and 4 (d), and collagen type III in passages 2, 4, and 6 (e). Concentrations of cytokines used: IL-1α 5 ng/ml, TNF-α 50 ng/ml. All experiments were performed in triplicate. The gene expression of each studied gene was normalized against GAPDH gene expression in the same sample using the $2^{-\Delta\Delta Ct}$ method. In every passage, expression levels of treated organoids were normalized against those of the control ones. Data are presented as the mean ± standard error of the mean (SEM).

1331 ± 129 pg/ml; 48 h: 2501 ± 317.3 pg/ml; and $p < 0.0001$; Figure 6(e)), while CXCL10 was unaffected. In passage 6, only CCL20 was upregulated (368.7 ± 33.59 pg/ml, $p < 0.05$; Figure 6(e)), after the 48 h IL-1α and TNF-α stimulation, while all the other chemokines were undetectable. In higher passages, none of the studied chemokines were traceable for both the stimulated and unstimulated organoids, while CXCL11 protein expression was absent in all passages and conditions.

In contrast, a different pattern was observed in the expression of tight junction molecules in response to inflammatory stimuli. CLDN1 and JAMA were upregulated in passage 2 in response to IL-1α and TNF-α (CLDN1: 2.03-fold, ±0.17, and $p < 0.05$; JAMA: 2.10-fold, ±0.23, and $p < 0.0001$, Figures 7(b) and 7(d)), but their expression was later returned to basal levels in passages 4 and 6. In passage 4, only OCLN and ZO1 showed a statistically significant mRNA upregulation that was abolished in later passages (OCLN: 1.28-fold, ±0.087, and $p < 0.01$; ZO1: 2.11-fold, ±0.13, and $p < 0.0001$, Figures 7(a) and 7(c)). In passage 8, only CLDN1 and JAMA were statistically significantly upregulated in response to IL-1α and TNF-α (CLDN1: 4.27-fold, ±0.55, and $p < 0.0001$; JAMA: 1.91-fold, ±0.04, and $p < 0.0001$, Figures 7(b) and 7(d)), while in passage 10, only JAMA remained upregulated in response to the two proinflammatory cytokines (1.63-fold, ±0.09, and $p < 0.0001$, Figure 7(d)), suggesting that structural molecules of the epithelium retain responsiveness to inflammatory stimuli in late passages despite the loss of mesenchymal responses. Finally, in passage 12, no effect in any studied tight junction molecule was observed, after the IL-1α and TNF-α stimulation.

4. Discussion

In this study, we show that HIOs mature and change through sequential passages, and their mesenchymal component gradually reduces with time. We have also observed that HIOs respond differently to the proinflammatory cytokines, IL-1α and TNF-α, depending on the passage, suggesting that the gradual loss of the stromal component reflects on the functionality of HIOs. Specifically, we showed that IL-1α and TNF-α stimulation upregulated the mRNA of various

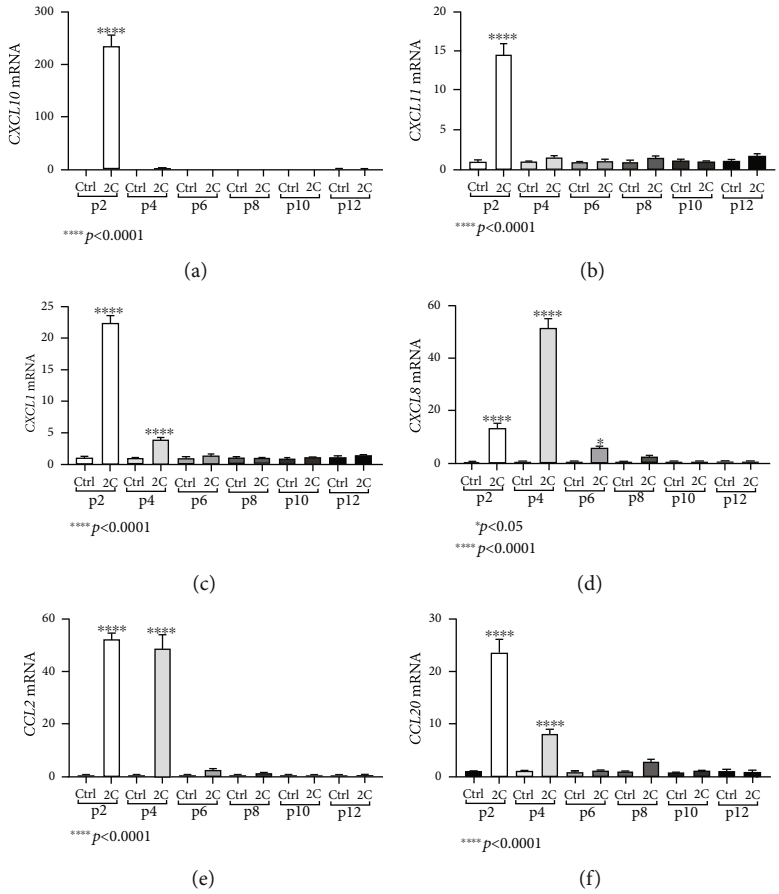

Figure 5: HIOs exhibit mesenchymal and epithelial chemokine mRNA responses to inflammatory cytokines. IL-1α and TNF-α (2C) induced the mRNA expression of CXCL10 in passage 2 (a), CXCL11 in passage 2 (b), CXCL1 in passages 2 and 4 (c), CXCL8 in passages 2, 4, and 6 (d), CCL2 in passages 2 and 4 (e), and CCL20 in passages 2 and 4 (f). Concentrations of cytokines used: IL-1α 5 ng/ml, TNF-α 50 ng/ml. All experiments were performed in triplicate. The gene expression of each studied gene was normalized against GAPDH gene expression in the same sample using the $2^{-\Delta\Delta Ct}$ method. In every passage, expression levels of treated organoids were normalized against those of the control ones. Data are presented as the mean ± standard error of the mean (SEM).

fibrotic and inflammatory factors in early, but not late, passages and this pattern was also observed at the protein level for the inflammatory chemokines CXCL10, CXCL1, CXCL8, CCL2, and CCL20. IL-1α and TNF-α stimulation had no effect on the HIO structure and growth rate in either the incubation period or the culture passage. In addition, we have also shown that HIOs maintain their structure through serial culture passages, and although their growth rate continues, it is probably attributed to the active proliferation state of their epithelial cells.

Organoids have been described as a more favorable *in vitro* tool for disease modeling for several reasons. Firstly, they can more accurately mimic the tissue architecture of the respective organ. As shown in our study, HIOs resemble the human intestinal tissue as they develop the villi, different types of epithelial cells including goblet and endocrine cells, and supporting stroma. Furthermore, they exhibit both the inflammatory and fibrotic responses to inflammatory stimuli similar to the intestinal tissue. Apart from HIOs, other types of organoids have been developed to accurately simulate different organs, such as lung organoids that are structured into alveolars, airways, and lung buds [20], liver organoids that consisted of hepatocytes and cholangiocytes that form a functional bile canalicular network [21], renal organoids that formed in most cases glomeruli and renal tubules [22], and many others [23]. Secondly, organoids can reduce the need for 2D cultures of primary cells; in most cases, they are difficult to isolate, characterize, and maintain in culture for prolonged periods of time [24]. Thirdly, HIOs contain healthy epithelial cells, which are easily studied and expanded, in contrast to primary epithelial cells which initiate apoptotic processes following isolation [25], and offer a more relevant human physiology model than using cultures of epithelial immortalized cell lines [26]. Fourthly, HIOs enable researchers to carry out high-throughput screening experiments without the need for large numbers of experimental animals (according to the Reduce-Replace-Refine principle). And finally, HIOs provide a more analytical approach by being able to separately study epithelial and mesenchymal responses from immune responses of the intestinal mucosa.

Apart from HIOs, which are pluripotent stem cell-derived organoids, there are also adult stem cell-derived 3D structures, called enteroids. Enteroids can be developed from isolated adult Lgr5$^+$ stem cells or intestinal crypts containing

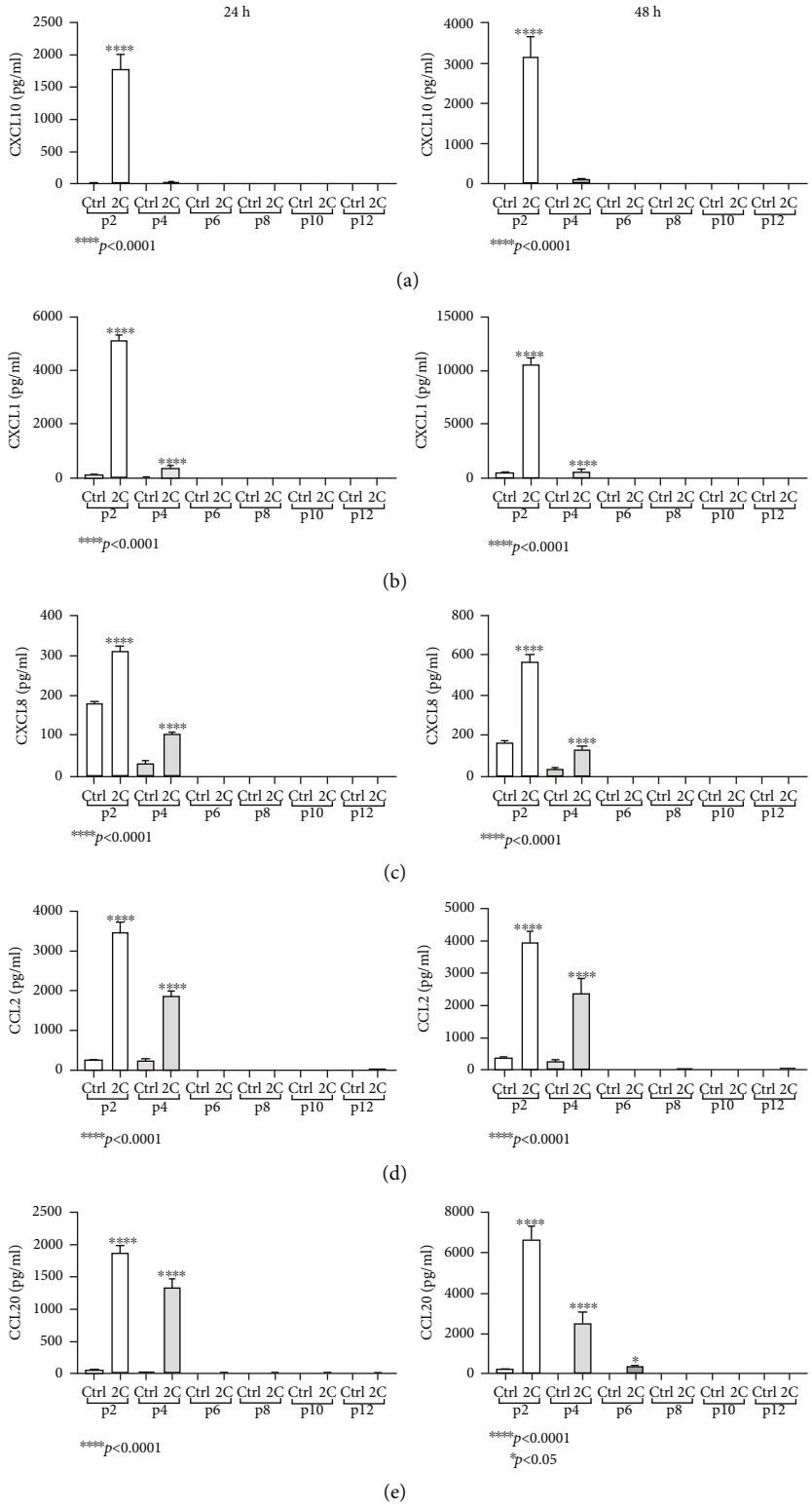

Figure 6: HIOs exhibit mesenchymal and epithelial chemokine protein responses to inflammatory cytokines. IL-1α and TNF-α (2C) 24 h and 48 h stimulation induced the protein expression of CXCL1 (b), CXCL8 (c), CCL2 (d), and CCL20 (e) in passages 2 and 4, while CXCL10 was induced only in passage 2 (a). CCL20 was also induced in passage 6, after 48 h stimulation. Concentrations of cytokines used: IL-1α 5 ng/ml, TNF-α 50 ng/ml. All experiments were performed in triplicate. Data are presented as the mean ± standard error of the mean (SEM).

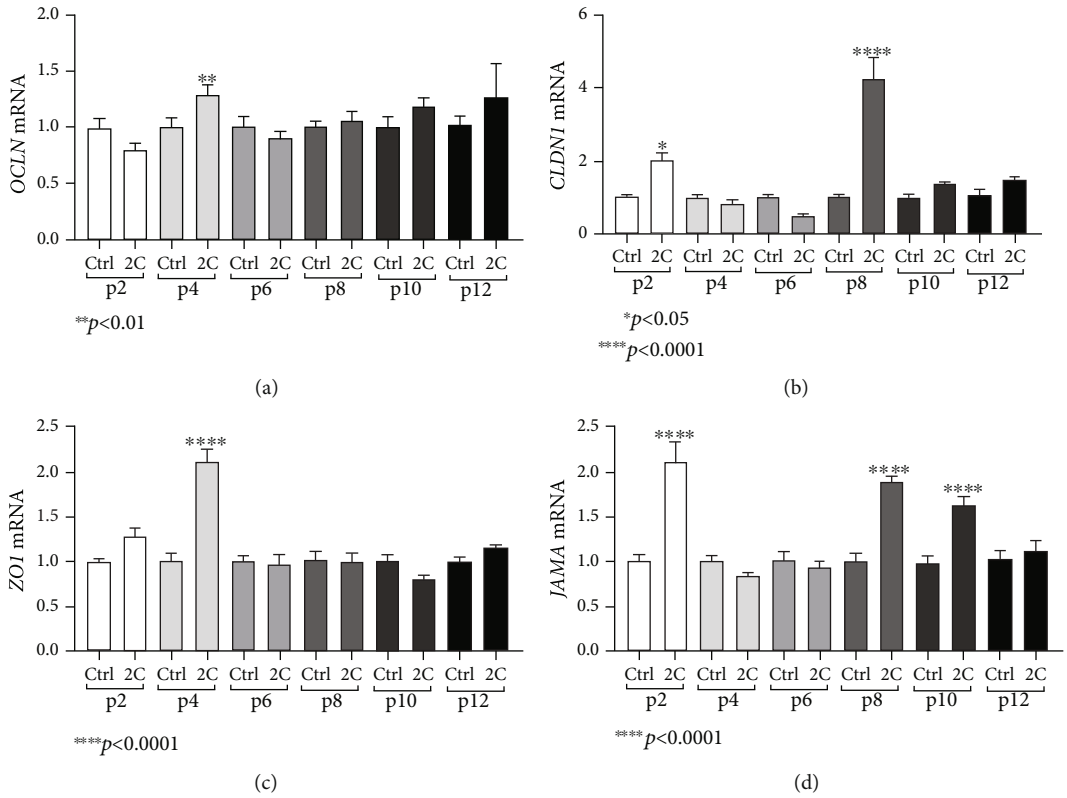

Figure 7: Epithelial responses to proinflammatory cytokines decrease during passaging. IL-1α and TNF-α (2C) induced the mRNA expression of OCLN in passage 4 (a), CLDN1 in passages 2 and 8 (b), ZO1 in passage 4 (c), and JAMA in passages 2, 8, and 10 (d). Concentrations of cytokines used: IL-1α 5 ng/ml, TNF-α 50 ng/ml. All experiments were performed in triplicate. The gene expression of each studied gene was normalized against GAPDH gene expression in the same sample using the $2^{-\Delta\Delta Ct}$ method. In every passage, expression levels of treated organoids were normalized against those of the control ones. Data are presented as the mean ± standard error of the mean (SEM).

these cells [27], using a more simplified and easier method than HIO development [28], and since they are developed from a less potent stem cell population, they are easier to maintain and culture [27]. Nonetheless, the main disadvantage of enteroids is that they only consist of epithelial cells, lacking the mesenchymal component [29], making HIOs the model of choice in studies investigating epithelial and mesenchymal interactions.

We showed that the mesenchymal component of HIOs, although gradually decreased over culture time, plays a significant role in both the inflammatory and fibrotic responses to proinflammatory stimuli, suggesting the importance of mesenchymal cells in organoid functional studies. Indeed, previous works from our group and others have highlighted the importance of mesenchymal cells in chronic intestinal inflammation and fibrosis. We have shown that intestinal subepithelial myofibroblasts (SEMFs) express various interleukin receptors, and stimulation with different Th-related cytokines leads to different fibrotic responses from SEMFs [9]. In this study, we concluded that the reduced responsiveness to IL-1α and TNF-α is possibly due to the mesenchymal component reduction and the consequent decrease of epithelial-mesenchymal crosstalk. We have previously shown that SEMFs interact with epithelial cells, as supernatants from previously stimulated HT-29 epithelial cells induce the expression of both the fibrotic and proinflammatory molecules, such as collagen and TL1A, respectively [30, 31]. Others have previously shown that high Oncostatin M (OSM) expression in patients with IBD is associated with failure to anti-TNF therapy and that high expression of its receptor is found in intestinal stromal cells, suggesting that mesenchymal cells have a significant role in IBD patient heterogeny to respond to anti-TNF agents [2]. SEMF-dependent IBD patient heterogeny is also highlighted in the research by Beswick et al. They showed that SEMFs isolated from inflamed intestinal regions of UC patients have a stronger capacity to suppress Th1 cell activity than CD or healthy SEMFs, as they overexpress programmed cell death protein 1 (PD-1), a molecule implicated in the regulation of Th immune responses [32]. In a recent study, Toll-like receptor 4 (TLR4) depletion in CCD-18Co cells, an intestinal fibroblast cell line, resulted in increased matrix metalloproteinase-1 (MMP-1) and decreased tissue inhibitor of metalloproteinase (TIMP) and collagen α1 expression [33], suggesting that innate immune responses directly regulate the fibrotic phenotype of SEMFs. More recent studies have also shown that the fibrotic phenotype of SEMFs depends on the tissue stiffness, as ileum isolated CD SEMFs have upregulated levels of the collagen crosslinking enzyme lysyl oxidase and lead to high ECM contraction [34], and this

may be regulated through endoplasmic reticulum stress-related gene overexpression [35].

In this study, we showed that early-passage HIOs overexpress fibrotic factors in response to inflammatory stimuli. In the same notion, Rodansky et al. were the first to show that HIOs are a promising fibrotic model, as HIOs overexpress several fibrotic factors in response to a dose-dependent TGF-β stimulation [36]. In a more recent study by the same research group, Steiner et al. utilized HIOs as a fibrotic model to prove that the inhibition of AXL, a receptor tyrosine kinase, could impede the TGF-β1-induced fibrotic overexpression [37]. Apart from the fibrotic responses, we have also shown that several chemokines are greatly overexpressed, at the mRNA and protein levels, when early-passage HIOs are stimulated with IL-1α and TNF-α. Other studies using induced pluripotent stem cell- (iPSC-) derived intestinal organoids as an *in vitro* inflammation model have reported similar results. Karve et al. observed that iPSC-derived intestinal organoids infected with a pathogenic strain of *Escherichia coli* produced elevated levels of IL-8 (CXCL8) and IL-18 [38]. Workman et al. showed that iPSC-derived intestinal organoids overexpressed the chemokines CXCL9, CXCL10, and CXCL11 in response to IFN-γ stimulation [39]. Finally, Onozato et al. reported that TNF-α induces the upregulation of TNF-α and IL-1β and abolishes the expression of Chromogranin A in iPSC-derived intestinal organoids. When TNF-α was combined with TGF-β, iPSC-derived intestinal organoids produced high levels of the profibrotic molecules, α-SMA, vimentin, collagen type I, and fibronectin, and the proinflammatory factors, TNF-α and IL-1β [40], suggesting that this is a promising model for studying inflammatory and fibrotic responses.

Overall, the novelty of our study lies in the fact that we show that there is a gradual downregulation of several fibrotic and mesenchymal markers, as HIOs progress from passage to passage, and there are different responses to proinflammatory cytokines depending on the passage. Other recent studies have also shown that organoids continue to mature and change throughout their culture, mimicking the process of embryonic to fetal and adult development [13–15]. Our results are in agreement with these studies and further verify the phenomenon of organoid maturation at late passages.

5. Conclusions

In conclusion, we show that embryonic stem cell-derived HIOs are supported by a mesenchymal component, which is gradually reduced over sequential passages. This mesenchymal component plays a significant role in both the epithelial and mesenchymal cell inflammatory and fibrotic responses, and its reduction leads to loss of functionality, as well as unresponsiveness to proinflammatory stimuli. Therefore, inflammatory and fibrotic studies employing HIOs should be focused on early passages. Further studies are needed to elucidate the mechanisms of HIO transformation and to identify the molecular pathways that are implicated in HIO maturation.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors have no conflict to declare.

Authors' Contributions

LK and EF wrote the main manuscript text. EF, LK, ID, KA, GB, VV, VP, and GK designed the study. EF, LK, and GT performed the experiments and analyzed the data. SD and MK assisted in imaging techniques. GB, VV, VP, and GK revised the final manuscript. All authors reviewed the manuscript. Leonidas Kandilogiannakis and Eirini Filidou contributed equally to this work.

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Supplementary Materials

Supplementary Figure 1: HIO growth rate in each passage and Ki67 proliferation marker staining. (A) Representative snapshots at culture days 0 and 10 in each passage, showing that the HIO intestinal structure remained stable. Magnification was set at 4x. (B) Semiquantitative calculation of the percentage of the organoid growth rate that shows an increase in diameter from day 0 to day 10, suggesting that HIOs continue to mature their luminal structures even in late passages. (C) Double staining for the expression of Ki67, a well-known proliferation marker, and EpCam, an epithelial marker, in a late-passage organoid. Ki67-positive expression is found in HIO epithelial cells. Representative 40x immunofluorescence snapshots are shown in (C). Supplementary Figure 2: characterization of the main developmental stages prior to HIO formation. (A) H1 pluripotent stem cell line stained against the embryonic stem cell markers, Nanog, SOX2, and OCT4. (B) Definitive Endoderm stained against SOX17 and FOXA2, two transcription factors required for the development of the definitive gut endoderm and the intestinal tissue, respectively. (C) Mid-/Hindgut spheroids stained against CDX2, an intestinal epithelial marker, and vimentin and E-cadherin, mesenchymal and epithelial markers, respectively. Representative 40x immunofluorescence snapshots are shown in (A–C). Supplementary Figure 3: IL-1α and TNF-α stimulation does not affect the HIO structure and growth rate. Representative snapshots of unstimulated (Ctrl) and IL-1α and TNF-α (2C)-stimulated HIOs in passages 2 (A and B), 6 (C and D), and 12 (E and F), showing no changes in the growth rate and structure. Magnification was set at 4x. *(Supplementary Materials)*

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Research Article

China's Stem Cell Research and Knowledge Levels of Medical Practitioners and Students

Deng Luo,[1] **Zihui Xu**,[1] **Zhongjing Wang**,[2] **and Wenzhuo Ran**[3]

[1]*Department of Endocrinology, Renmin Hospital of Wuhan University, Wuhan, China*
[2]*Department of Endocrinology, The Central Hospital of Wuhan, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China*
[3]*Department of Clinical Laboratory, Wuhan No. 1 Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China*

Correspondence should be addressed to Wenzhuo Ran; 34230270@qq.com

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Over the last few decades, China has greatly expanded its scope of stem cell research, generating various scientific advances and medical applications. However, knowledge of the extent and characteristics of domestic stem cell development, particularly medical workers' opinions, is lacking. This study's purposes were to analyze the growth trends of China's stem cell community and identify the knowledge and attitudes held by Chinese medical workers regarding stem cell research. We found that there are currently 13 high-quality stem cell research centers with more than 400 PhD-level researchers across Mainland China. These centers feature many high-caliber scientists from the stem cell research community. From 1997 through 2019, the National Natural Science Foundation of China allocated roughly $576 million to 8,050 stem cell programs at Chinese universities and research institutions. China's annual publications on stem cells increased from less than 0.6% of the world's total stem cell publications in 1999 to more than 14.1% in 2014. Our survey also revealed that most participants held positive attitudes toward stem cell research, supported further funding, and had high general awareness about stem cells.

1. Introduction

Stem cells are immature cells capable of becoming any cell type through the process of differentiation [1]. When injuries occur, these super cells can replicate rapidly and then mature into different cells needed around the body to repair and rebuild damaged tissues [2, 3]. Growing interest in the future medical application of stem cell technology is leading to the emergence of a new field called stem cell science. Scientists advocate that stem cells could tackle major degenerative diseases, such as arthritis, stroke, heart disease, diabetes, cancer, Alzheimer's disease, and Parkinson's disease [4–6]. In addition, it may be possible to use stem cells to treat debilitating spinal cord injuries and other structural injuries [7, 8]. Indeed, a recent clinical trial of combination therapy in patients with newly diagnosed multiple myeloma by using patients' own stem cells is a prominent and early success [9]. Besides, stem cells will also have applications in the discovering and testing of new drugs [10]. Therefore, stem cell research holds great promise in future medicine.

Because the work has the potential to revolutionize the way that human diseases are treated, many nations, including the USA, the UK, and Japan, have invested heavily in stem cell research and its applications [11–13]. China has also increased funding in the field via multiple sources since 1997 [14–16]. According to a report by the UK-China Stem Cell Partnership Development Initiative, the awards will be jointly funded by the UK Medical Research Council (MRC) and the National Natural Science Foundation of China (NSFC): "Funding is available for the research project as well as for the essential partnership activities required to support delivery of the collaborative research program." Up to £2 million is available from the MRC, and roughly 3 million RMB is provided by the NSFC [17].

China is emerging in the stem cell science [18]. However, knowledge of the extent and characteristics of the domestic

stem cell research community, particularly China's public opinion on stem cells, is lacking. In this project, we collected and reviewed historical investigation data on the development of stem cell science in China from 1997 through 2019, as well as a survey of 32 universities and institutions conducted from December 2013 through August 2019 as part of the National Social Survey Program, which is a cross-country collaboration between universities and research institutions. Exact numbers of high-quality scientists and research centers will provide more specific or detailed information to help integrate resources and promote collaboration and communication among international and local researchers. Furthermore, by analyzing the respondents' survey results, we are helping to evaluate the levels of knowledge that clinical practitioners and medical students have about the potential of stem cells.

2. Materials and Methods

2.1. Data Collection. To explore the current state of stem cell research in China, we collected publicly accessible data from university and faculty websites about the composition of well-known stem cell laboratories at leading academic institutions. Elite scientists who worked for each research center were identified from public departmental listings or their laboratory's website. The NSFC-allocated funds and the number of programs were downloaded from http://www.nsfc.gov.cn/. The number of articles published was obtained from the ISI Web of Knowledge database. The survey study was launched by Renmin Hospital of Wuhan University. A total of 32 universities representing China's geographical regions participated; a questionnaire was distributed to the selected medical colleges' practitioners and students randomly. Survey responses were anonymous and 2,310 responses were received.

2.2. Statistical Analysis. The mean, range, and shape of the distribution were examined for each continuous variable, with frequencies and percentages (%) tabulated for each categorical variable. All analyses were performed using SPSS Statistics 22.0.

3. Results

3.1. China's Top Stem Cell Research Centers and Currently Most Influential Scientists. Twenty years ago, few scientists were involved in the field of stem cells in China; today, more than 400 Chinese PhD-qualified researchers are working on a variety of stem cells, and there are 13 high-quality stem cell research centers (Figure 1), including Peking University's Stem Cell Research Center and the Institute of Zoology at the Chinese Academy of Sciences (CAS) (both in Beijing); the National Engineering and Research Center of Human Stem Cells at Changsha's Xiangya Medical College; the National Engineering Research Center of Stem Cells, which is affiliated with the Chinese Academy of Medical Sciences; Guangzhou's Center for Stem Cell Biology and Tissue Engineering at Sun Yat-Sen University; Tongji Medical College's Stem Cell Research and Application Center in the Wuhan Union Hospital at Huazhong University of Science and Technology (HUST); Renji-Med X Clinical Stem Cell Research Center at Renji Hospital, Shanghai Stem Cell Institute of Shanghai Jiao Tong University (SJTU) School of Medicine, and Tongji Hospital's Translational Center for Stem Cell Research at Tongji University School of Medicine (all in Shanghai); the South China Institute for Stem Cell Biology and Regenerative Medicine, the Guangzhou Institutes of Biomedicine and Health; the Center for Stem Cell Biology and Regenerative Medicine, which is affiliated with Tsinghua University; the South China Research Center for Stem Cell & Regenerative Medicine, which is affiliated with the Academy of Military Medical Sciences (AMMS); and Zhejiang University's Stem Cell and Tissue Engineering Center.

These China's top research centers and laboratories have a number of local stars of the Chinese stem cell research community: Hongkui Deng at Peking University, Guangxiu Lu at Xiangya Medical College, Peng Xiang at Sun Yat-Sen University, Zhongchao Han at the Chinese Academy of Medical Sciences and Peking Union Medical College, Ying Jin at the Shanghai Stem Cell Institute (affiliated with SJTU), Weiqiang Gao at Renji Hospital (also affiliated with SJTU), Qi Zhou at the Institute of Zoology, Shiang Huang at HUST, Duanqing Pei at the CAS, Qimin Zhan at the Chinese Academy of Medical Sciences and Peking Union Medical College, Yi Sun at Tongji University School of Medicine, Duanqing Pei at the Guangzhou Institutes of Biomedicine and Health (affiliated with the CAS), Wei Guo at Tsinghua University, XueTao Pei at the AMMS, and HongWei Ouyang at Zhejiang University. Fanyi Zeng at Shanghai Stem Cell Institute of SJTU is mainly engaged in developmental biology and medical genetics, with reprogramming cells and molecular mechanisms in mammalian reproduction and development as the core. Zeng's research has been published in more than 40 papers in *Nature*, *PNAS*, and other authoritative academic journals [19–21].

3.2. The Pattern of Funding Growth in China for Stem Cell Research. For more than a decade, China's government has devoted the majority of its science funding to stem cell research. The NSFC is a funding organization responsible for the management of the National Natural Science Fund and is aimed at promoting and financing basic research and its applications in China. To understand better the pattern of funding growth, the NSFC's data of stem cell funding from 1997 to 2019 were collected. According to the NSFC, the total amount of government spending on stem cell research from 1997 to 2019 has been about 3.7 billion yuan (roughly $576 million). Funding on a yearly basis increased dramatically, rising from 0.69 million yuan in 1997 to about 504 million yuan in 2014. Growth is slow and steady at an early developmental stage, but there is a fast growth between 2006 and 2012. Our analysis revealed that the number of yearly research programs increased over fivefold from 156 in 2006 to 839 in 2012. However, both the program numbers and funding amount were decreased after 2014 (Figure 2). This reflects that China's stem cell research has got into an adjustment period (2015-2019).

China's leading universities and research institutions are taking steps to enhance their stem cell research. The NSFC

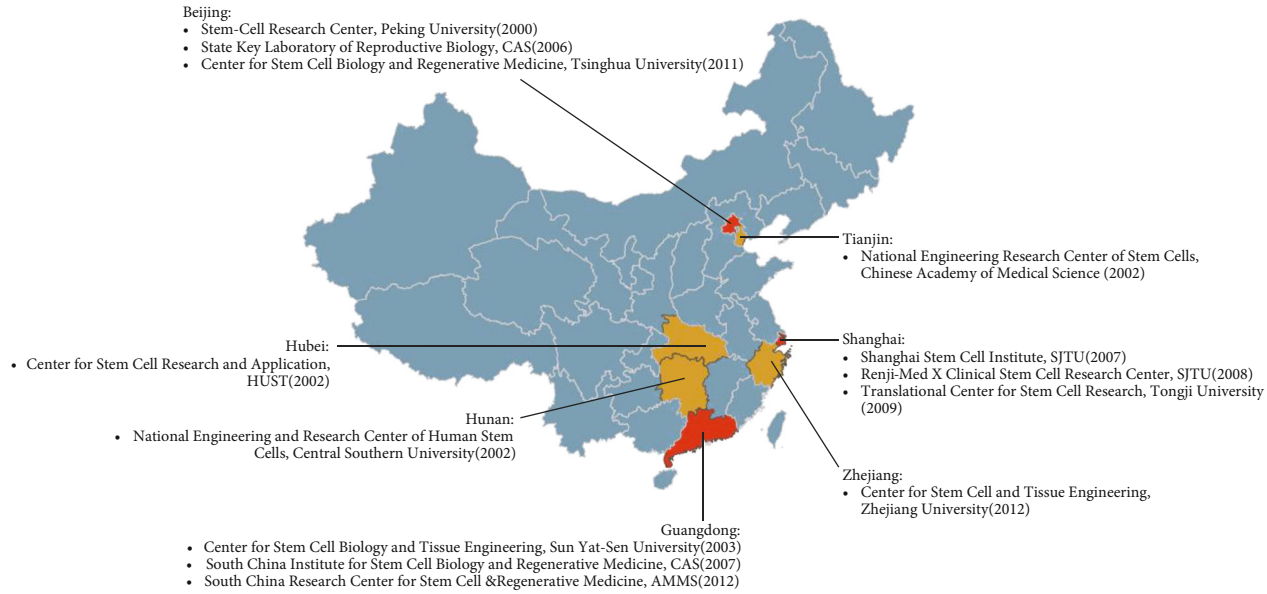

Figure 1: Leading stem cell research centers in mainland China. Brackets show the year of establishment.

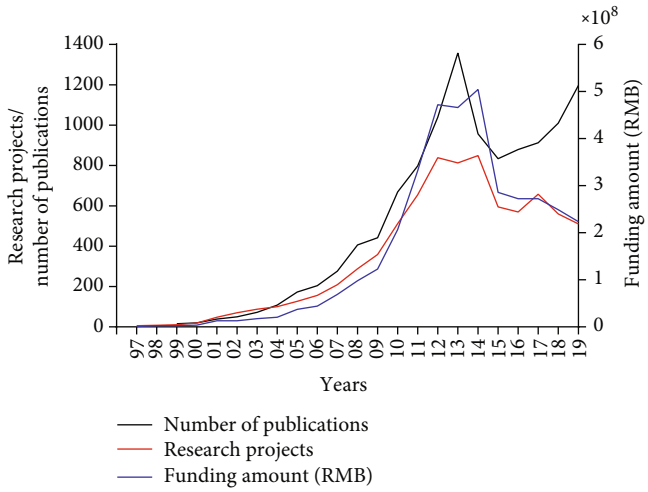

Figure 2: Publications and funding on stem cells in China from 1997 to 2019. Black line shows the trend of publications. Red line shows the trend of research projects. Blue line shows the trend of funding amount.

allocated 295 stem cell programs to SJTU between 1997 and 2019, which were 3.7% out of the whole programs ($n = 8{,}050$). Besides, Sun Yat-Sen University, the CAS, and the Third Military Medical University were 284, 248, and 243, respectively. However, the CAS was allocated the most funding amount of over 190 million RMB (Figure 3).

3.3. Trends of Publications on Stem Cells by Chinese Scholars. From 1999 to 2014, China's annual publications on stem cells jumped from 16 to 957; this represents a growth from 0.6% to more than 14.1% of the world's total stem cell research publications. Although the number of publications has a short decline in 2014 and 2015, there is a recovery growth in the adjustment period (2015-2019) (Figure 2).

In addition, we also analyzed the publications on stem cells of China's top universities and agencies. We compared the relative contribution of various institutions in different periods. In the early period from 1997 to 2002, the Chinese University of Hong Kong and its affiliated Prince of Wales Hospital occupied the top 2 positions in stem cell publications. However, mainland Chinese universities and agencies developed rapidly and replaced top positions in the next period. The CAS topped the list, followed by SJTU and Peking University, while Chinese Academy of Medical Science, Sun Yat-Sen University, Zhejiang University, Fudan University, and the Military Medical Universities also show high levels of activity in stem cell research (Figure 3). Being overtaken by mainland universities indicated that China funding reforms had promoted a shift in research emphasis.

In this study, we also performed an analysis of the trends of research categories in stem cells (Figure 4), for example, the clinical applications or molecular mechanisms of stem cells. The stem cell research responsible for molecular mechanisms increased quickly from 1997 to 2019, but publications focused on clinical applications remained relatively unstable during the same period. In particular, after 2012, clinical application publications decreased gradually.

3.4. China's Organoids Research. Organoids research is a new field of stem cell science. Organoids are self-renewing and self-organizing 3-dimensional cellular structures that resemble organs in function and structure. They can be derived from embryonic stem cells, induced pluripotent stem cells, or adult stem cells [22]. From 2011 through 2019, the NSFC has allocated 29.68 million RMB to 45 organoids projects. In general, the number of projects and publications showed a fast upward trend. In 2011, China funded the first organoids project. In 2016, 2017, 2018, and 2019, NSFC funded 3, 8, 12, and 21 organoids projects, respectively (Figure 5(a)). This increasing trend is highly consistent with the development

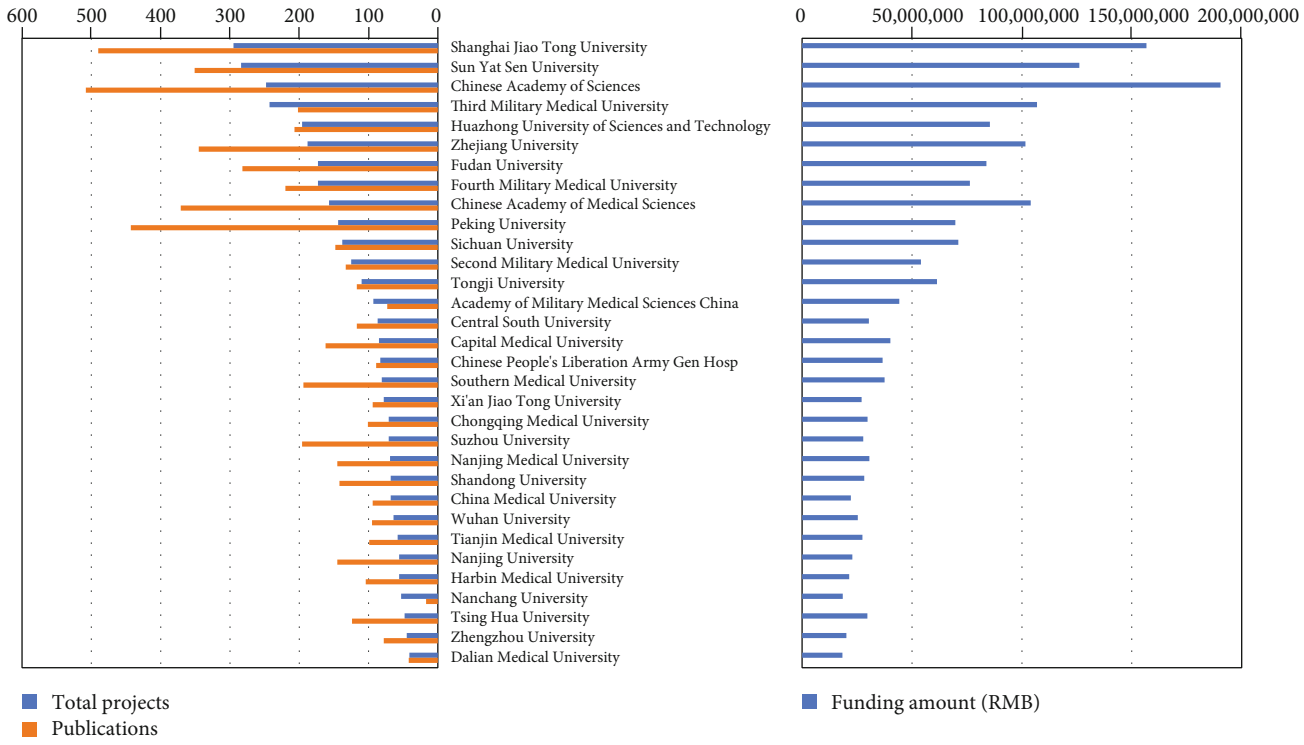

Figure 3: Top 32 China's universities and institutions in stem cell research, 1997–2019. Left histograms show their projects (blue) and publication (orange) on stem cells. Right histograms show their funding amount on stem cells.

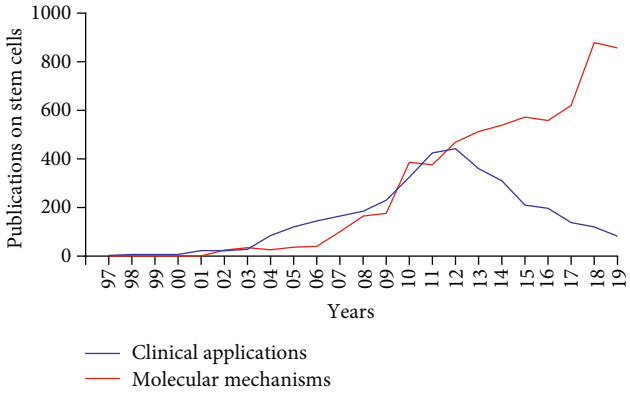

Figure 4: Trends of publications on stem cell clinical applications and molecular mechanisms by Chinese scholars from 1997 to 2019. Red line shows the trend of publications on molecular mechanisms. Blue line shows the trend of publications on clinical applications.

of organoids research in the world. In these 45 projects, the 8 most studied organs are the liver ($n = 8$), followed by brain ($n = 5$), intestine ($n = 3$), heart ($n = 2$), uterus ($n = 2$), ovary ($n = 2$), kidney ($n = 2$), and lung ($n = 2$) (Figure 5(b)). The 5 most allocated universities and institutions are Zhejiang University (8.365 million RMB), Second Military Medical University (3.165 million RMB), CAS (2.41 million RMB), Chongqing Medical University (2.4 million RMB), and Fudan University (2.35 million RMB). It shows that Chinese scholars have done a lot of research on in vitro 3D culture of different types of stem cells to establish organoid models, which are mainly used for in vitro drug screening. In terms of uses, precision medicine, tumor research, and personalized medicine dominate.

3.5. Participants' Knowledge Levels and Opinions of Stem Cell Research. A total of 1,668 (72.2%) medical students and 642 (27.8%) clinical practitioners participated in the National Stem Cell Research Survey (Table 1). Participants were recruited from 32 universities with varying levels of academic performance in 24 cities that are representative of China's different geographical regions (Figure 6). Of the 2,310 participants, 1,183 (51.2%) were men and 2,132 (92.3%) were under 30 years of age. Most of the participants have a bachelor's degree or higher: doctoral degree (12.3%), master's degree (48.1%), and bachelor's degree (39.5%). Among the 2,310 participants, 2,105 were in clinical medicine (91.1%), 156 were in basic medicine (6.8%), 34 were in public health (1.5%), 9 were in other majors, and 6 were from unknown departments.

For all participants, 99.1% knew stem cells, and the media (Internet, newspapers, magazines, and TV/radio) were the most common ways to acquire knowledge on stem cells. A total of 78.9% had a great interest in stem cells, while only 21.1% had no interest. Only 20.6% of participants were familiar with stem cell transplantation for human diseases, but 79.4% had low or moderate self-estimated knowledge concerning stem cell transplantation. Roughly 63.5% accepted medical research using human embryonic stem cells; 30.8%

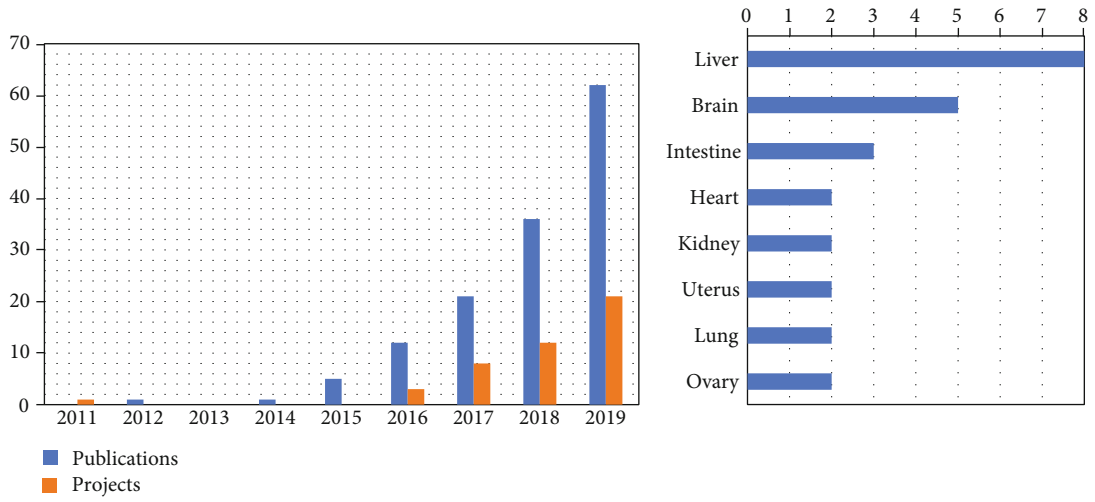

Figure 5: China's organoids research. (a) The trends of publications (blue) and projects (orange) on organoids research in mainland China from 2011 to 2019. (b) The organs and their frequencies involved in these organoids projects.

Table 1: A questionnaire on participants' knowledge levels and opinions of stem cell research.

Item	Questions
1	Have you heard of stem cells before?
2	Are you interested in research on stem cells?
3	What type of stem cells have you heard of?
4	How much do you know about stem cell transplantation?
5	What kind of attitude do you think our country should have towards the development of stem cell research in clinical treatment?
6	Do you think the country should increase funding for stem cell research?
7	Do you think it is necessary to conduct medical science popularization on stem cells among the general public?
8	Will you actively promote the application and prospects of stem cells to your patients and their families?
9	Do you support the use of stem cells derived from human embryos for basic research on clinical diseases?
10	IPS technology is a major breakthrough in the field of stem cell research. It solves difficult ethical disputes and immune rejection issues and makes stem cells a major step forward for clinical applications. How do you see its application prospects in China?
11	Have you participated in an academic conference on the application of stem cells in the field of clinical diseases?
12	Are you willing to take (or accept) continuing education courses related to stem cells?
13	Do you think stem cell clinical disease treatment has application prospects in my country?
14	What is your personal attitude towards the clinical translational treatment of clinical diseases by stem cells?
15	Do you have any concerns about the safety of stem cell transplantation to treat clinical diseases?
16	Before starting the treatment of stem cell clinical diseases, do you think it should go through a complete animal experiment demonstration?
17	If you can get a higher legal income, would you give priority to providing or recommending stem cell/regenerative treatment technologies to patients?
18	Do you agree and support the establishment of a dedicated human stem cell bank for the treatment of clinical diseases?
19	What are your relative concerns about stem cell transplantation for the treatment of clinical diseases?
20	Do you think there are any difficulties you may encounter in the actual operation of stem cell transplantation to treat clinical diseases?
21	If you have the opportunity to donate stem cells to save the lives of others, what kind of return do you expect?
22	If you are willing to donate raw materials for stem cell extraction, which of the following samples would you prefer to donate?
23	Are you willing to donate urine for basic research on clinical diseases?
24	What is your opinion on the basic research and clinical application of stem cells derived from urine?
25	Do you think that there should be corresponding remuneration for donating stem cells for clinical treatment?
26	What do you expect to maintain the effectiveness of stem cell treatment of clinical diseases?
27	If you are willing to donate stem cells, what do you want to know most?

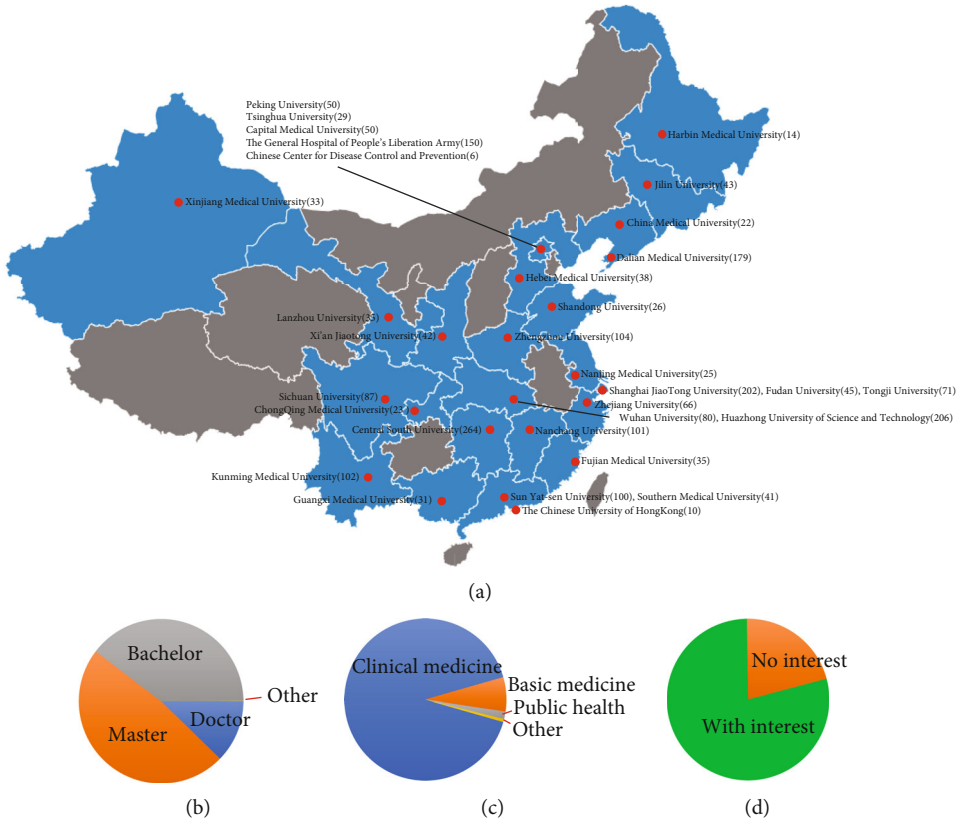

FIGURE 6: Participants' knowledge levels and opinions on stem cell research. (a) Surveyed 32 universities and institutions from 24 cities in different regions of China. Brackets show numbers of participants. (b) Educational background of participants. (c) Majors of participants. (d) Participants' interest in stem cells.

considered this morally unacceptable. In addition, 54.2% supported the research of induced pluripotent stem cells upon explanation of the nature. 71.6% supported the clinical translation of stem cells, and only 1.1% of participants were opposed. Finally, it is worth mentioning that 89.9% supported increased funding for stem cell research; fewer than 2% of those questioned were against any stem cell research funding.

4. Discussion

Stem cell technologies are often described as scientific breakthroughs that could potentially revolutionize medicine [23]. This study's aim was to examine the levels and characteristics of stem cell research in mainland China. First, we ascertained the number of top stem cell research centers and top-tier scientists in mainland China today. Second, we collected detailed information to investigate the historical growth trend in government funding since 1997. Third, we summarized the stem cell research publications by Chinese scholars between 1997 and 2019. Then, we reviewed the organoids research in China. Finally, we obtained valuable information on the knowledge levels and opinions of China's clinical practitioners and medical students using questionnaires. In this study, we found that China's stem cell research improved significantly from 1997 to 2019. Most of the surveyed participants expressed support for stem cell research. These findings might reflect China's efforts to provide infrastructure that supports stem cell and regenerative medicine.

According to a report in 2006, the total numbers of stem cell researchers and laboratories in China were relatively small. [24] However, after 8 years of development, the number of Chinese stem cell centers had doubled in 2014, according to detailed information from public departmental listings or the laboratory's websites. We examined the top stem cell laboratories in China and found 13 high-caliber laboratories with more than 400 PhD-qualified researchers. These research centers have many competitive scientists from the stem cell research community. With the efforts by national and local governments, universities, ministries, and agencies to provide financial and research incentives, the number of high-caliber talents returning from abroad has increased dramatically. They have cooperated with international and local partners to enhance the influence of stem cell research in China.

The rapid development of China's stem cell field depends largely on the increase in governmental funding. Since 1997, China has increased funding significantly to the field of stem cell science through multiple sources. Our results found that, between 1997 and 2019, the NSFC has allocated roughly $576 million to 8,050 stem cell programs among universities and research institutions. Growth is slow and steady at the early developmental stage from 1997 through 2006, but increased rapidly from 2006. The number of annually funded programs

increased over fivefold from 2006 to 2014. Additionally, the Ministry of Science and Technology of China has also provided significant research funding through National High-tech R&D Program (863 Program) and National Program on Key Basic Research Projects (973 Program). Although precise figures are hard to come by, it appears that China provided roughly $5 million to each of the 2 major programs in stem cell basic research and applications since 2002. The bulk of the spending in this field reflected China's determined efforts to advance stem cell science. While China has the scientific infrastructure to excel in this field, the importance of international collaboration for the field's development has also been underlined. In December 2013, the UK-China Stem Cell Partnership Development Initiative was launched to deliver significant 3-year research funding for internationally competitive and innovative collaborative projects between scientists from China and the UK, which allowed the pursuit of shared research interests.

China's stem cell research was generally strongly supported and can be divided into three stages. From the 1990s to 2008, China began to vigorously support stem cell research, and the supervision was relatively loose. From 2009 to 2014, the stem cell policy of China emphasized safety, and the policy orientation was based on stability. From 2015 to 2019, China's stem cell research has been further improved. Besides the fast development of basic research, the supervision of stem cell clinical research is more stringent. As new funding programs have been added over the years, competitive funding has become divided among some 100 competitive schemes overseen by about 30 different governmental departments. The Chinese government announcement noted that wastefulness and fragmented management has led to overlaps and inefficient use of funds for science and technology. On 2016, the Chinese government announced a passage of reform plans and eliminated the 863 Program and the 973 Program that fundamentally reshape research in the country.

We also evaluated the scientific production of stem cell research for the past 23 years and provided insights into the characteristics of the stem cell research publications. Data are based on the online version of the Science Citation Index (Web of Science) from 1997 to 2019. The number of stem cell papers published by Chinese scholars increased markedly between 1999 and 2014, which increased from less than 0.6% to more than 14.1% of the total publications in the world. According to an international report released by Elsevier, EuroStemCell, and Kyoto University's Institute for Integrated Cell-Material Sciences [25], the total publication volume is strikingly similar to that of the USA today. China has been the second most productive country regarding the volume of stem cell papers. Most of the papers were published mainly by Hong Kong's universities and research institutions from 1997 through 2006. However, Mainland China overtakes Hong Kong in number of published articles on stem cells between 2007 and 2014. The CAS topped the list, followed by SJTU and Peking University. This change in output suggests that the numerous government investments in education and infrastructure have improved China to the forefront of international scientific productivity.

Alongside their high general awareness of stem cells, the participants appear positive about the level of progress in stem cell research and support further funding. More than half of all respondents had a great interest in stem cell research; fewer than 2% of those questioned were against stem cell research and governmental funding. Despite China's widespread support, there are still a number of major hurdles on the stem cell research path in China. On the one hand, most of Chinese scholars are keen to publish their work in English language journals and present their findings at international conferences rather than local ones. This means that the exchange of views for promoting collaboration among local researchers is woefully inadequate, due to the lack of systematic data about the number of stem cell researchers and laboratories. On the other hand, China remains a developing country, with a per capita annual income of only $10,121 in 2019, ranking 72nd in the world. Government funding of stem cell science in China is relatively limited. Moreover, we fear that the output of stem cell papers is not always matched with the quality of Chinese research. Scientific corruption and fraud by minor scientists appear to have risen and led to additional negative publicity. Thus, there is also a need for the progressive development of appropriate legal and regulatory frameworks to allow China's stem cell research to move forward.

5. Conclusion

This study provided an analysis of the extent and characteristics of stem cell research development from data obtained between 1997 and 2019. The stem cell research landscape has changed considerably over these years. A critical acceleration began in 2006 when China began making rapid strides toward understanding stem cell science and the ways in which medicines can be used to treat illnesses. Furthermore, our survey of 2,310 highly educated participants also identified high-level support for stem cell research. Therefore, China could be considered a powerhouse in the international stem cell enterprise and will continue to apply research findings to clinical practice. Although we may not directly benefit from the survey's results, they will be used to help scientists, partners, policymakers, physicians, and medical students improve the awareness and resources that they have identified as being important to them.

Data Availability

All data included in this study are available upon request by contact with the corresponding author.

Consent

Informed consent was obtained from all individual participants included in the study.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Authors' Contributions

Deng Luo and Zihui Xu contributed equally to this work.

Acknowledgments

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Research Article

Exosomes from Kartogenin-Pretreated Infrapatellar Fat Pad Mesenchymal Stem Cells Enhance Chondrocyte Anabolism and Articular Cartilage Regeneration

Jiahua Shao, Jun Zhu, Yi Chen, Qiwei Fu, Lexiang Li, Zheru Ding, Jun Wu, Yaguang Han, Haobo Li, Qirong Qian, and Yiqin Zhou

Department of Orthopedics, Shanghai Changzheng Hospital, Naval Medical University, Shanghai 200003, China

Correspondence should be addressed to Qirong Qian; qianqr@163.com and Yiqin Zhou; drzhouyiqin@163.com

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Objective. To evaluate the effect of Kartogenin-pretreated exosomes derived from infrapatellar fat pad mesenchymal stem cells on chondrocyte in vitro and articular cartilage regeneration in vivo. *Methods*. Infrapatellar fat pad mesenchymal stem cells (IPFP-MSCs) were isolated from rabbits to harvest exosomes. After identification of mesenchymal stem cells and exosomes, rabbit chondrocytes were divided into three groups for further treatment: the EXO group (chondrocytes treated with exosomes isolated from infrapatellar fat pad mesenchymal stem cells), KGN-EXO group (chondrocytes treated with exosomes isolated from infrapatellar fat pad mesenchymal stem cells pretreated with KGN), and control group. After processing and proliferation, phenotypic changes of chondrocytes were measured. In the in vivo study, 4 groups of rabbits with articular cartilage injury were treated with KGN-EXO, EXO, IPFP-MSCs, and control. Macroscopic evaluation and histological evaluation were made to figure out the different effects of the 4 groups on cartilage regeneration in vivo. *Results.* The proliferation rate of chondrocytes in the EXO or KGN-EXO group was significantly higher than that in the control group ($P < 0.05$). The qRT-PCR results showed that the expression of Sox-9, Aggrecan, and Col II was the highest in the KGN-EXO group compared with the EXO group and the control group ($P < 0.05$). The results of Western blot were consistent with the results of qRT-PCR. In vivo, the cartilage defects in the KGN-EXO group showed better gross appearance and improved histological score than those in IPFP-MSC groups, EXO groups, and control groups ($P < 0.05$). At 12 weeks, the defect site in the KGN-EXO group was almost completely repaired with a flat and smooth surface, while a large amount of hyaline cartilage-like structures and no obvious cracks were observed. *Conclusion*. Our study demonstrates that the exosomes isolated from infrapatellar fat pad mesenchymal stem cells pretreated with KGN have potent ability to induce chondrogenic differentiation of stem cells, effectively promoting the proliferation and the expression of chondrogenic proteins and genes of chondrocytes. The KGN-EXO can also promote the repair of articular cartilage defects more effectively, which can be used as a potential therapeutic method in the future.

1. Introduction

Osteoarthritis (OA) is one of the most common diseases encountered in the field of orthopedics. OA is a chronic degenerative joint disease that commonly causes pain and limited mobility. Furthermore, the treatment costs associated with OA are high [1]. OA is characterized by the loss of extracellular matrix and the destruction of articular cartilage [2, 3]. There are many risk factors associated with OA, including genetic factors, female gender, a history of trauma, age, and obesity [4]. Currently, there are approximately 237 million people worldwide suffering from OA [5, 6]. The main pathologic change of OA is articular cartilage lesion. Promoting articular cartilage repair or regeneration is the key to prevent OA progress. However, it is a worldwide challenge to promote articular cartilage regeneration or repair through current clinical methods, such as medications, physical therapy, arthroscopy, microfracture, or cartilage transplantation [7, 8].

For the past few years, there have been significant advancements in the field of tissue engineering and regenerative medicine. These forms of biological treatment could represent a novel and promising way to help regenerate articular cartilage. Several studies have shown that lesions in articular cartilage could be regenerated effectively by biological intervention *in vitro* or *in vivo*, including mesenchymal stem cells (MSCs), biological growth factors, or other tissue engineering methods [9, 10]. In particular, MSCs have been proved to be particularly promising for the repair of lesions in articular cartilage lesion, as demonstrated by a combination of both basic and clinical research. Although some of these previous studies have reported exciting results, there are still significant problems remaining if we are to apply MSCs to the treatment of patients in the early stage of OA, including inconsistent data, the poor quality of autologous MSCs, ethical issues relating to xenogenous MSCs, and the risk of tumorigenicity or infection [11]. Collectively, these issues create a significant limitation to the widespread and consistent application of MSCs in the clinical treatment of OA.

Recently, Murphy et al. suggested that the mechanism underlying the use of MSCs to repair damaged tissues is not mainly related to their capacity to promote the differentiation of MSCs but rather via the paracrine pathways associated with these cells [12]. Extracellular vesicles secreted by the paracrine pathway release a variety of cytokines by binding to target cells. These cytokines subsequently regulate tissue regeneration [13, 14]. Existing research indicates that exosomes are the most important form of these extracellular vesicles. Exosomes are 40 to 120 nm in diameter and contain a large number of proteins, nucleic acids, lipids, and other components secreted by cells. There are extensive differences in the components of exosomes secreted by different cell types and even by the same cell type under different conditions [15, 16]. Some studies have found that exosomes extracted from cells have a more targeted effect when pretreated by specific methodology [17]. Kartogenin (KGN) is a small molecular compound that was identified in over 22,000 heterocyclic drug molecules by Johnson et al. [18]. In their research, KGN could effectively promote the differentiation of MSCs specifically into chondrocytes. So, it is quite interesting to explore the paracrine changes of MSCs pretreated by KGN. Thus, in this study, MSCs derived from the infrapatellar fat pad of rabbits were pretreated with KGN and their exosomes were extracted for comparison with MSCs-exosomes without treating. The main aim is to investigate the role of two kinds of exosomes in the promotion of cartilage repair *in vitro* and *in vivo*.

2. Methods and Materials

2.1. Ethics Statement. All animal procedures of this study were conducted with the approval of the Ethics Committee of Second Military Medical University and in compliance with the Institutional Animal Care and Use Committee (IACUC), following international guidelines for animal treatment.

2.2. Preparation of MSCs and Chondrocytes. MSCs and chondrocytes were obtained from New Zealand white rabbits (aged less than 6 months and weighing between 2 kg and 2.5 kg). From each rabbit, we removed the infrapatellar fat pad and knee cartilage block. Next, we removed blood vessels and connective tissue and used enzymatic methods to isolate infrapatellar fat pad mesenchymal stem cells (IPFP-MSCs) and chondrocytes, as described previously [19]. The MSCs were then subjected to flow cytometry to detect a variety of surface antibodies (CD34, CD45, CD73, CD90, and CD105); IgG1-PE was used as a negative control to exclude potential interference from fluorescein. In addition, MSCs were cultured in three stages of differentiation: osteogenic, adipogenic, and chondrogenic stages, in order to identify their relative potential to differentiate in different directions.

2.3. Western Blotting. Western blotting was carried out as described previously [20]. In brief, PMSF-RIPA lysis buffer was added to the cells and the resultant lysate was centrifuged at 12,000 rpm for 5 mins to permit collection of the supernatant. The concentration of each protein was measured by the BCA method and the concentrations were adjusted to separation by SDS-PAGE. For each sample, we loaded 15 μl (50 μg) per well. Following separation, proteins were transferred to the nitrocellulose membrane. Membranes were then incubated with primary antibodies (anti-CD9, CBL162, Sigma-Aldrich; anti-TSG101, SAB2702167, Sigma-Aldrich; anti-GAPDH, G8795, Sigma-Aldrich; anti-PPARγ, MAB3872, Sigma-Aldrich; anti-Col2, CP18, Sigma-Aldrich; anti-Runx2, AV36678, Sigma-Aldrich; anti-Sox9, AV37986, Sigma-Aldrich; and anti-Aggrecan, MABT83, Sigma-Aldrich) at 1 : 1,000 and secondary antibodies at 1 : 5,000 (goat anti-rabbit (ab6721, Abcam) or goat anti-mouse (ab97023, Abcam) horseradish peroxidase- (HRP-) conjugated secondary antibody), and positive binding was visualized using a ChemiDoc™ XRS imaging system (Bio-Rad, Beijing, China). The immunoreactive bands were analyzed with ImageJ software (National Institutes of Health, Bethesda, MD, USA).

2.4. Real-Time Reverse Transcription-Polymerase Chain Reaction (qRT-PCR). Total RNA was extracted from cells using TRIzol (Invitrogen, Shanghai, China), in accordance with the manufacturer's instructions. RNA was then reverse transcribed using qScript cDNA SuperMix reagent (Quanta BioSciences, Beijing, China), and relative gene expression was determined by qRT-PCR and the $2^{-\Delta\Delta CT}$ method. The primer sequences were as follows: *SOX9* (5′-AGCAAGAACAAGCCCCACGTC-3′, 5′CCTGCCCATTCTTCACCGACT-3′); *ACAN* (5′-CATCTGGAGTTCTTTTTGGGAG-3′, 5′-CAGGTCAGGGATTCTGTGTGTC-3′); *COL2A1* (5′-GAAGACACCAAGGACTGCCTG-3′, 5′-GCACCCTTTTCGCCTTTGTCA-3′); *PPARγ* (5′-TGCAGGAGCAGAGCAAAGAAG-3′, 5′-GAGGCCAGCATGGTGTAGATG-3′); and *Runx-2* (5′-TGATGACACTGCCACCTGTG-3′, 5′-ACTCTGGCTTTGGGAAGAGC-3′). Each experiment was repeated in triplicate.

2.5. Extraction, Identification, and Measurement of Exosomes. IPFP-MSCs from passage 3 were selected and cultured in two groups. One group was cultured normally, while the other group was cultured with 5 μl of 10 mmol/L KGN in 5 ml of medium as a pretreatment. After 72 hours of culture, the supernatant was extracted and stored at −80°C. Exosomes (MSC-EXOs) were then extracted using multiple rounds of centrifugation. First cells were centrifuged at 300 g for 10 minutes. The supernatant was then collected and centrifuged at 2,000 g for 10 minutes. Again, the supernatant was collected and centrifuged at 10,000 g for 30 minutes. The supernatant was collected and then recentrifuged at 100,000 g for 70 minutes. The supernatant was then discarded, and the pellet was resuspended with phosphate-buffered saline (PBS); this was then centrifuged at 100,000 g for 70 minutes. Finally, the supernatant was discarded, and the precipitate was resuspended in 200 μl of PBS and stored at −80°C. For analysis, 10 μl of exosome solution was then added to a copper mesh and examined by electron microscopy. We then used Western blotting to determine the expression of CD9 and TSG101 on the surface of the extracted exosomes. The size distribution of the extracted exosomes was then determined using a NanoSight NS300 system (Malvern Panalytical, Malvern, UK).

2.6. The Effect of Exosomes on Cell Proliferation. Next, we selected chondrocytes showing good rates of growth from passage 3 and divided these into three groups. Chondrocytes in the EXO group were treated with 1×10^8 IPFP-MSCs. Chondrocytes in the KGN-EXO group were treated with 1×10^8 IPFP-MSC exosomes and KGN as a pretreatment. Finally, chondrocytes in the control group were treated with PBS as a blank control. CCK-8 reagent was subsequently used to detect cell proliferation in each group for 7 consecutive days.

2.7. The Effect of Exosomes on the Phenotype of Chondrocytes. Chondrocytes from passage 3 were selected and divided into the same three groups as described above and cultured at a 37°C temperature for 14 days. Chondrocytes were then collected from each of the three groups. We then determined the relative expression levels of Sox-9, Aggrecan, Col-II, PPARγ, and Runx-2, by Western blotting and qRT-PCR.

2.8. The Establishment of the Rabbit Articular Cartilage Injury Model. Forty-eight healthy New Zealand white rabbits (aged 5–6 months and weighing between 2 and 2.5 kg) were used for the in vivo study. All animals were treated with care at all times, and all experimental procedures were approved by the ethics committee and carried out in strict accordance with the ethical rules governing animal experimentation. For consistency, the right knee was selected as the experimental surgical site in order to create a rabbit model of knee cartilage injury. The 48 rabbits were divided into 4 groups at random. The control group received an intra-articular injection of 0.5 ml PBS, the IPFP-MSC group received an intra-articular injection of cell suspension containing 1×10^7 IPFP-MSCs, the EXO group received an intra-articular injection of suspension containing 1×10^{10} Exos, and the KGN-EXO group received an intra-articular injection of suspension containing 1×10^{10} KGN-Exos. The rabbit model of knee cartilage injury was created as follows. First, all rabbits were anesthetized by slowly injecting sodium pentobarbital into the ear vein. Then, penicillin was slowly administered to prevent infection. Rabbits were then placed in a supine position and a medial parapatellar approach was used to open the joint capsule. The patella was then pulled laterally to expose the femoral trochlea. A cartilage defect (4 mm in diameter and 1.5 mm in depth) was then drilled into the center of the femoral trochlea using a sterile electric drill. Thereafter, penicillin sodium was injected daily into the gluteus maximus to prevent infection for the first 3 days after surgery. During this time, rabbits were not restricted and were allowed to be active. Six experimental animals from each group were sacrificed at 4 and 12 weeks after surgery for analysis.

2.9. Macroscopic Evaluation. Rabbits were sacrificed by an intravenous injection of sodium pentobarbital. The surgical site was then exposed and harvested. The cartilage defect sites were then photographed and evaluated in a blinded manner in accordance with the International Cartilage Repair Society (ICRS) scoring system (Table 1). Scoring was carried out independently by three investigators.

2.10. Histological Evaluation. Specimens were fixed in 4% paraformaldehyde for 36 hours and then decalcified with 20% EDTA solution at room temperature for 4–6 weeks. Samples were then measured by needle punching every 2 weeks until the needle could be easily inserted into the bone tissue, thus indicating that decalcification was complete. The samples were then dehydrated with a gradient series of alcohols, embedded in paraffin, and sectioned to create histological sections that were 4 μm thick. Sections were then stained with HE and Safranin O/Fast Green. In order to achieve consistent and objective results, the sections were then evaluated using the modified O'Driscoll histological score (Table 2) [21].

3. Results

3.1. Characterization of IPFP-MSCs. Primary IPFP-MSCs were extracted using the method described above and evaluated by microscopy each day thereafter. After 24 hours, we observed a small amount of adherent cellular growth. After 2 weeks, the cells had reached 80% confluency. The cells were then passaged at a ratio of 1 : 3; this allowed the MSCs to proliferate rapidly after subculture, showing a fusiform fibroblast-like appearance (Figure 1(a)). Since cells aged after multiple passages, we selected cells from passage 3 (P3) for experimentation. Flow cytometry results showed that 99% of cells expressed CD73, CD90, and CD105, while <1% of cells expressed CD34 and CD45 (Figure 1(b)). These results indicated that the extracted MSCs were consistent with previous publication standards [22]. Three-line differentiation experiments were then carried out and alizarin red staining was performed 4 weeks after osteogenic induction culture. Microscopic observation revealed the presence of scattered

Table 1: International Cartilage Repair Society macroscopic evaluation of cartilage repair.

Categories	Score
Degree of defect repair	
In level with surrounding cartilage	4
75% repair of defect depth	3
50% repair of defect depth	2
25% repair of defect depth	1
No repair of defect depth	0
Integration to border zone	
Complete integration with surrounding cartilage	4
Demarcation border < 1 mm	3
Three-quarters of graft integrated, one-quarter with a notable border > 1 mm in width	2
One-half of graft integrated with surrounding cartilage, one-half with a notable border > 1 mm	1
From no contact to one-quarter of graft integrated with surrounding cartilage	0
Macroscopic appearance	
Intact smooth surface	4
Fibrillated surface	3
Small, scattered fissures or cracks	2
Several small or few large fissures	1
Total degeneration of grafted area	0
Overall repair assessment	
Grade I: normal	12
Grade II: nearly normal	8–11
Grade III: abnormal	4–7
Grade IV: severely abnormal	0–3

calcium nodules and calcified matrix. After 4 weeks of adipogenic induction culture, oil red O staining was performed; this showed that lipid droplets had formed and fused into a sheet. After 4 weeks of cartilage-induced culture, we observed the formation of cartilage pellets. Following alcian blue staining, we were able to visualize the cartilage matrix around the cells and a large amount of mucopolysaccharide (Figure 1(c)). Collectively, these results indicated that the extracted cells expressed surface proteins that were specific to MSCs and exhibited the potential to differentiate in multiple ways. Consequently, these cells were proved to be MSCs derived from IPFP (IPFP-MSCs).

3.2. Characterization of MSC-EXOs. Exosomes were isolated by collecting and ultracentrifuging the supernatant collected during the culture of MSCs. Transmission electron microscopy revealed that these exosomes were flat and disc shaped with a double-sided concave structure. The diameter of these cells was 40–120 nm (Figure 2(a)), thus concurring with the expected shape characteristics of exosomes. Western blotting showed that these exosomes were positive for the exosome-specific surface proteins CD9 and TSG101 (Figure 2(b)). NTA further showed that the size of the particles within the precipitate were predominantly 40–100 nm in diameter (Figure 2(c)). These results indicate that the exosomes we isolated exhibited the characteristics of exosomes and could be used for subsequent experiments.

3.3. Exosomes Promoted the Proliferation of Chondrocytes. CCK-8 assays showed that the proliferation of chondrocytes in the Exo group and the KGN-Exo group increased significantly compared with the control group. There were statistical differences between the KGN-EXO group and the control group ($P < 0.01$) and also between the EXO group and the control group. There was no statistical difference between the KGN-EXO group and the EXO group ($P > 0.05$) (Figure 3). These results suggested that exosomes enhanced the proliferation of chondrocytes with or without KGN pretreatment.

3.4. Exosomes Induced Phenotypic Changes in Chondrocytes In Vitro. In order to verify whether exosomes could influence the expression of intracellular proteins and genes associated with cartilage, we conducted several *in vitro* experiments. Western blotting showed that the expression levels of Sox-9, Aggrecan, and Col II increased significantly after treatment with EXO and KGN-EXO when compared with the control group. Moreover, the KGN-EXO treatment was more effective than the EXO treatment alone ($P < 0.05$, Figure 4). qRT-PCR showed that the expression levels of *Sox-9*, *Aggrecan*, and *Col II* were significantly increased after treatment with KGN-EXO and EXO (Figure 5). For all three genes, there were statistical differences between the KGN-EXO group and the control group ($P < 0.05$) and statistical differences between the KGN-EXO group and the EXO group

Table 2: The modified O'Driscoll histologic score.

Characteristic		Score
% hyaline cartilage	80–100	8
	60–80	6
	40-60	4
	20-40	2
	0-20	0
Structural characteristics		
Surface irregularity	Smooth and intact	2
	Fissures	1
	Severe disruption, fibrillation	0
Structural integrity	Normal	2
	Slight disruption, including cysts	1
	Severe lack of integration	0
Thickness	100% of normal adjacent cartilage	2
	50% to 100% or thicker than normal	1
	0–50%	0
Bonding to adjacent cartilage	Bonded at both ends of graft	2
	Bonded at one end/partially both ends	1
	Not bonded	0
Freedom from cellular changes of degeneration	Normal cellularity, no clusters	2
	Slight hypocellularity, <25% chondrocyte clusters	1
	Moderate hypocellularity, >25% clusters	0
Freedom from degenerate changes in adjacent cartilage	Normal cellularity, no clusters, normal staining	3
	Normal cellularity, mild clusters, moderate staining	2
	Mild or moderate hypocellularity, slight staining	1
	Severe hypocellularity, slight staining	0
Reconstitution of subchondral bone	Complete reconstitution	2
	Greater than 50% reconstruction	1
	50% or less reconstruction	0
Bonding of repair cartilage to de novo subchondral bone	Complete and uninterrupted	2
	<100% but >50% reconstruction	1
	<50% complete	0
Safranin O staining	>80% homogeneous positive stain	2
	40%–80% homogeneous positive stain	1
	<40% homogeneous positive stain	0
	Total score	Max 27

($P < 0.05$). Although the expression levels of the three genes were increased in the EXO group compared with the control group, there was no statistical difference between these two groups ($P > 0.05$). qRT-PCR found no significant difference in the gene expression of *PPARγ* and *Runx-2* when compared with that across the three groups. Western blotting also showed that the expression levels of PPARγ and Runx-2 proteins were similar across the three groups. Collectively, these results showed that KGN-EXO and EXO did not improve the expression of genes or proteins related to osteogenesis or adipogenesis.

3.5. Macroscopic and Histological Evaluation. Four weeks after surgery, there was almost no repair tissue in either the control group or the IPFP-MSC group; the boundary with the surrounding normal cartilage tissue was obvious. The defect area was extremely uneven and no new cartilage had been formed. A small number of cartilage-like structures had formed in the defect area of the EXO group; this formed a connection with the surrounding normal articular cartilage tissue and gathered towards the center. More cartilage tissue had formed in the KGN-EXO group; the surface was relatively flat and was well connected with the surrounding normal articular cartilage thus showing good levels of repair (Figure 6(a)). H&E staining indicated that almost no cartilage-like structures had formed in the control group and the IPFP-MSC group. Only a small amount of cartilage-like tissue had formed at the bottom of the defect

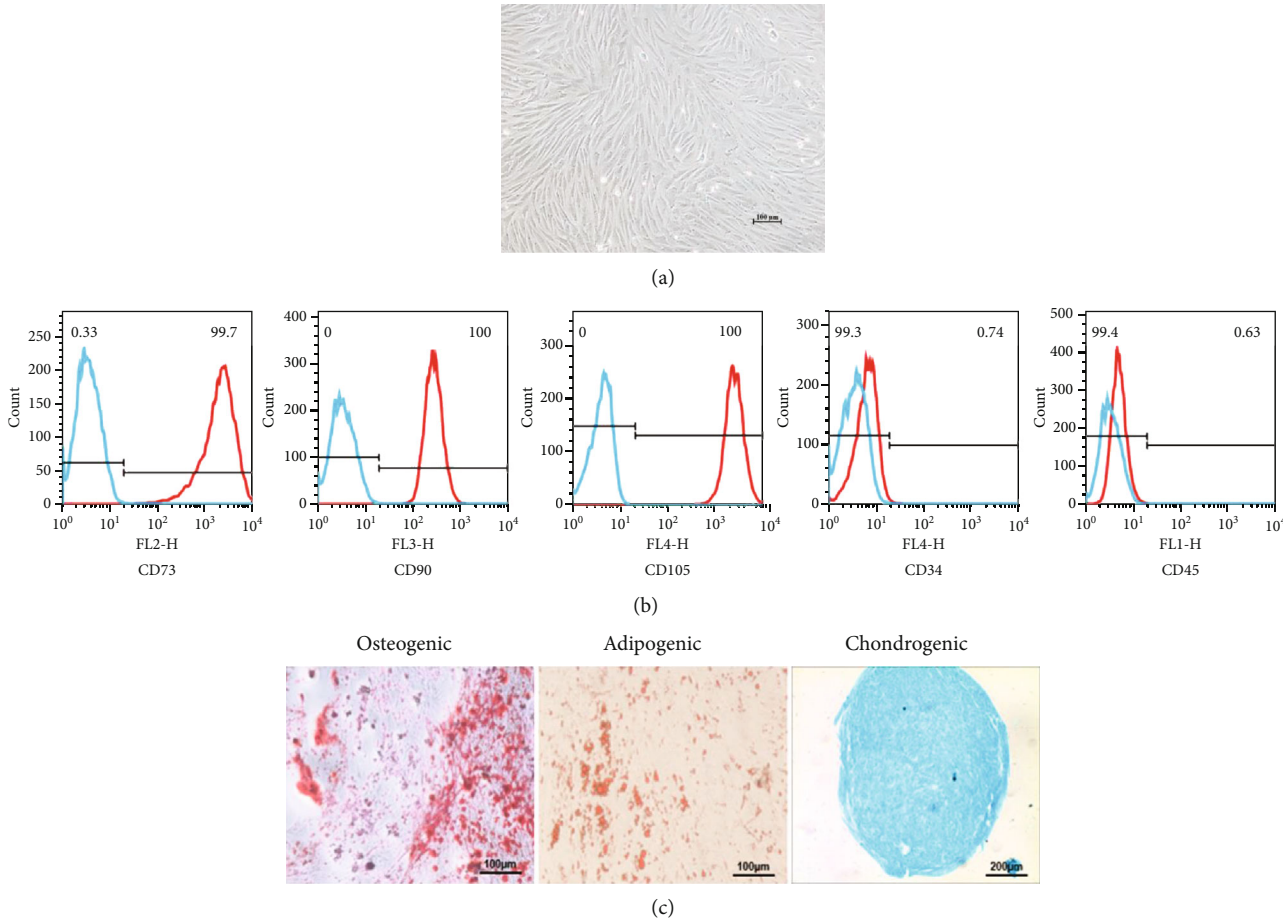

Figure 1: Isolation and identification of IPFP-MSC. (a) The P3 generation IPFP-MSC. (b) The results of flow cytometry. Blue line: negative control; red line: IPFP-MSCs. (c) Three-line differentiation experiments.

site in the EXO group; the thickness of the regenerated tissue was less than 50% than that of the normal cartilage in the surrounding area. Hyaline cartilage had formed in the KGN-EXO group; the thickness of the regenerated tissue was significantly greater than that of the other three groups. S&F staining showed that only the regenerated tissues in the KGN-EXO group showed strong, positive, and uniform Safranin O staining, thus suggesting that the proteoglycan content in the regenerated tissue of the KGN-EXO group was similar to that of normal cartilage (Figure 6(a)).

Twelve weeks after surgery, the control group showed almost no regeneration of cartilage tissue and the defect area was clearly evident. The surface was uneven with poor levels of integration with the surrounding normal articular cartilage tissue. The defect area in the IPFP-MSC group was predominantly filled with fibrous connective tissue; a small amount of cartilage tissue had formed at the edge of the defect, and the surface was uneven. The repaired tissue was partially connected with the surrounding normal articular cartilage tissue. In the EXO groups, the defect area was filled by a large area of regenerated cartilage-like tissue. The defect site had been filled with small cracks evident in the repaired tissue. The surface of this regenerated tissue was relatively flat and the repaired tissue had integrated with the surrounding normal articular cartilage, although the boundary was still obvious. The surface of the defect site in the KGN-EXO group was smooth and flat and was almost covered by regenerated cartilage tissue. Very few cracks were evident in the repaired tissue, which showed good integration with the surrounding normal cartilage; it was difficult to determine the boundary (Figure 6(b)). The ICRS scores for the KGN-EXO group (9.94 ± 0.87) were significantly higher than those for the other three groups ($P < 0.01$; Figure 6(c)). The ICRS scores for the EXO group (6.56 ± 1.10) were significantly higher than those for the control group (1.33 ± 0.84) and the IPFP-MSC group (3.00 ± 0.69) ($P < 0.01$). The ICRS scores for the IPFP-MSC group were higher than those for the control group, although the difference was not statistically significant ($P > 0.05$). These results suggested that the KGN-EXO treatment had the strongest ability to repair cartilage defects *in vivo* and was significantly better than any of the other three groups. H&E staining showed that a small amount of nonchondroid tissue had formed in the control group. There were only very minimal amounts of hyaline cartilage structure in the repaired tissue and the Safranin O staining was not significantly positive. A large number of cracks were observed in the regenerated tissue which showed poor integration with the surrounding normal cartilage. In

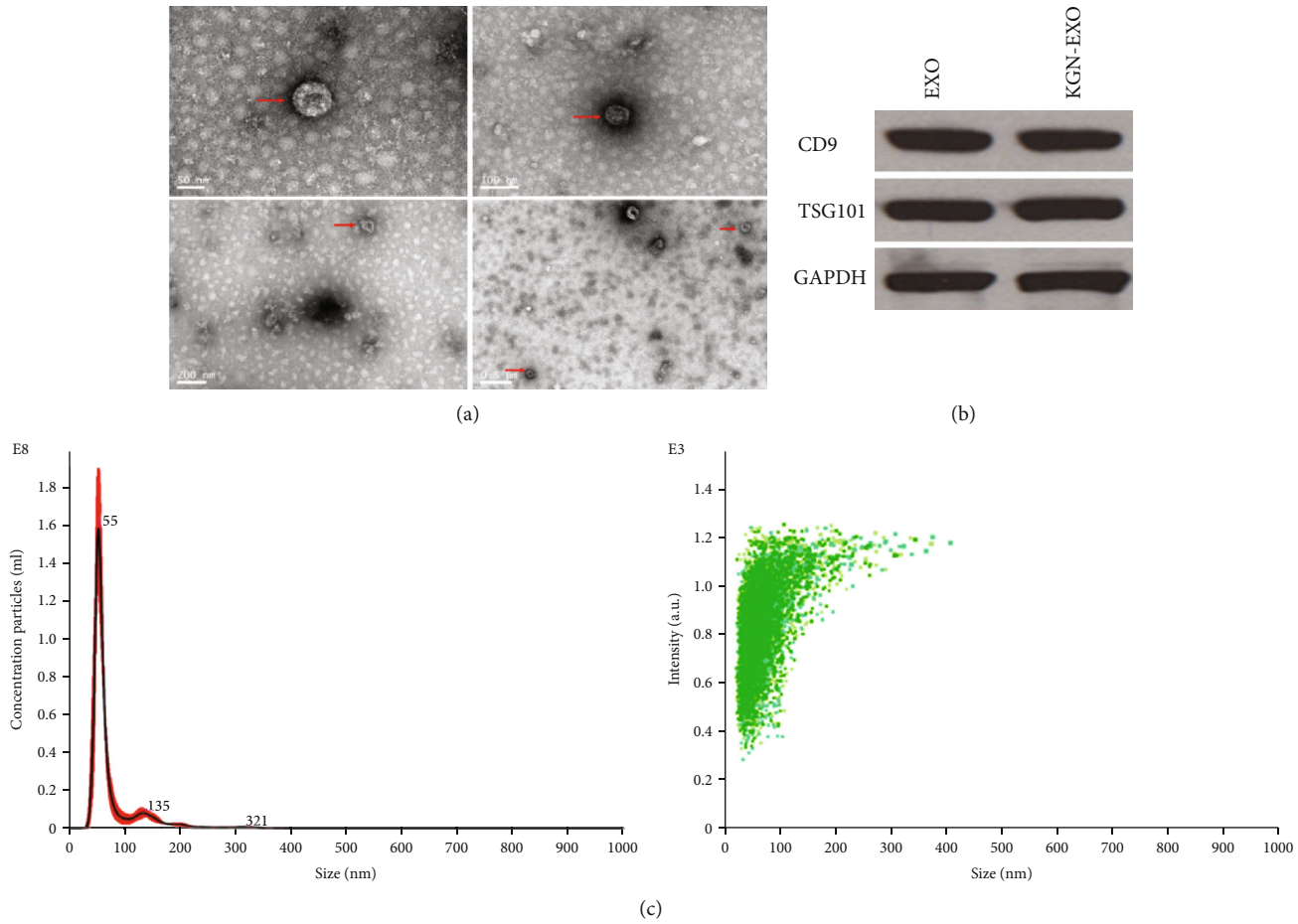

Figure 2: Isolation and identification of exosomes. (a) TEM results. The red arrow indicates exosomes. (b) Identification of exosome surface proteins. Western blotting showed that these exosomes were positive for the exosome-specific surface proteins CD9 and TSG101. (c) NTA test results. The size of the particles within the precipitate were predominantly 40–100 nm in diameter. Red line: range of particle concentration at different diameter sizes; black line: average concentration of particles at different diameter sizes.

the IPFP-MSC group, we observed moderate tissue regeneration; the regeneration has occurred unevenly and the hyaline cartilage structure was thinner than that in the KGN-EXO group; many cracks were evident in this tissue. Safranin O staining was little and not fully integrated with the surrounding normal cartilage. More explant tissue regeneration was evident in the EXO group; more than 70% of the defect area had been filled. Hyaline cartilage tissue was visible inside the defect area and there were few cracks. Safranin O staining was very prominent, indicating that this tissue contained a significant proportion of proteoglycans. The regenerated tissue was well integrated with the surrounding normal cartilage, and a clear boundary was evident. In the KGN-EXO group, the defect site had been almost completely repaired; the surface was flat and smooth. We also observed a notable proportion of hyaline cartilage-like tissue with no obvious cracks. Safranin O staining showed uniform and strong positive staining, suggesting that this tissue contained a large proportion of proteoglycans. Furthermore, the repaired tissue was fully integrated with surrounding normal cartilage and it was difficult to visualize the boundaries. Histological scoring (Figure 6(c)) at 12 weeks after surgery showed that the histological scores of the KGN-EXO group (20.56 ± 1.91) were significantly higher than those of the other 3 groups ($P < 0.01$). The histological scores of the EXO group (15.44 ± 1.79) were also significantly higher than those of the control group (3.94 ± 1.43) and the IPFP-MSC group (6.89 ± 1.49) ($P < 0.01$). The score of the IPFP-MSC group was higher than that of the control group, but this was not statistically significant ($P > 0.05$). Collectively, these results showed that the exosomes derived from IPFP-MSCs that had been pretreated with KGN possessed a strong ability to promote the repair of cartilage defects *in vivo*.

4. Discussion

In this study, we used KGN to pretreat IPFP-MSCs and then successfully isolated exosomes from the supernatant of MSCs by ultracentrifugation. We then evaluated the characteristics of the isolated exosomes by transmission electron microscopy, surface protein identification, and NTA assays. *In vitro* experiments demonstrated that the exosomes derived from MSCs could significantly enhance the proliferation of chondrocytes, but KGN pretreating method could not increase

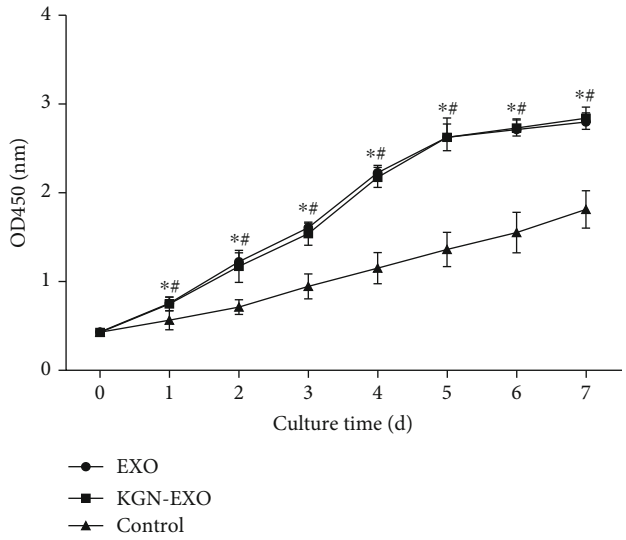

Figure 3: The proliferation of chondrocytes after treatment with exosomes. The proliferation of chondrocytes in the EXO group and the KGN-EXO group increased significantly compared with that in the control group. *Significant difference between the EXO group and the control group ($P < 0.05$). #Significant difference between the KGN-EXO group and the control group ($P < 0.05$). d: days.

the capacity of MSC exosomes to promote proliferation additionally. The MSC exosomes pretreated with KGN could significantly promote the expression of cartilage-associated proteins and genes compared with non-KGN-pretreated exosomes. In the *in vivo* experiments, better cartilage repair and a large amount of hyaline cartilage-like tissue regeneration in the defect site were found in the KGN-EXO treatment group compared with the other three groups. The surface of the repaired tissue was smooth, flat, and well integrated with the surrounding normal cartilage. No obvious boundary was observed, and Safranin O staining was strong and positive. Macroscopic evaluation and histological scoring also proved that the KGN-EXO group had the best overall efficacy with regard to the repair of cartilage defects in our rabbit models.

MSCs are commonly used for the regeneration of lesions in articular cartilage. Indeed, results derived from both basic science and clinical research have reported promising results using this technique [23]. The most commonly used MSCs in earlier researches were BM-MSCs (bone marrow mesenchymal stem cells), AD-MSCs (adipose-derived stem cells), SMSCs (synovial mesenchymal stem cells), and UC-MSCs (umbilical cord mesenchymal stem cells). The infrapatellar fat pad is a type of fat that is situated under and behind the patella bone within the knee and has traditionally been considered as a cushion to buffer forces in the joint. However, an increasing body of evidence now supports the fact that mesenchymal stem cells can be extracted from the infrapatellar fat pad and exhibit far better chondrogenic ability than other forms of MSCs [24]. Koh and Choi were the first to use infrapatellar fat pad-derived mesenchymal stem cell therapy to treat knee osteoarthritis; over the short-term, the results from this study were encouraging and demonstrated that the injection of IPFP-MSCs was safe and could reduce pain and improve knee function [25]. In another study, Dragoo and Chang used arthroscopic techniques to harvest the IPFP and successfully isolate adipose-derived MSCs, thus making it easier for the application of IPFP-MSCs in clinic. Neri et al. subsequently used *in vitro* experiments to demonstrate that IPFP-MSCs derived from patients with OA still met the criteria to be considered as MSCs and were suitable and safe for the regeneration of cartilage [26]. Initially, it was thought that cell replacement therapy would be the best protocol to apply MSCs to repair cartilage lesions. This concept was based on the ability of these cells to undergo chondrogenic differentiation and to secrete PGs and collagen II, which are the essential components of tissue [12, 27]. It also indicates that MSCs possess immunomodulatory properties that may help to reduce the loss of cartilage [28]. However, recent studies showed that the principal source of repair tissue is derived from endogenous cells following the intra-articular transplantation of MSCs, thus implying that paracrine effects may be predominantly responsible for the manner in which MSCs induce cartilage regeneration [27, 29].

The paracrine effects of MSCs predominantly include soluble factors and extracellular vesicles. In particular, exosomes released by MSCs have been shown to influence cartilage regeneration [13, 30, 31]. Given that there is still many limitations relating to the use of MSCs in clinics to treat cartilage lesions, such as ethical issues and policy limitations, it is quite necessary and important that we continue to seek a cell-free treatment to promote cartilage repair, which could be easier to translate for clinical application [11]. Therefore, exosomes represent a promising MSC-based cell-free method to induce cartilage regeneration. In a previous study, Wang et al. reported that secretory factors from UC-MSCs could regulate the differentiation of MSCs [32]. In a subsequent paper, Huang et al. proposed that exosomes derived from MSCs could represent an alternative treatment for cartilage repair in the form of a cell-based tissue engineering strategy [33]. Zhang et al. subsequently demonstrated that exosomes from ESCs could promote osteochondral regeneration [30] while Cosenza et al. showed that exosomes from BM-MSCs could protect the cartilage and bone from degradation in OA [34]. A subsequent study by Tao et al. found that exosomes from SMSCs had significant potential to prevent the progression of OA and that the efficacy of this technique could be significantly enhanced by the overexpression of miR-140-5p in SMSCs [35]. In another paper, Wu et al. suggested that miR-100-5p-abundant IPFP-MSC-EXOs could protect articular cartilage by inhibiting mTOR in OA [36]. Qi et al. proved that exosomes from BMSCs could inhibit mitochondrial dysfunction-induced apoptosis in chondrocytes via p38, ERK, and Akt pathways [37]. Thus, many studies have demonstrated the advantages of MSC-EXOs for cartilage repair and the prevention of OA. However, the components and function of exosomes are quite susceptible for variability and can differ when extracted from different cell types or the same cell types under different conditions [13, 29]. This inconsistency makes it difficult to consider the

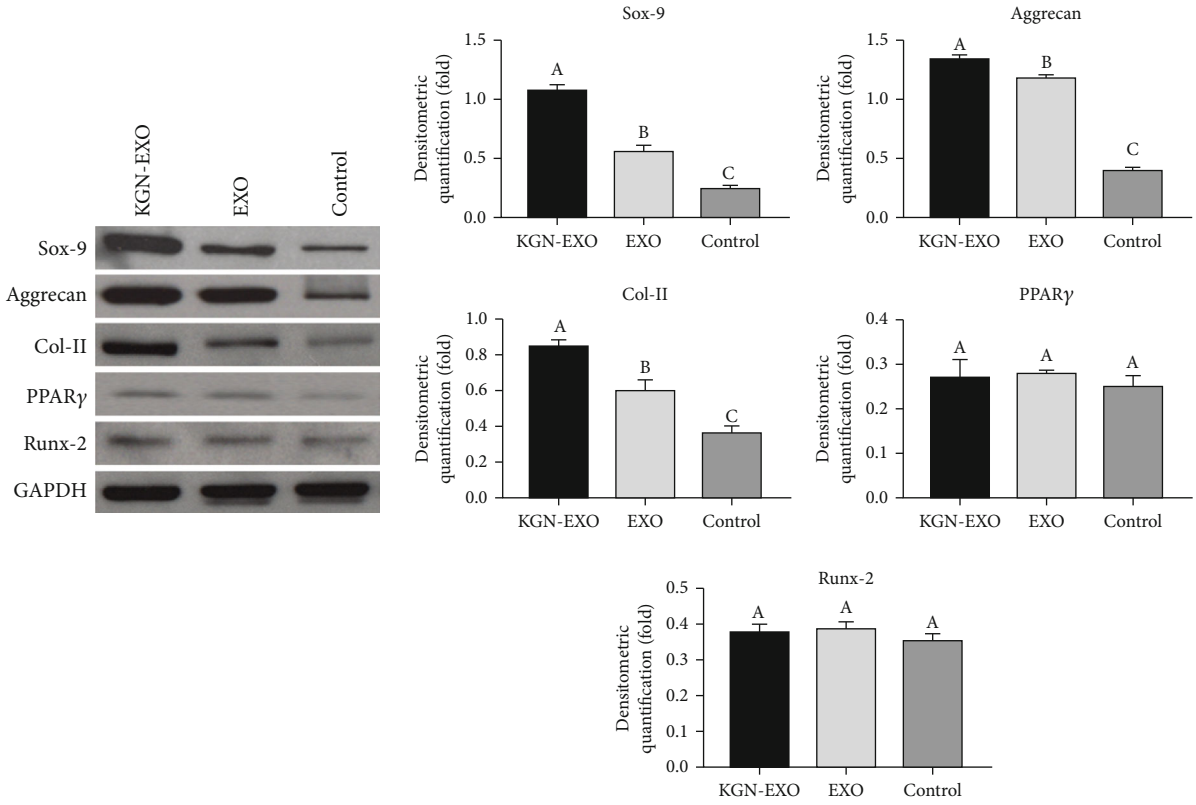

Figure 4: The Western blotting results showed that the expression levels of Sox-9, Aggrecan, and Col II, increased significantly after treatment with EXO and KGN-EXO when compared with those in the control group. Moreover, the KGN-EXO treatment was more effective than the EXO treatment alone ($P < 0.05$). Note that different letters above bars indicate significant differences ($P < 0.05$) while matched letter means no significant difference in each comparison among the KGN-EXO/EXO/control groups.

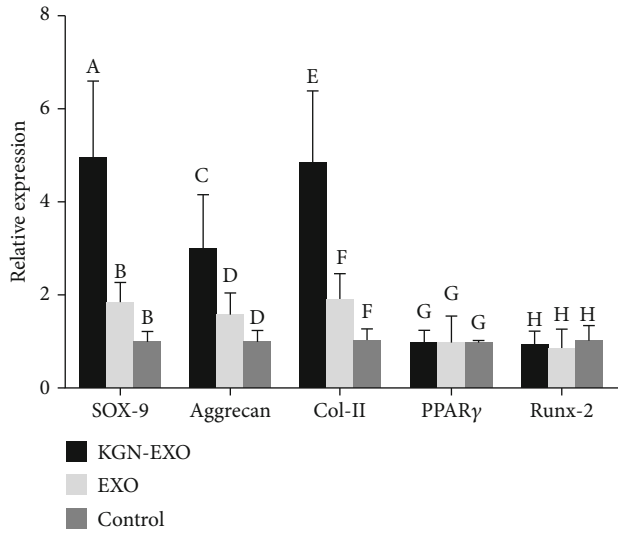

Figure 5: qRT-PCR results showed that the expression levels of Sox-9, Aggrecan, and Col II were significantly increased after treatment with KGN-EXO and EXO. For all three genes, there were statistical differences between the KGN-EXO group and the control group ($P < 0.05$) and statistical differences between the KGN-EXO group and the EXO group ($P < 0.05$). Note that different letters above bars indicate significant differences ($P < 0.05$) while matched letters mean no significant difference in each comparison among the KGN-EXO/EXO/control groups.

results arising from previous studies, but also gives us a chance to pretreat the original cells to enhance the subsequent function of the exosomes. For example, Kato et al. used IL-1 to stimulate synovial fibroblasts and found that exosomes isolated from IL-1-stimulated synovial fibroblasts could induce more osteoarthritic changes in articular chondrocytes than those without IL-1 stimulation, thus proving that the function of exosomes can be regulated by pretreatment [38]. KGN is a small heterocyclic compound that exhibits a strong ability to induce MSCs to differentiate into chondrocytes [18, 39]. In the present study, we used KGN-pretreated IPFP-MSCs to investigate whether this action influenced the ability of the exosomes to induce the differentiation of chondrocytes. We found that exosomes from IPFP-MSCs could significantly promote the proliferation of chondrocytes, thus concurring with previous reports [40]. No significant change was observed when IPFP-MSCs were pretreated with KGN or not. We also found that exosomes derived from IPFP-MSCs could enhance the anabolic effects of chondrocytes and reduce catabolic effects by increasing the expression of SOX-9, Aggrecan, and COL-2 and by reducing the expression of MMPs, which also concurred with previous studies [34, 40, 41]. Furthermore, exosomes derived from KGN-pretreated IPFP-MSCs could significantly enhance anabolic effects and reduce catabolic effects when compared with exosomes derived from IPFP-MSCs without

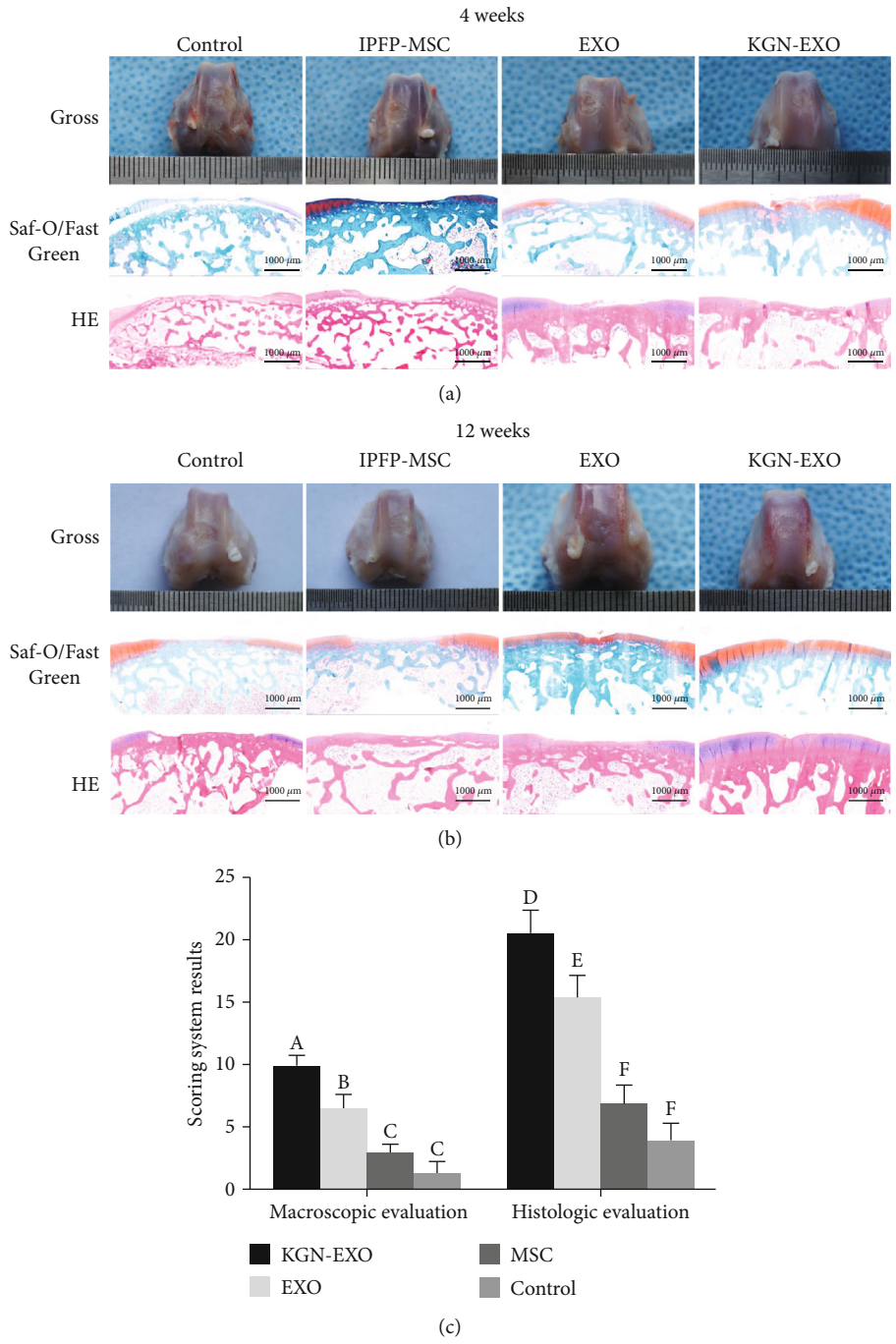

Figure 6: In vivo cartilage repair at 4 and 12 weeks after surgery. (a) The gross appearance, Saf-O/Fast Green and HE staining at 4 weeks. (b) The gross appearance, Saf-O/Fast Green and HE staining at 12 weeks. (c) The ICRS and histological scores at 12 weeks.

pretreatment. This implies that exosomes derived from KGN-pretreated MSCs may be more beneficial for cartilage repair. We also performed *in vivo* experiments to further verify our hypothesis.

There are some limitations associated with this study that need to be considered. We observed that KGN pretreatment could enhance the function of MSC-EXO for cartilage regeneration, but we were not able to elucidate the specific mechanisms responsible for this effect. Future studies would be needed to identify such mechanisms. The results of previous studies imply that microRNA changes may be the most likely mechanism [35, 36, 42]. Furthermore, we directly injected MSC-EXO without a carrier. Future studies will need to identify a reliable carrier for EXO in order to promote their sustained release, although exosome could be a promising cell-free therapy used in clinical practice without many policy limitations like cell therapy. However, exosome-related research still mainly stay in the laboratory. It should not be used in clinical practice until the safety and efficacy of exosome is verified in clinical trials. Also, it should not be ignored that the cost of exosome therapy

is even more than the cost of cell therapy, which could be the potential limitation to translate the exosome method into clinical practice.

5. Conclusion

Our study demonstrated, for the first time, that exosomes isolated from infrapatellar fat pad mesenchymal stem cells can be pretreated with KGN to induce stronger chondrogenic capability. These exosomes effectively promoted the proliferation and expression of chondrogenic proteins and genes in chondrocytes. These exosomes were also able to promote the repair of articular cartilage defects in a very effective manner. We propose that these exosomes can be used as a potential therapeutic method in the future.

Data Availability

All data is available from the corresponding author YZ (drzhouyiqin@163.com) when requested.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Authors' Contributions

Jiahua Shao, Jun Zhu, and Yi Chen contributed equally to this work.

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Review Article

Clinical Application of Human Induced Pluripotent Stem Cell-Derived Organoids as an Alternative to Organ Transplantation

Gabriella Shih Ping Hsia,[1] **Joyce Esposito**,[1] **Letícia Alves da Rocha**,[1,2] **Sofia Lígia Guimarães Ramos**,[1] **and Oswaldo Keith Okamoto**[1,3]

[1]*Human Genome and Stem Cell Research Center, Departamento de Genética e Biologia Evolutiva, Instituto de Biociências, Universidade de São Paulo, SP, Brazil*
[2]*Genetics Unit, Instituto da Criança, Hospital das Clínicas, Faculdade de Medicina, Universidade de São Paulo, SP, Brazil*
[3]*Hemotherapy and Cell Therapy Department, Hospital Israelita Albert Einstein, São Paulo, SP, Brazil*

Correspondence should be addressed to Oswaldo Keith Okamoto; keith.okamoto@usp.br

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Transplantation is essential and crucial for individuals suffering from end-stage organ failure diseases. However, there are still many challenges regarding these procedures, such as high rates of organ rejection, shortage of organ donors, and long waiting lines. Thus, investments and efforts to develop laboratory-grown organs have increased over the past years, and with the recent progress in regenerative medicine, growing organs *in vitro* might be a reality within the next decades. One of the many different strategies to address this issue relies on organoid technology, a miniaturized and simplified version of an organ. Here, we address recent progress on organoid research, focusing on transplantation of intestine, retina, kidney, liver, pancreas, brain, lung, and heart organoids. Also, we discuss the main outcomes after organoid transplantation, common challenges faced by these promising regenerative medicine approaches, and future perspectives on the field.

1. Introduction

Organ transplantation is still an important and necessary procedure that increases overall survival of many patients with organ failure diseases. It has been largely reported that organ transplantation improves the quality of life (QoL) of these patients. For instance, kidney transplantation provides more benefits and a better QoL for patients compared to hemodialysis [1–7]. Even though medicine and technology have advanced greatly over the past years, organ transplantation still faces many issues: ethical and religious concerns (since many organs are derived from brain-dead or non-heart-beating donors); organ trafficking; elevated risk of organ rejection, the possibility of health complications for living donors and receptors posttransplantation; the necessity of additional tests before transplantation; continuous use of immunosuppressive drugs/medications; and psychological impacts [8, 9]. Even when most conditions are favorable for transplantation, the number of available donors usually does not cover the number of patients in need of a donation. For instance, in the United States, a survey conducted in 2013 revealed that more than 116,000 patients were on the waiting list for transplantation, but only 28,000 underwent the procedure [10–15].

Some of these issues are the reason for decreased patients' QoL posttransplantation [16–19]. Thus, there has been an urgent need for new strategies for tissue repair and organ replacement. Over the past years, the development of laboratory-grown organs has been the focus of many types of research.

In 2006, a big step towards this goal was made by Yamanaka and collaborators [20] with the advent of induced pluripotent stem cells (iPSCs), opening many new possibilities for the emergence of other technologies, such as 3D bioprinting and organoid development, making the production of organ-like structures in the laboratory a close reality.

Here, we discuss how these novel technologies have evolved towards organoid development, new insights in the transplantation of different types of organoids, its outcomes, and challenges.

2. Manipulating Cell Identity: The Foundation

Cell manipulation is an essential tool to provide efficient and reliable biological information, allowing the study of various human diseases through a system that mimics *in vivo* physiological conditions [21, 22]. The first attempt of cell manipulation dates back to 1907, when Ross Harrison not only developed an innovative *in vitro* method, isolating frog embryo nerve fibers, but also maintained them successfully in culture [23]. Later, in 1955, King and Briggs developed a method to transfer the nuclei of embryonic cells into enucleated frog eggs [24]. In 1962, Gurdon demonstrated that cell specialization is a reversible process; the immature cell nucleus of a frog egg cell was replaced by a mature intestinal cell nucleus, generating a zygote-like cell that successfully developed into a normal tadpole [25]. In 1981, Evans and Kaufman obtained embryonic stem cells (ESCs) from mouse embryos [26], and in 1995, Thomson et al. isolated the first ESCs from primates [27].

These achievements contributed to the development of methods to derive and cultivate ESCs from human embryos, which started in 1998 [28] and continues until nowadays, leading to major breakthroughs, such as the discovery of Yamanaka and colleagues in 2006 on how to reprogram somatic cells into pluripotent stem cells (PSCs) [20]. The authors discovered that the ectopic expression of four defined factors, *Oct3-4*, *c-Myc*, *Sox2*, and *Klf4*, was necessary and sufficient to reprogram human adult cells into a pluripotent state, producing iPSCs [20]. This revolutionary technology opened a myriad of possible applications impacting personalized medicine, drug screening, and human disease modeling, without ethical hurdles imposed by therapeutic cloning and the use of human embryos. Furthermore, due to the possibility of generating patient-specific cells from iPSCs, this discovery also brought a possible solution to circumvent immune rejection, one of the main complications in transplantation.

3. Organoids: Why Use a Tridimensional System?

Many clinically oriented cell therapy studies have reported controversial results about therapeutic evidence and adverse events [29, 30]. Most early studies rely on two-dimensional cultures, which fail to replicate biological interactions among cells and between cells and the extracellular matrix (ECM), which occur in native tissues [31]. Conversely, tridimensional (3D) cell culture systems can mimic *in vivo* conditions involving cell-cell and cell-matrix interactions, such as dynamic regulation of signaling pathways and paracrine signals. Some examples of 3D culture systems include spheroids, tissue engineering constructs, and organoids [32].

Organoids are arranged structures, typically originated from stem cells, composed of multiple cell types that self-organize in culture, partly recreating tissue native architecture, morphology, and several biological interactions occurring *in vivo* [33, 34]. Although this research field has developed a lot in the last decade, especially after the iPSC development, organoid research dates back to the beginning of the 20th century. In 1910, Wilson demonstrated that disassociated adult cells contain enough information to reaggregate and self-reorganize into a specific multicellular structure resembling the original organ, without extracellular influence [35].

Organoid formation depends on the recapitulation of self-patterning, morphogenetic, and architectural rearrangements through manipulation of physical properties of the culture environment; endogenous and exogenous signals; and starting cell type culture with appropriate conditions [36]. During human embryonic development, there is a highly and tightly orchestrated differentiation process from zygote to self-organization of cells. In order to reproduce this process *in vitro*, iPSCs are induced to differentiate in specific lineages to form tissue-specific organoids with 3D biochemical cues [31].

Several parameters are controlled to stimulate self-renewal, differentiation, and self-organization [31]. The chosen organoid derivation method depends mainly on organoid type, on the required tissue differentiation, and on what is the ultimate practical application.

Organoids can be produced by self-assembly, when suspended cells self-organize in culture by cell aggregation through endogenous signals. Other strategies include starting induction with exogenous signals and then allowing self-organization of cells or providing exogenous factors continuously [36]. Differentiated stem cells can be seeded along with other cell types, such as endothelial and mesenchymal cells that, in combination, may form a 3D structure. In 2015, Takebe et al. published a generalized method for organ bud production from different types of tissues, in which mesenchymal stem cells (MSCs) were included into constructs. MSC-driven contraction was essential for organoids self-condensation, which could be reproduced for many cell types, such as liver, lung, heart, brain, and intestine cells [37]. In fact, the mesenchymal niche seems important for organoid engraftment and maturation after transplantation [38].

One important component of the organoid system is the ECM, which must support cell proliferation and enable cell adherence, diffusion of nutrients, and growth factors [39]. Stem cells must be in strict contact with ECM components, such as laminin, collagen, and fibronectin, important regulators of stem cell behavior, migration, and differentiation, especially through interaction with integrin receptors [40]. Matrigel, derived from murine cancer cell secretome [41], is widely used as a source of ECM for organoid manufacturing. However, there is a lot to lot variation, which brings an additional difficulty in standardizing culture conditions, and it may also trigger immunologic reactions. Some alternatives to delivery vehicles for organoid transplantation are being proposed, such as four-arm poly(ethylene glycol) (PEG) [42, 43] and Poloxamer 407, a triblock copolymer consisting of a central hydrophobic block of polypropylene glycol flanked by two hydrophilic blocks of PEG [44]. Single-cell genomics and clonal genome editing have made it possible

to better understand cell behavior, cell-cell interactions, cell migration, and tissue organization, contributing to the generation of new ECM components compatible with organoid systems [45].

The immediate application for organoid technology is disease modeling and drug screening. The ultimate goal, given the promising application of organoids in regenerative medicine, is to perform transplantation of tissue-specific organoids to recover or improve tissue function. In this regard, some initial studies have been evaluating organoid transplantability. Transplants are being tested in mouse models, in which tissue engraftment, biocompatibility, and functionality are evaluated. Here, we review the main published works in this area, highlighting the main outcomes of intestinal, retinal, kidney, liver, pancreas, lung, brain, and heart organoid transplantation (Tables 1 and 2).

4. Transplantation of Organoids

4.1. Intestinal Organoids. In 2009, Clever and colleagues employed for the first time the concept of organoids when they noticed the proliferation and self-organization capacity of adult intestinal stem cells *in vitro* to form genomically stable 3D structures [73]. Ever since, there has been an increased investment in intestinal organoid production and optimization of culture condition differentiation and self-organization and many efforts to enable its transplantability, as numerous diseases, such as short bowel syndrome, Crohn's disease, and genetic intestinal diseases, can be treated by intestinal transplantation. However, there are still considerable issues, such as graft rejection, surgical complications, and risk of infection [74], revealing the need to create new strategies for intestinal organ replacement.

Many studies have attempted to evaluate the transplantability of intestinal organoids derived from adult or fetal mouse/rat intestinal cells [46, 47] or differentiated cells from PSCs [38, 49–51]. Intestinal epithelial organoids derived from mouse or rat adult intestine were orthotopically transplanted and showed successful engraftment and presence of enterocytes, enteroendocrine cells, Paneth cells, and goblet cells and reepithelization of damaged ileal mucosa [46]. Organoids derived from enhanced green fluorescent protein ($EGFP^+$) mice, which were administered to immunocompromised mice with induced acute colitis, proved to be successful as it formed invaginated linings, cystic structures, and interacted with the mouse epithelium. Also, $EGFP^+$ organoid transplantation regenerated colonic injured epithelium, improved body weight, and was capable of recovering the epithelial barrier function [47]. In this same study, it was demonstrated that $EGFP^+$ mouse crypt cell organoids, derived from a single leucine-rich repeat-containing G-protein coupled receptor 5 ($Lgr5^+$) stem cell, could engraft into mouse colon and remain with proliferative and cell differentiation capacity [47].

One of the first works reporting a functional human intestinal organoid transplantation using PSCs was done by Watson and collaborators in 2014. An intestinal organoid transplanted under the kidney capsule showed great engraftment and maturation, increasing in size and volume, and considerable vascularization. In addition, they reported an increased villus height, smooth muscle layer thickness, and crypt fission and depth, due to the release of humoral factors after ileocecal resection [38], hence proving that intestinal organoids respond to humoral factors released by the host and epithelium was capable of peptide uptake and presented an intestinal barrier. In 2015, using the same methodology for organoid production as Watson, Finkbeiner et al. performed a transcriptome-wide unbiased analysis of intestinal organoids, demonstrating successful engraftment *in vivo* and high expression of maturation markers (presence of Paneth cells and expression of *OLFM4*). Also, organoids acquired intestine architecture with villi containing lamina propria and had mesenchymal cells similar to adults [48].

In 2017, using an alternative source of ECM, Cruz-Acuña and collaborators developed an intestinal organoid with four-arm PEG macromer, with maleimide groups at each terminus, which, after 12 weeks, showed organoid growth (10- to 40-fold larger than the initial organoids), crypt-villus architecture, and regeneration of colonic wound, similar to results observed when these organoids were cultivated with Matrigel™ [49]. Moreover, to track the fate of intestinal organoids after transplantation, engraftment was evaluated by promoter-reporter biosensor in the lumen of mouse small intestine, using $KLF5^{mCherry}$ or ISX^{eGFP} reporters that allow the monitoring of cell fate and differentiation *in vivo*. Results revealed fluorescent signals after three hours and as long as one week after transplantation, indicating successful organoid engraftment [50].

Most intestinal transplantation studies were performed using the kidney capsule as the transplantation site. However, mesentery transplantation of intestinal organoids represented a more physiologic strategy as it was observed 85% of engraftment into the host [51]. Also, a comparison between transplanted organoids after ten weeks and their *in vitro* counterpart revealed that organoid size and volume, as well as elements from epithelium, mesenchyme, and muscular layers, were larger. Histologically, organoids resemble human intestinal tissue, with specific cell lineages, subepithelial elements, and muscle, expressed intestinal maturation markers, and received vascular ingrowth from mesenteric vessels. This study was an important advance in this area as it created a model that may facilitate translational studies of intestinal organoid transplants [51].

Despite all the advances in the development of intestinal organoids, studies have mentioned that there are still limitations to overcome regarding intestinal organoid transplantation, such as (1) variation between intestinal organoid transplantation results from different rodents or species; (2) necessity to improve engraftment, intestine debridement, and organoid optimization; (3) difficulties to directly compare two models of transplantation (orthotopic versus ectopic); and (4) problems with functional significance of gene expression comparisons between distinct developmental stages.

4.2. Retinal Organoids. Retinal disorders (RD) are the main cause of vision loss and impairment, which are caused by loss/damage of photoreceptors. Over the years, many RD-

Table 1: Description of main studies performing organoid transplantation.

Organ	Ref.	Cell source	Receiver	Extracellular matrix	Time of evaluation after transplantation
Intestine	[46]	Rat/mouse neonatal small bowel	Adult male Lewis rat or wild-type mice	Extracellular matrix gel	2, 3, or 6 weeks
Intestine	[47]	$EGFP^+$ mouse crypt cells	Immunocompromised $Rag2^{-/-}$ mice	Matrigel-containing PBS	6 d, 16 d, 4 weeks
Intestine	[38]	Human ESCs or iPSCs	NSG IL2Rg-null mice	Type I collagen	6 weeks
Intestine	[48]	H9 human ESCs	NSG IL2Rg-null mice	Type I collagen	16 weeks
Intestine	[49]	Human ESCs or iPSCs	NSG IL2Rg-null mice	PEG-4MAL	12 weeks
Intestine	[50]	Human iPSCs	NSG IL2Rg-null mice	Matrigel	1 week, 3 h
Intestine	[51]	H1 ESC	NSG IL2Rg-null mice	Matrigel	10 weeks
Pancreas	[52]	hPSCs	Immune-deficient mice	Matrigel	5 weeks
Pancreas	[53]	hESC	Nude mice	Growth factor-reduced Matrigel	30 d, 60 d, 90 d
Pancreas	[54]	ICs and hAECs	Diabetic SCID mice	Agarose	1 month
Liver	[37]	hiPSC, HUVEC, MSC	NOD/SCID mice	Matrigel diluted with EGM	Multiple time points, ranging from 0 to 60 days
Liver	[44]	iPS-H and stromal cells	C57BL/6 mice	2D: Matrigel; 3D: Pluronic f127; and transplant: alginate to encapsulate	Twice a week, 3 d postoperation until day 24
Liver	[55]	hiPSC endoderm, EC and MSC	Alb-TRECK/SCID mice	Growth factor-reduced Matrigel diluted with SFD medium	Every 5 d until the 20th day
Retina	[56]	Wild-type E14TG2a mES	$Prom1^{-/-}$ and tg(Cpfl1;$Rho^{-/-}$) mice	Growth factor-reduced Matrigel	3 to 4 weeks
Retina	[57]	mESC (E16 CEE and Crx-GFP line)	Wild-type and $Aipl1^{-/-}$ mice	Growth factor-reduced Matrigel	3 weeks
Retina	[58]	hESC	SD-Foxn1 Tg(S334ter)3Lav	Growth factor-reduced Matrigel	54 to 300 d
Kidney	[59]	Single-cell suspensions derived from E11.5 CD1 mouse kidneys	Male athymic nude rats	—	3 and 6 weeks
Kidney	[60]	hESC and hPSC	NOD/SCID mice	Vitronectin-coated culture dishes	7 d and 28 d
Kidney	[61]	hPSC	CAM of 7-day-old chick embryos	Vitronectin-coated culture dishes	3 to 5 d
Kidney	[62]	E11.5 mouse embryonic kidneys	NOD/SCID mice	Atelocollagen membranes	7 d
Brain	[63]	hPSC	NOD/SCID mice	Matrigel	0.5–8 months
Brain	[64]	hESC or hiPSC (H9 hES cells, WAe009-A)	P8-P10 CD1 mice	Matrigel	In 2 and 4 weeks
Brain	[65]	hESCs	Sprague-Dawley rats	Matrigel	4 weeks
Brain	[66]	hESCs and hiPSCs	SCID mice	—	1–5 months
Heart	[67]	hESC coculture with hESC-MSC, CPC, and EC	Male nude mice (25–30 g, B6NU)	Matrigel	12.5 d, 4 weeks
Lung	[68]	hESCs	NSG mice	With or without PLG and/or Matrigel	4, 6, 8, 12, or 15 weeks
Lung	[69]	hESCs and iPSCs	NSG mice	Matrigel	1.5, 5, or 7 months
Lung	[70]	HBEpC, HMVEC-L, and HLF	NSG mice	Matrigel	1 or 6 weeks
Lung	[71]	$CD45^-$ $EPCAM^+\beta4^-$ AT2 cells	Influenza-infected mice	Matrigel	13 d
Lung	[72]	hESCs	NSG mice	PEG, PLG, and PCL	Between 1 and 8 weeks

Abbreviations: (h/m) ESC: human/mouse embryonic stem cells; $Aipl1^{-/-}$ mice: a model of end-stage retinal degeneration; AT2: alveolar type 2 cells; CAM: chick chorioallantoic membrane; Crx-GFP ESC lines: ESC lines of transgenic mouse line expressing GFP with control of endogenous photoreceptor-specific promoter Crx; d: day(s); EC: endothelial cells; h: hour(s); hAECs: human amniotic epithelial cells; HBEpC: human bronchial epithelial cells; HLF: human lung fibroblasts; HLO: hPSC-derived lung organoids; HMVEC-L: human microvascular lung endothelial cells; ICs: islet cells; iPS-H: human induced pluripotent stem cell-derived hepatocyte-like cells; iPSCs: induced pluripotent stem cells; MSCs: mesenchymal stem cells; NSG mice: nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice; PBS: phosphate-buffered saline; PCL: polycaprolactone; PEG: poly(ethylene glycol) hydrogel; PEG-4MAL: four-arm poly(ethylene glycol) (PEG) macromer with maleimide groups at each terminus; PLG: poly(lactide-co-glycolide) scaffolds; $Prom1^{-/-}$ mice: prominin1-deficient mice; PSC: pluripotent stem cells; SC: superior colliculus; SD-Foxn1 Tg(S334ter)3Lav: severe retinal degeneration immunodeficient nude rat; tg(Cpfl1;$Rho^{-/-}$) mice: cone photoreceptor function loss 1 (Cpfl1) crossed with rhodopsin knockout mice ($Rho^{-/-}$).

Table 2: Description of main studies performing organoid transplantation.

Organ	Ref.	Methods to evaluate engraftment, maturation, organoid behavior, and physiologic responses	Site of transplantation (orthotopic or ectopic)	Limitations
Intestine	[46]	Bile acid uptake; HS; IHC; GFP^+ mouse-derived organoids	Orthotopic—omentum	Variation between different rodents or species; improvement of engraftment and intestine debridement needed
Intestine	[47]	MV; TRITC-dextran analysis; $EGFP^+$ cells; IM; body weight	Orthotopic—colon	Optimization needed
Intestine	[38]	MV; HS; IM; qPCR; TEM; LGR5 reporter; permeability; peptide uptake	Ectopic—kidney capsule	—
Intestine	[48]	qPCR; HS; TEM; IM; RNAseq	Ectopic—kidney capsule	It is unclear if gene expression variation between distinct development stages has truly functional significance
Intestine	[49]	MV; FM of mCherry expressing organoids; HS; IM; wound closure quantification; *in situ* hybridization	Ectopic—kidney capsule	—
Intestine	[50]	iPSC lines expressing reporters for *ex vivo* FI; HS; live-cell imaging	Ectopic—kidney capsule/orthotopic—intestinal lumen	—
Intestine	[51]	Survival rate; percent of engraftment and size of organoids; IHC; HS	Orthotopic—mesentery	Impossibility to directly compare two models of transplantation; level of organoid functionalization and maturation was not evaluated
Pancreas	[52]	MV; IF for human origin marker; trilineage differentiation potential; HS; IF for acinar and ductal markers	Orthotopic	
Pancreas	[53]	Insulin IHC; human C-peptide serum measurement in PO, ES-PP, and ECM; vessel area of harvested grafts and vessel numbers	Ectopic—intraperitoneal cavity	—
Pancreas	[54]	Blood glucose measurements; IM; qPCR; IHC; human C-peptide serum measurements	Ectopic—under the kidney capsule	Significant islet loss in the early posttransplant period
Liver	[37]	MV; dextran infusion at day 3; connections' visualization among HUVECs and host vessels; quantification of human vessels; functional vessel length between human iPSC-LB x HUVEC human MSC transplants	Ectopic	—
Liver	[44]	ELISA; qPCR; IM; IHC	Ectopic—intraperitoneal cavity	Cell encapsulation did not completely eliminate the immune responses induced by foreign cells; fibrosis was reported. Further work is needed to develop iPS-H for clinical uses
Liver	[55]	MV; ELISA; IHC; IF analysis; cytochrome P450 3A4 and urea assay	Ectopic—renal subcapsule space	Further efforts are necessary to evaluate the use of SDC-LOs in clinical treatment
Retina	[56]	IHC; IM assays; retinal sections; expression of phototransduction and synaptic markers; ERG measurements	Orthotopic—subretinal space	Photoreceptor replacement procedures need to be optimized; risk of initiating tumor growth; proper differentiation and sorting methods aimed at specific target cell types are needed, as well as long-term studies to assess safety, and development of strategies to promote synapse formation and potential functional repair
Retina	[57]	IM assays; FC; GFP measurement	Orthotopic—superior and inferior hemispheres of the eye (subretinal space)	Further investigation of potential functionality of the transplanted cells

Table 2: Continued.

Organ	Ref.	Methods to evaluate engraftment, maturation, organoid behavior, and physiologic responses	Site of transplantation (orthotopic or ectopic)	Limitations
Retina	[58]	OKT response testing and SC electrophysiological recording; IHC for donor and retinal markers; spectral-domain OCT imaging and quantification	Orthotopic—subretinal space	Improve retina transplant lamination
Kidney	[59]	IHC; IF; IM assays; molecules' expression to assess maturation; VEGF injection; CM	Orthotopic—beneath the renal capsule	Ethical concern regarding the use of exogenous spinal cord cell layer; draining collection system is needed, as well as further maturation techniques to obtain a more robust collecting system and excretory function
Kidney	[60]	IF; nanoelectron microscopy; *in vivo* imaging; IM; SEM analysis; repeated intravital multiphoton imaging; TEM	Orthotopic—under renal capsule	Development of a glomerular filtration unit is needed
Kidney	[61]	*In vivo* injection of dextran–FITC into the CAM; IF analysis; IHC; TEM analysis	Ectopic—CAM of chick embryos	Development of methods to improve organoid differentiation (*in vivo* or *in vitro*), such as biomimetic approaches, is needed
Kidney	[62]	Whole-mount and section staining; FC	Orthotopic—under renal capsules	Formal proof using dye injection into the host circulation and examination of physiological functions in reconstituted kidneys are needed; differences between transplanted organoids and branching patterns of intrarenal arterioles from *in vivo* kidneys
Brain	[63]	GFP^+ detection; neuroepithelial ventricular zone analysis; level of gliogenesis; IM; axonal outgrowth and synaptic connectivity analysis; cranial glass window; two-photon calcium imaging; electrophysiological with cross-correlation; optogenetic control	Orthotopic—retrosplenial cortex	Improvements in vascular system, neuronal circuits, and immune system are needed, as well as understanding the complex physiological context of the brain
Brain	[64]	Fluorescent protein; ICC; GPF expression; cerebral organoid and the graft area measurements; blood vessels and microvasculature quantification; IHC; IM; neuronal differentiation	Orthotopic—frontoparietal cortex	Technical difficulties or increased cell death before engraftment; controlling stem cell proliferation after engraftment and developing a more complex cerebral organoid are needed; ethical concerns
Brain	[65]	IF; IHC; behavior tests (dysfunction, mNSS); image quantification; measurement of neural connectivity and brain functionality	Orthotopic—middle cerebral artery	—
Brain	[66]	HS; IM; FI; cell morphology; photostimulation of grafted cells	Orthotopic—medial prefrontal cortex	—
Heart	[67]	Beating; voltage-sensitive dye imaging; vasculogenesis; neovascularization; IM; organization of sarcomeric structures; RT-qPCR	Ectopic—internal abdominal muscle with a basket	Maturations details (pre- and posttransplant)
Lung	[68]	IM	Ectopic—kidney capsule, omentum, or fat pad	Additional cues for tissue maturation are needed, as well as variability across transplants
Lung	[69]	IF; HS; dot blot	Ectopic—kidney capsule	Terminal maturation; branching seems random; nature of mesenchyme is unclear; *in vitro* culture biases to restricted cell types
Lung	[70]	IM; size evaluation; proliferation	Ectopic—kidney capsule	Ectopic transplantation is limited and does not resemble true regenerative potential

Table 2: Continued.

Organ	Ref.	Methods to evaluate engraftment, maturation, organoid behavior, and physiologic responses	Site of transplantation (orthotopic or ectopic)	Limitations
Lung	[71]	IM; pulse oximetry; qPCR	Orthotopic	Better elucidation regarding transcriptional changes and signals in AT2 transplanted organoids; better optimization of organoid transplant
Lung	[72]	IHC; H&E; imaging	Ectopic—epididymal blood vessels and fat pad	PEG did not support maturation over the 8 weeks; increase in immune cell recruitment in PEG scaffolds due to hydrogel swelling

Abbreviations: CM: confocal microscopy; ERG measurements: electroretinogram; FC: flow cytometry; FI: fluorescence imaging; FITC: fluorescein isothiocyanate; FM: fluorescence microscopy; (E)GFP: (enhanced) green fluorescent protein; H&E: hematoxylin and eosin staining; HS: histology; ICC: immunocytochemistry; IF: immunofluorescence; IHC: immunohistochemistry; IM: immunostaining; LGR5: leucine-rich repeat-containing G-protein coupled receptor 5; MV: macroscopic view; OKT: optokinetic response; qPCR: quantitative polymerase chain reaction; RNAseq: RNA sequencing; SEM: scanning electron microscopy; TEM: transmission electron microscopy; TRITC: tetramethylrhodamine isothiocyanate.

related studies have been performed, especially with retinitis pigmentosa (RP) and age-related macular degeneration (AMD) [75]. Since there is no cure and accessible treatments for this type of disorder, there has been a great interest in developing methods for the transplantation of photoreceptor precursors or retina derivatives.

Many works were performed involving the transplantation of pluripotent cell derivatives (iPSC-derived retinal cells or human embryonic stem cell retina (hESC-retina)), most of which with promising and feasible results [76–83]. In this context, 3D cell culture systems have emerged as a model enabling the development of retinal tissue, grafts, and its derivative cells in substantial quantities for clinical transplantation tests [82–84].

The first protocol of retinal organoid was derived from mouse ESC by Eiraku and collaborators in 2011 [85, 86]. Later on, in 2012, Nakano and colleagues developed an ESC-derived retinal organoid, in which they not only reported that hESC-derived optic cup was larger than the one derived from mouse ESC (mESC) but also reported that hESC-derived neural retina grows into multilayer tissue containing rods and cones, while cone differentiation is rare in mESC culture [87].

Later on, with the advent of iPSC and 3D culture systems, the production of diverse retinal 3D structures from both mouse and human pluripotent cells was significantly improved [75, 84, 88–90]. In 2013, Gonzalez et al. performed transplantation of retinal organoids differentiated from embryoid bodies (EB) in *Gnat1*$^{-/-}$ mice (which exhibits stationary night blindness). In 2014, Assawachananont et al. performed the first transplantation of 3D retina sheets, derived from mESC and mouse iPSC, in *rd1* mice (a model with rapid and progressive RP). In the same year, Decembrini et al. developed a mESC 3D culture system to produce large amounts of photoreceptors. Once transplanted, 3D retina structures demonstrated maturation, morphological integration, production of new photoreceptors, integration with the outer nuclear layer (ONL) and outer segments, expression of phototransduction pathway proteins, and formation of synaptic connections [84, 89–91].

In 2016, Santos-Ferreira et al. developed mESC-derived retinal organoids, which were transplanted in the subretinal space of mice with either mild or severe cone-rod degeneration: *Prom1*$^{-/-}$ (prominin1-deficient) and *tg*(*Cpfl1;Rho*$^{-/-}$) mice (a model generated from the crossing of cone photoreceptor function loss one mouse—*Cpfl1*—with rhodopsin knockout mice—*Rho*$^{-/-}$), respectively. Organoids were capable of producing rod photoreceptors that, when transplanted in *Prom1*$^{-/-}$ mice, were able to integrate with the host's ONL, to maturate, survive, and express important proteins of the phototransduction pathway, as well as synaptic markers. On the other hand, in *tg*(*Cpfl1;Rho*$^{-/-}$) mice, transplanted photoreceptors expressed rod markers but not synaptic markers and did not reach morphological maturation [56]. In 2017, Kruczek et al. produced organoids to obtain cone receptors, which are responsible for mediating high acuity and color vision during daylight. These mESC-derived organoids produced cone receptors that were transplanted into the subretinal space of *Aipl1*$^{-/-}$ mice (a model of end-stage retinal degeneration). Cone photoreceptors generated *in vitro* not only matured and survived within host eyes of both healthy and *Aipl1*$^{-/-}$ mice but also apparently made physical contact with inner retinal neurons. They also expressed synaptic transmission markers, as well as phototransduction-related proteins [57]. In 2018, McLelland et al. generated hESC-derived retinal organoid sheets, which were then placed within the subretinal space of *SD-Foxn1 Tg(S334ter)3Lav* (a model of severe RD immunodeficient nude rat). These transplanted retina organoid sheets exhibited maturation, integration, differentiation, production of functional photoreceptors and other retinal cells, synaptic activation, extensive transplant projections within the host RD retina, and improvement of PSC visual acuity and light sensitivity [58].

Even though these preclinical studies presented promising and extremely valuable results, they also pointed out limitations: (1) retinal organoids are composed of heterogeneous cell populations, which may represent a risk for tumor formation, cell contamination, and acute immune responses [56]; (2) the need for further investigation regarding the physiological functions of retina organoid-derived photoreceptors [57]; and (3) the absence of transplantation studies involving retina organoids derived from human iPSCs [75].

4.3. Kidney Organoids. A large number of patients with end-stage kidney disorders are dependent on hemodialysis and kidney transplantation [92]. Therefore, it is extremely relevant to invest in the production of transplantable kidney organoids. The kidney is a very complex organ, composed of many different cell types that, in order to perform its adequate function, need a complex 3D structure; thus, the development of organoids represents a valid investment [93].

One of the first attempts to transplant a kidney organoid dates back to 2012, when Xinaris and colleagues produced renal organoids derived from single-cell suspensions of E11.5 mouse kidneys and implanted them beneath the renal capsule of male athymic nude rats. These implanted kidney organoids exhibited formation of vascularized glomeruli with fully differentiated capillary walls, maturation of erythropoietin-producing cells, and physiological functions, including glomerular filtering and tubular reabsorption functions [59].

In 2014, Taguchi derived metanephric mesenchyme (MM) from mouse PSCs, which is responsible for generating many kidney components. This MM formed *in vitro* kidney 3D structures, such as vascularized nephric glomeruli and tubules [94]. Still in 2014, Takasato et al. differentiated hESCs into an *in vitro* self-organized nephron structure through simultaneous induction of MM- and ureteric bud-like (UB) progenitors [95]. In 2015, Morizane et al. developed multipotent hPSC-derived nephron progenitor cell differentiation, which were able to form nephron-like structures in both 2D and 3D culture systems. These organoids expressed podocytes, proximal tubules, Henle's loop, and distal tubule markers, resembling *in vivo* nephrons [96]. Next, in 2015, Takasato et al. generated kidney organoids containing nephrons with collecting duct network, early loops of Henle, and podocyte glomeruli [97]. In 2017, Taguchi et al. generated a kidney organoid derived from mPSC and hPSC by induction of MM and UB. This method enabled the development of a high-order architecture kidney organoid, which included peripheral progenitor niche and internally differentiated and interconnected nephrons [98].

Studies involving kidney organoid transplantation have started only recently. In 2018, hPSC-derived kidney organoid was transplanted under the renal capsule of immunodeficient mice. The transplanted kidney organoids exhibited maturation of podocytes, glomeruli vascularization, functional glomerular perfusion, and connection with preexisting vascular networks. Organoids, in the absence of any exogenous vascular endothelial growth factor, developed host-derived vascularization [60]. In 2019, Garreta et al. transplanted hPSC-derived kidney organoids into the chorioallantoic membrane (CAM) of chick embryos. CAM demonstrated to be a good microenvironment to study vascularization since it is not only a highly vascularized naturally immunodeficient soft environment but also easily manipulated and monitored. Besides, in parallel, hydrogel was also used, and they observed that kidney organoids transplanted into these soft environments stimulated organoids' differentiation and growth. CAM-transplanted organoids exhibited successful engraftment, vascularization, multiple blood vessels, and blood circulation [61]. Also, in 2019, Murakami et al. transplanted kidney organoids derived from mouse embryonic kidneys, under the renal capsules of immunodeficient mice. Transplantation results showed *in vitro* vascular development together with extensive UB branching and glomerulus formation, as well as formation and reestablishment of arteriolar network [62].

Although kidney organoid transplantation studies are still scarce, some challenges have already been pointed out and should be taken in consideration for future translational studies, such as (1) organoid size, as kidney organoids produced with larger amounts of cells presented higher survival rates [59]; (2) necessity to examine physiological functions (vascularization flow and urine production) in reconstituted kidneys [62]; and (3) the fact that the kidney is a highly complex and metabolic organ, therefore bioenergetics analysis should be considered with transplantation of kidney organoids [61].

4.4. Liver Organoids. The first functional liver organoid derived from pluripotent cells was made by Takebe et al. in 2015 [37]. The researchers used a coculture of hiPSC, human umbilical vein endothelial cells (HUVECs), and MSCs, which enabled the recapitulation of cell interactions during organogenesis, allowing them to self-organize into a 3D structure, resembling liver buds (iPS-LB) at the embryonic stage. When transplanted into nude mice, these liver buds exhibited quick and functional vascularization of the construct after 48 h of transplantation, evidenced by dextran infusion, showing functional human vessel formation and connections among donor and host cells. They also evaluated the number of vessels, which had already increased three days after transplantation, and the area of vessels, which was similar to the human liver. In addition, they evaluated drug metabolism activity, and the results were positive for this essential hepatic function and have rescued the drug-induced lethal liver failure model.

Despite their promising results, Song et al. (2015) argued that, for clinically relevant purposes, there was a need for researchers to use immunocompetent mice. Therefore, they decided to generate liver organoids with a slightly different protocol, combining initial 2D culture, to ensure homogenous distribution of nutrients and differentiation factors, with 3D culture, which allows complex interactions between cell-cell and cell-matrix to induce maturation. In order to transplant organoids into immunocompetent animals, they encapsulated the aggregates into biocompatible materials, such as alginate capsules. These capsules prevented direct immune cell rejection but did not eliminate immune response, as evidenced by detection of *Il-2*. Nevertheless, it did not compromise organoid function, maturation, and survival, as seen by the presence of albumin secretion and mature hepatic marker expression. However, one concern is fibrosis, which indeed occurred in a fraction of implanted capsules [44].

In 2018, Nie et al. investigated whether organoids could be used to treat acute liver failure in mice [55]. Considering future clinical applications, the group developed the liver organoid using three cell types originated from the same donor, unlike other published works that used different

donors with different human leukocyte antigen types. After transplantation, organoids were able to perform hepatic functions and promote recovery from acute liver failure. Although very promising, further efforts are necessary to evaluate the use of single-donor cell-derived liver organoid for clinical treatment.

4.5. Pancreas Organoids. The development of pancreas organoids could represent a possible treatment for type I diabetes mellitus, an autoimmune disease in which destruction of pancreatic β cells results in insulin deficiency. However, most of the studies focus on cell therapy using only β cells. The generation of acinar and ductal cells from pluripotent cells, although poorly understood, has been successfully achieved through production of pancreatic organoids (PO) that were capable of expressing pancreatic markers and were functionally and ultrastructurally similar to the pancreas [52]. Orthotopic transplantation of these organoids exhibited engraftment after five weeks, neovascularization in the grafts, and expression of ductal and acinar markers and also validated the use of pancreas organoids to model cystic fibrosis.

Recently, Soltanian et al. proposed a strategy using PO to enhance maturation of pancreatic progenitors (PP) [53]. The PO was placed in a 3D-printed tissue trapper and heterotopically implanted into the peritoneal cavity of immunodeficient mice, and the results indicated that, in contrast to corresponding early PP transplants, 3D PO developed more vascularization as indicated by greater area and number of vessels, containing higher number of insulin-positive cells and displaying improved human C-peptide secretions. In another study, Lebreton et al. demonstrated that combining dissociated islet cells (ICs) with human amniotic epithelial cells (hAECs) into an organoid improves its vascularization, engraftment, and function *in vivo* [54].

4.6. Lung Organoids. Transplantation of lung organoids is a promising tool for airway diseases, such as asthma. These organoids can be formed by a 3D assembly of lung epithelial progenitor cells with or without mesenchymal cells [99], as well as by using adult stem cells and PSCs [70].

The first attempt to transplant lung organoids from human PSCs was performed by Dye et al. (2016), in which different conditions for transplantation were tested. Most of the transplants showed huMITO$^+$ NKX2.1$^+$ immature airway-like structures. The most successful transplants, in terms of organoid maturation, were lung organoids cultivated for one day in microporous polylactide-co-glycolide (PLG) scaffolds, which were able to engraft *in vivo*, differentiate into a similar airway epithelium, and generate secretory lineages, resembling the adult human lung [100].

The combination of adult bronchial epithelial cells, lung endothelial cells, and lung fibroblasts creates a human airway organoid suitable for ectopic transplantation: one week after lung organoid transplantation into the kidney capsule, Tan et al. (2017) observed proliferation of host cells in organoids' border and presence of human endothelial cells. Organoids reduced in size after six weeks; the vascular network was mainly of host origin, and *in vivo* environment stimulated maturation and switched to a nonproliferating status [70]. Similarly, Chen et al. were able to generate organoids with branching morphogenesis and proximodistal specification [69]. After 1.5 months of ectopic transplantation, lung organoids showed growth, tubular structure, and an airway epithelium formation. Branching structures and epithelial cells were observed after 5 months, and histology revealed multiciliated cells and similar morphology to proximodistal specification in lung branching.

In 2019, Weiner et al. developed an alveolar type 2 (AT2) organoid, which was then transplanted to influenza-infected mice. Thirteen days after transplantation, analysis revealed that AT2 organoids presented good engraftment *in vivo* and retained the AT2 fate. However, these organoids did not elevate the capability of oxygen exchange in the infected receiver mice and sometimes they adopt a dysplastic fate upon engraftment [71]. Dye and collaborators (2020) studied the efficiency and physicochemical properties of lung organoids generated in three different scaffolds: PLG scaffolds, PEG hydrogel, and polycaprolactone scaffolds. Although some scaffolds present some advantages compared to others, for instance, organoids developed in PEG scaffolds did not support maturation over eight weeks and increased immune cell recruitment, overall, lung organoid maturation is supported by multiple microporous scaffolds. The conclusion was that manipulation of scaffolds' physicochemical properties influences the explant's properties, directing tissue formation, and may be used for modeling normal development or disease states [72].

Some challenges of lung organoids transplantation are related to poor cell maturation, branching morphogenesis which appears to be random, and the mesenchyme nature and patterns that are not well understood [69].

4.7. Brain Organoids. One of the most difficult systems to understand is the cerebral, as it is a highly complex organ with many functionalities. Also, regular cell culture systems do not capture the organ's complexity and the access to material is difficult [101]. Therefore, the production of brain organoids is a promising tool to study and treat cerebral diseases, such as neurological diseases and mental disorders [102, 103].

In 2013, Lancaster and collaborators (2013) were able to derive brain tissue *in vitro* through a 3D culture system to study microcephaly. Previously, studies were performed with only neural tissue *in vitro*, and differently from other organs, there were no studies using whole-brain organoids until then [102].

After this study, many others were developed in order to enable the transplantation of brain organoids. In 2018, Mansour et al. generated the GFP hESC line from lentivirus-transduced human ESCs, which originated brain organoids after 40–50 days of culture. Only organoids that passed the quality criteria were implanted into a cavity in the retrosplenial cortex of nonobese diabetic-severe combined immunodeficient (NOD-SCID) mice [63, 102, 104]. Eight months after transplantation, cell differentiation and progressive maturation were observed, as well as synaptic connectivity between human axons and the host brain and axonal outgrowth in cerebral organoids. Researchers were

also able to prove the organoid's successful vascularization through a cranial glass window that allowed tracing blood vessels. With these results, they were able to directly analyze the impact of environment and vascularization towards the brain organoids and verify their *in vivo* viability. The conclusion is that human brain organoids successfully interact with the mouse brain and present integration, maturation, and neuronal differentiation, which are promising for future human brain disorder treatment [63].

In 2018, Daviaud et al. compared cerebral organoids with neuronal progenitor cells (NPC), both derived from hESC. These organoids and NPCs were transplanted into the frontoparietal cortex of postnatal day P8-P10 mice. After two and four weeks of transplantation, they showed that brain organoids presented better results than NPCs, when comparing vascularization, graft survival, neural differentiation, and cytoarchitecture [64].

In 2019, Wang et al. developed and used cerebral organoids in the attempts of reversing damage after stroke. Parameters evaluated included the cerebral organoid volume, function recovery, effectiveness, and viability. Organoids were transplanted at 55 days in the rat middle cerebral artery occlusion, and results, 6 h–24 h later, demonstrated that cerebral organoids were able to differentiate and migrate into different brain regions. Also, they observed reduced brain damage volume, synaptic reconstruction, and neurological motor function recovery, among other neurological improvements, likely due to cell survival and vascularization, cell multilineage differentiation, and cellular replacement after stroke [65].

Recently, Dong et al. developed a protocol for the generation of small human brain organoids. After transplantation into the mouse medial prefrontal cortex, the authors observed that organoids survived and matured, extending 4.5 mm in length during the first engraftment. Differentiation of human cells into cortical neurons *in vivo* and electrophysiological activity affecting behavior were observed a few months posttransplantation. Organoid graft and host mouse brain interaction was also observed, involving synaptic connections and a possible functional integration between them [66].

Even though many improvements towards transplantation of cerebral organoids have been made, there are still some concerns, such as (1) the ethical implications related to the creation of brain chimeras that, somehow, could be responsible for "humanization" of host animals, raising questions about brain development and function [105]; (2) limited formation of neuronal circuits, microenvironment, immune system, and vascular circulation, as the absence of oxygen can interfere in the neuronal development and migration [63]; and (3) difficulty of tissue cross-communication and organization of the brain shape and structure [31].

4.8. Heart Organoids. Cardiac organoid production is still an area poorly explored. One advantage of 3D cultures for cardiac disease treatment is the possibility of observing tissue dynamics and organ physiology.

In 2019, Varzideh et al. developed the first hiPSC-derived cardiac organoid for transplantation. After 24 h of organoid formation, the presence of three different cell types was observed, cardiac progenitor cells (CPC), MSCs, and endothelial cells. These cells started to self-organize into 3D organoids after 72 h, and after one week, cardiac organoids presented a homogeneous beating, which maintained organoids mechanically stable for transplantation [67]. Detection of cardiomyocyte (CM) maturation markers and electrophysiological activity study were also evaluated before transplantation. To assist *in vivo* transplantation, a two-piece basket was fabricated using a 3D printer, and collagen type I was used to encompass the cardiac organoids, which were then transferred into the basket [67]. The transplantation was performed on the internal abdominal muscle of male nude mice, and four weeks later, organoids revealed extensive neovascularization, highly organized sarcomeric structures, CM marker expression, and electrophysiological activity. This *in vivo* transplantation induced structural organization of myofibrils, enhanced gene expression, and excitation-contraction coupling. CPCs interacting with mesenchymal cells developed into CMs and other specialized cells, allowing primary heart organogenesis. To facilitate organogenesis and because of their immunomodulatory and anti-inflammatory properties, MSCs were also included [67]. COs from transplanted mice were detached from the basket and transferred to a chick embryo to complete the lymphoid system development [67].

In conclusion, complex organoids are a promising tool to model heart diseases for regenerative medicine and drug testing, but further challenges still need to be overcome, due to (1) heart system complexity and diversity; (2) functional human cardiac organoids requiring at least three cell types: cardiomyocytes, cardiac fibroblasts, and cardiac endothelial cells [106]; and (3) improvement of cell maturation, as iPSC-derived cardiomyocytes, even after *in vitro* differentiation, still have embryonic properties [107].

5. Challenges on Organoid Transplantation

Current strategies for treatment of organ failure diseases involve transplantation of existing organs, cell therapy, and regenerative medicine concepts. The organoid system has arrived as an important alternative that is capable of recapitulating embryonic development, creating a favorable microenvironment to derive complex and functional structures resembling an organ. Here, we have reviewed the first attempts to generate different organoid systems, using animal models to evaluate their transplantability.

In general, preclinical evidence supports positive engraftment of organoids after transplantation, once it has been observed that these 3D structures integrated, maturated, vascularized, and developed specific targeted tissue physiological functions. Nonetheless, there are important subjects that must be taken into account before their application in organ failure diseases [45].

5.1. Organoid Size. One crucial issue regarding organoids for transplantation purposes is their small size. Thus far, organoids measure typically 10 μm to 1 mm in diameter, but there have been some attempts to make them bigger.

One approach to solve this issue is using spinning bioreactors, thereby facilitating oxygen and nutrient absorption, to make larger brain organoids resembling more of a human organ, for instance [102, 108]. Another option is combining small organoids to make a larger one as it was made for epithelia-only gut organoid [109].

The size is a major concern in some specific organs, such as kidney organoids, in which bigger organoids, produced with more precursor cells, had more chances of survival and growth than the smaller ones [59]. In contrast, the large size of organoids may be a problem. Human cerebral organoids seem fragmented after two weeks, maybe because of disparity in the size of organoid and host brain or due to hypoxia [64].

5.2. Cell Maturation. Cell maturation is important to ensure organoids will execute tissue-specific functions and guarantee their safety and efficiency after *in vivo* engraftment. For example, in some cases, differentiation protocols yield cells more similar to fetal than to adult ones, which might not be suitable for tissue replacement intents [44].

On the other hand, it seems that organ buds formed by less mature tissues might be a better strategy toward regeneration after transplantation, which is shown by some of the reviewed works: with kidney organ bud experiments [37], with intestinal organoid [38], and with heart organoid [67]. It occurs because the *in vivo* environment provides biochemical and physical signals from multiple sources, as well as vascularization and innervation networks that are difficult to completely reproduce *in vitro*. Besides, transcriptome-wide comparisons between intestinal organoids cultivated only *in vitro* or transplanted to NSG mice showed that *in vivo* engraftment improved cellular differentiation and organoids resemble mature adult-like intestine tissue, while *in vitro* organoids were more similar to fetal tissue [48].

Another aspect that influences cell maturation is the microenvironment in which the organoids are cultivated. For instance, Garreta et al. demonstrated that kidney organoids in soft environments, such as hydrogels or CAM, enhanced its formation and growth [61]. Also, in Völkner et al.'s study, the authors mentioned that several processes, such as progenitor proliferation and cell differentiation, are potential sources for organoid variation [110].

5.3. Animal Models. Several preclinical trials are required to confirm the true potential of organoids as a medical device to replace or improve organ function. However, these *in vivo* tests involve many concerns and difficulties in translation for human application. For example, according to Avansino et al., there is considerable variation between distinct rodent models and species, which makes it difficult to establish an ideal animal model [46]. Translational studies are needed to achieve successful clinical application, and it is important to count with larger animal models to better reproduce human conditions [51].

In addition, most of these studies still rely on immunodeficient models, because in general, organoids are derived from human cells, and this could introduce an important experimental variation, since mice's immune system would most likely reject the transplanted organoid. Only one out of the articles reviewed here used an immunocompetent animal and encapsulated the organoids in alginate which partially avoided immune system cell attack [44]. Nevertheless, this approach is still a xenotransplant and cannot simulate the clinical scenario of allogeneic transplantations.

One strategy to overcome this limitation is the use of humanized animal models, which have already been developed elsewhere [111]. Also, it is important to use larger animal models, such as pigs, to better understand possible outcomes of organoid transplantation [45].

5.4. Site of Transplantation. The site of transplantation must be chosen carefully. Fetal intestine organoids did not survive transplantation under the kidney capsule, showing that orthotopic transplantation could be more suitable [100]. Also, lung organoids did not survive after transplantation into the kidney capsule [68].

On the other hand, the kidney capsule is often chosen, because it is an isolated location, with a certain degree of immune privilege, good accessibility, and transplantation which is usually well tolerated by the host [51]. However, as discussed by Cortez and collaborators, some limitations regarding the kidney capsule for intestinal organoid transplantation made them search for closely related sites for intestinal transplantation, in this case, the mesentery [51].

In two works related to kidney and heart organoids, the site of transplantation differed from the usual, kidney capsule [61, 67]. They used chick CAM, which demonstrated to be highly vascularized, as well as a naturally immunodeficient and easier to monitor microenvironment [61].

Further alterations were done to facilitate organoid transplantation and recovery. For example, in heart organoids, they used a 3D printed basket [64], and for pancreas organoids, tissue trapper was used [53].

5.5. Vascularization and Innervation. Organoid vascularization is a critical issue because the absence of vascular networks limits organoid growth and factor exchange, reducing nutrient distribution [31, 93]. Using endothelial cells as an organoid component is a suitable strategy. HUVECs present in the liver bud organoids were capable of engrafting and forming blood vessels [112]. However, most *de novo* vascularization that occurs into organoids after transplantation is derived from host cells.

One option to investigate vascularization was transplanting organoids into CAM. Both studies that used CAM generated positive results, since immunofluorescence analysis and fluorescent isothiocyanate-dextran confirmed the presence of chick blood vessels and blood circulation [61, 67].

An important aspect that was not investigated by either of the works presented here is innervation, which is essential for the proper control of organ functions.

5.6. Follow-Up after Transplantation. An important matter for organoid transplantation technology is tracking organoids *in vivo* to evaluate their behavior, engraftment, vascularization, and function. Development of iPSC-expressing fluorescent biosensors through lentiviral vector infection, for example, enable the visualization and study of organoids

inside the host, creating an efficient and informative tracking system using tissue-specific promoters [50].

Another crucial aspect of organoid transplantation safety is to make sure that no tumor is formed, since tumorigenicity is a clinical hurdle for PSC-based therapies [113]. In some cases, fibrosis formation was a concern, in particular in those protocols that used encapsulation of organoids using biocompatible materials [44].

Despite all of these challenges, organoid transplantation represents a growing promising system for regenerative medicine application. The first-in-human trial of intestinal organoids is being planned to be carried out by Tokyo Medical and Dental University (TMDU) for treatment against inflammatory bowel disease. Besides that, the INTENS team is leading a research with adult stem cells to treat short bowel syndrome (SBS). In the meantime, diagnostic tools have been developed by a group called Hubrecht Organoid Technology (HUB). The purpose of these tools is to link patient-specific genetic and phenotypic information. A center in Yokohama City University (YCU) was investing in a treatment of pediatric metabolic liver disease. Also, in Cincinnati Children's Hospital Medical Center, a Center for Stem Cell and Organoid Medicine (CuSTOM) was created, encompassing various collaborations focused on organoid research [45].

6. Final Remarks and Conclusions

Organoids are promising tools for disease modeling, drug screening, and personalized medicine. The ultimate application of organoid technology is to use them for organ regeneration and replacement therapies, reducing whole organ transplant requirements and improving the life quality of patients. The therapeutic use of organoids would be an alternative to the challenging transplantation of organs with a short period of viability outside the body, such as the heart and lungs. In particular, organoids should highly impact regenerative treatments of organs that remain technically nontransplantable, such as the brain. The recent development of edited pluripotent stem cells with targeted disruption of HLA genes by CRISPR/Cas technology should also facilitate the generation of immunocompatible healthy organoids for widespread therapeutic purposes.

Compared with typical cell cultures, organoids better reproduce the structural complexity of a real organ, recreating tissue native architecture, morphology, and several biological interactions occurring *in vivo*. Despite being still in its infancy, organoid transplantation for the intestine, retina, kidney, liver, brain, heart, pancreas, and lung seems feasible and safe, based on preclinical evidence showing engraftment and great biocompatibility. After transplantation, studies have shown that organoids generate differentiated and functional cells that are capable of interacting with other host cells. Taken together, the good outcomes of these initial studies encourage the exploration of organoids for regenerative medicine purposes. However, relative organoid graft immaturity compared with host natural organ, incomplete functional tissue integration, and possible occurrence of heterotypic cell interactions are some of the remaining challenges to overcome before clinical application.

Conflicts of Interest

The authors declare the absence of any conflicts of interest.

Authors' Contributions

Gabriella Shih Ping Hsia, Joyce Esposito, Letícia Alves da Rocha, and Sofia Lígia Guimarães Ramos contributed equally to this work.

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Review Article

Enteropathogenic Infections: Organoids Go Bacterial

Viktoria Hentschel, Frank Arnold, Thomas Seufferlein, Ninel Azoitei, Alexander Kleger, and Martin Müller

Department of Internal Medicine I, Ulm University, 88079 Ulm, Germany

Correspondence should be addressed to Alexander Kleger; alexander.kleger@uni-ulm.de
and Martin Müller; martin.mueller@uniklinik-ulm.de

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Enteric infections represent a major health care challenge which is particularly prevalent in countries with restricted access to clean water and sanitation and lacking personal hygiene precautions, altogether facilitating fecal-oral transmission of a heterogeneous spectrum of enteropathogenic microorganisms. Among these, bacterial species are responsible for a considerable proportion of illnesses, hospitalizations, and fatal cases, all of which have been continuously contributing to ignite researchers' interest in further exploring their individual pathogenicity. Beyond the universally accepted animal models, intestinal organoids are increasingly valued for their ability to mimic key architectural and physiologic features of the native intestinal mucosa. As a consequence, they are regarded as the most versatile and naturalistic *in vitro* model of the gut, allowing monitoring of adherence, invasion, intracellular trafficking, and propagation as well as repurposing components of the host cell equipment. At the same time, infected intestinal organoids allow close characterization of the host epithelium's immune response to enteropathogens. In this review, (i) we provide a profound update on intestinal organoid-based tissue engineering, (ii) we report the latest pathophysiological findings defining the infected intestinal organoids, and (iii) we discuss the advantages and limitations of this *in vitro* model.

1. Introduction

The human intestinal tract can be affected by a myriad of infectious diseases ultimately impairing the intestinal mucosa's capability of regulating the net water absorption, electrolytes, and nutrients, while retaining its function as a physical barrier. In particular, infections of bacterial, viral, or protozoan origin rate among the most common causes of diarrheal diseases, both in resource-rich and -poor countries. Such infections are frequently linked to low hygienic standards and improper handling of food and drinks, as well as occupational exposure to domestic livestock [1]. Often, patients experience watery diarrhea as the only or leading symptom at a varying level of severity, optionally accompanied by hematochezia, abdominal cramps, emesis, or febrile temperatures [2]. Most of the cases of intestinal infectious diseases usually resolve spontaneously or can be treated with exclusively supportive measures such as rehydration and correction of serum electrolytes. However, health care systems across the globe continue to face recurrent infectious disease outbreaks, mostly resulting from the coincidence of several contributory factors: increased transmission rates due to crowded living conditions, limited access to sanitation, and a shortage of public health institutions to put effective prevention and control measures in place [3]. Particularly in developing countries, poor planning and/or poor implementation of health policies and programs negatively impacts on the availability, accessibility, affordability, and sustainability of a healthcare service. In these countries, it is estimated that around 10% of hospitalized patients acquire an infection during their stay. This is intensified by inaccurate diagnoses, medication errors, inappropriate or unnecessary treatment, and inadequate or unsafe clinical facilities or practices. For example, the inappropriate administration of antibiotics over the past decades has led to an accumulation of highly resistant and difficult-to-treat bacterial pathogens [4]. This

worrying development has prompted increased efforts both to devise alternative therapeutic strategies and to constantly deepen our current knowledge about pathogen-specific transmission routes, modes of intracellular replication and propagation, and reactive defense mechanisms of the infected host cell. In the past, intestinal cell lines were widely used to construct *in vitro* models of human infectious diseases and to gain insight into their molecular pathomechanisms. However, compared to nontransformed intestinal epithelium, established cell lines usually originate from a cancerous clone with abnormal growth and differentiation behavior as well as altered physiological features, which substantially limit their potential to recreate *in vivo* conditions.

In recent years, intestinal organoids have emerged as a promising tool, allowing researchers to establish long-lasting stem cell-based cultures dedicated to the intestinal epithelium in the absence of feeder cells. Cell proliferation and the growth of organoid culture systems are thereby sustained by adding appropriate stem cell niche factors to the culture medium. Intestinal organoids may emanate either from pluripotent stem cells of embryonic origin (ESC) or be reprogrammed by overexpression of pluripotency genes (c-MYC, OCT3/4, KLF4, SOX2) in somatic cells (iPSC). Alternatively, they may be derived from multipotent organ-committed leucine-rich repeat-containing G-protein coupled receptor 5 + (Lgr5+) crypt columnar base intestinal stem cells (ISC) (Figure 1). With regard to the latter, suitable tissue material can be obtained either from human donors undergoing endoscopy-guided biopsy or surgical resection or can be extracted from the whole murine intestine of sacrificed animals. The foundation of this fascinating tissue engineering technique was laid by Hans Clevers and his research group, who for the first time allowed the implementation of a robust 3D culture system of the intestinal epithelium originating from a single ISC [5]. Reproducible cultivation methods, amenability to experimental genetic manipulation, and conserved primary cell biology have all contributed to predestine intestinal organoids as an extremely useful tool to model host-pathogen interactions in human-relevant diseases. Embedding in an extracellular matrix-like scaffold and supplementation with the essential niche factors, epidermal growth factor (EGF), Noggin, R-Spondin 1, and Wnt3a drive proliferation and asymmetric division of the ISC to yield the rapidly cycling transit amplifying compartment. Next, the already lineage-committed progeny starts to form immature spheroids which are subsequently transformed into mature intestinal organoids with distinct crypt-villus compartmentalization [5, 6]. The luminal surface facing the inside is lined by a monolayer of polarized columnar epithelial cells which recapitulate the diversity of highly differentiated intestinal cell types typically encountered throughout the intestinal tract. Absorptive enterocytes account for the most prevalent cell type and are principally engaged in the regulation of water and electrolyte balance as well as the absorption of nutrients [7]. As a prerequisite for charge- and size-selective permeability, paracellular diffusion is restricted by an intercellular network of tight junctions. Besides absorptive enterocytes, the intestinal epithelium is interspersed with the following highly specialized cell types. (i) Goblet cells produce a viscid mucus rich in complex glycoproteins (mucins) which functions as a physical barrier between the host epithelium and the luminal microbiota. Goblet cells are perceived as an adjunct to innate immunity, as they produce various antimicrobial proteins such as angiogenin 4 [8], chemokines, and cytokines [9–12]. (ii) Paneth cells originate from and remain in the close vicinity of the ISCs, whose capacity for self-renewal largely depends on the juxtacrine secretion of the growth-promoting niche factors, namely, transforming growth factor, EGF, and Wnt3a from the Paneth cells. Additionally, they support local immune defense by excreting antimicrobial peptides such as lysozyme and α-defensins/cryptdins [13, 14]. (iii) Microfold (M) cells are a specialized cell type of the follicle-associated epithelium (FAE) responsible for luminal antigen sampling and trafficking to the underlying lymphoid tissue, thus contributing to mucosal immune surveillance [15]. Under steady-state conditions, the occurrence of this rare cell type is confined to the FAE, where its differentiation mainly depends on the receptor activator of nuclear factor-κB (NF-κB) ligand exclusively secreted by the underlying subepithelial stromal cells [16, 17]. (iv) Tuft cells represent another rare epithelial cell lineage which has been implicated in assisting innate lymphoid cells (ILC) to fight helminthic infections by supplying interleukin (IL) 25. Conversely, exposure to IL 13 derived from activated ILC has been shown to induce tuft cell hyperplasia [18, 19]. (iv) Another epithelial cell subpopulation is represented by the numerically small entity of enteroendocrine cells, among which the enterochromaffin cells constitute the most abundant cell type [20]. Their principal secretory product, serotonin, functions as a regulator of coordinated propulsive gut motility and intestinal fluid secretion [21, 22].

A considerable contribution to early immune response is made by the heterogeneous epithelial cell population of the intestine arguing in favor of the use of intestinal organoids as a stand-alone *in vitro* system for modeling enteric infections (Figure 2). The host immune response is further shaped by various local immune effector cells which can optionally be integrated into the organoids to achieve a more truthful adaptation to *in vivo* conditions. Within recent years, the primary cell-based origin of organoids and their versatility in many fields of application has encouraged the establishment of a series of infection models collectively adding to the pathophysiological understanding of clinically relevant human enteropathogens. The approaches addressed in this review illustrate the latest achievements in generating pathogen-specific intestinal co-cultures for advanced disease modeling and drug screening and outline particular results that have been ascertained (Table 1).

2. Bacterial Enteropathogens and Their *In Vitro* Replicas

2.1. Vibrio cholerae. Cholera is a diarrheal disease affecting mainly malnourished patients in resource-poor countries with reduced access to clean water and inadequate sanitation. The majority of epidemic outbreaks are caused by the serogroups O1 and O139 of the *Vibrio cholerae* (*V. cholerae*)

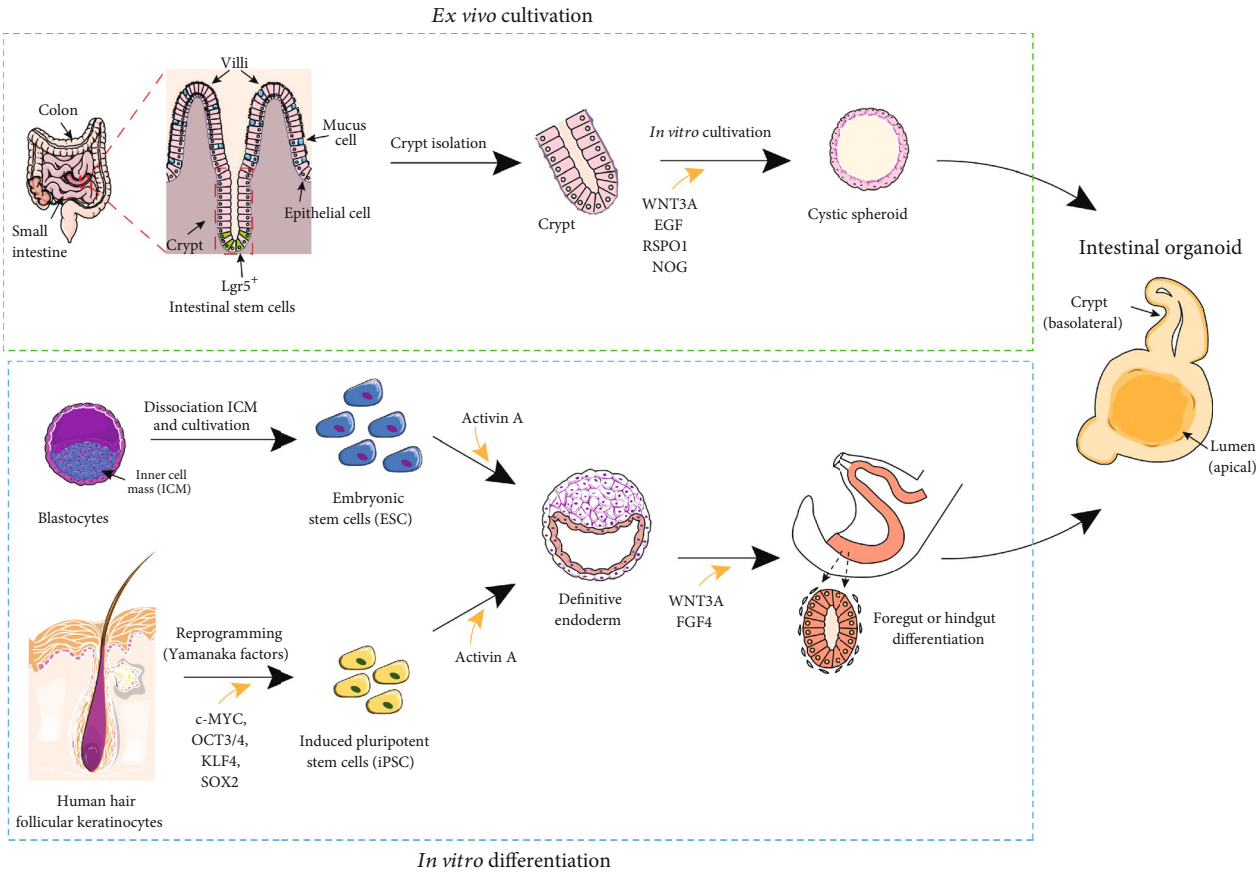

Figure 1: Generation of intestinal organoids from multipotent intestinal stem cells (ISC), embryonic stem cells (ESC), and induced pluripotent stem cells (iPSC). The protocols illustrated above are routinely applied in our laboratories.

bacterium, whose pathogenicity relies on the production of AB5 cholera toxin (CT). The toxin consists of an A subunit localized at its core, which is surrounded by a pentameric B subunit [23]. The B subunit encompasses an anchoring element with high affinity to the ganglioside molecule GM1. Despite its sparse expression on the host enterocyte surfaces, GM1 is considered a crucial receptor for CT [24]. It has been shown to promote endocytotic absorption of the holocomplex toxin into the host cell where the A subunit triggers adenylate cyclase activity. This results in augmented intracellular levels of the second-messenger molecules cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP). Subsequent activation of principal ion selective transport channels, such as the cystic fibrosis transmembrane conductance regulator (CFTR), leads to a dramatic rise in the luminal secretion of chloride followed by a passive efflux of water [25]. The cytotoxic effect can be reliably reproduced *in vitro* by the exposure of intestinal organoids to CT causing a dose-dependent quantifiable enlargement of the organoid volume. For the first time, the intestinal organoid-based swelling assay was validated as a preclinical screening tool for multivalent CT inhibitors by Zomer-van Ommen et al. [26]. By employing human rectal organoids, Haksar et al. not only identified a range of efficacious and at the same time cost-effective compounds featuring metanitrophenyl α-galactoside, a well-known ligand to CT [27] but also different polymer organic scaffolds derived from linear polyacrylamide, dextran, and hyperbranched polyglycerol. All compounds tested proved to inhibit CT attachment to and entry into the intestinal cells in an equipotent manner compared to synthetically produced GM1 oligosaccharide [28]. To mimic the fecal-oral infection route of *V. cholerae* and create a physiological model of enteric disease, Kane et al. used intact bacteria for microinjection into the lumen of iPSC-derived small intestinal organoids [29].

2.2. Enterotoxigenic Escherichia coli. Enterotoxigenic *Escherichia coli* (ETEC) is one of the leading causes of the usually self-limiting traveler's diarrhea and sudden-onset diarrheal illness in areas with low hygienic status. It is mainly caused by the secretion of the heat-labile and heat-stable toxins (LT and ST) which display structural similarity to the CT [30, 31]. Effective colonization of the mucosa, allowing immediate toxin delivery to the intestinal epithelium, is optimized by plasmid-encoded adhesive fimbriae and adhesins. Among the latter, EtpA, a high molecular weight adhesin, has been assigned a key role in promoting colonization of the host epithelium [32, 33]. This adhesin molecule attracted attention when it became evident that human volunteers challenged with oral ingestion of ETEC strain H10407

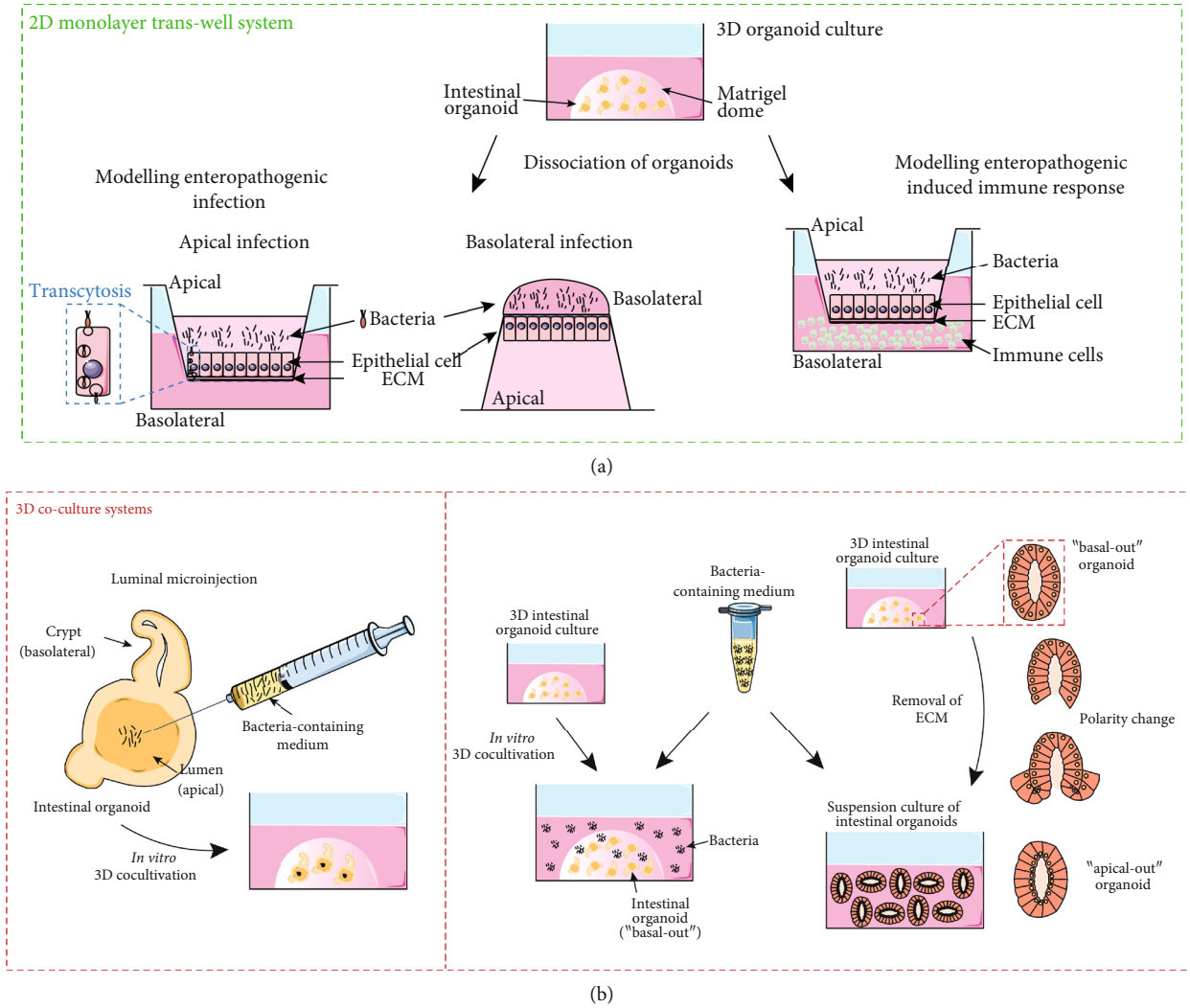

Figure 2: *In vitro* modeling of enteropathogenic infection. (a) 2D intestinal coculture models: bacteria are seeded onto the apical or basolateral surface of the intestinal epithelial monolayer (adapted from: Ranganathan et al., 2019 [38], Koestler et al., 2019 [39]). Optionally, immune cells are added to the basolateral compartment of infected intestinal epithelium (adapted from: Noel et al., 2017 [31], Karve et al., 2017 [49]). (b) 3D intestinal coculture models: bacteria are either introduced into intestinal organoids via luminal microinjection (adapted from: Karve et al., 2017 [49]) or added to the culture medium of "basal-out" or "apical-out" intestinal organoids (adapted from: Co et al., 2019 [59]).

suffered from diarrhea of substantially varying severity, suggesting the influence of at least one host factor on disease manifestation [34, 35]. Large-scale glycan assays probed with recombinant EtpA revealed preferential binding to N-acetylgalactosamine, a terminal sugar residue pertaining to blood group A. To create a model closely resembling *in vivo* conditions, small intestinal organoids from human donors of each major AB0 blood group were incubated with purified EtpA and EtpA-expressing wild type (WT) H10407 ETEC, respectively. In both cases, epithelial cells bearing blood group A glycoproteins were recognized with higher affinity compared to small intestinal organoids derived from blood group B or 0 donors, suggesting the role of EtpA as a pathovar-specific lectin. In accordance with this finding, adhesion of EtpA-mutant ETEC to blood group A small intestinal organoids occurred more hesitantly compared to EtpA-expressing WT H10407 ETEC. Intracellular levels of cAMP, reflective of toxin-dependent adenylate cyclase activity, were significantly reduced in EtpA-mutant-infected small intestinal organoids, while production of ST did not differ between both groups. These findings indicated that EtpA in the capacity of a pathovar-specific lectin ensures stable binding preferentially to blood group A-glycosylated epithelial surfaces, thereby rendering toxin delivery more efficient [36].

A complex *in vitro* co-culture involving ETEC strain H10407 and peripheral blood monocyte-derived macrophages is aimed at modeling the host innate immune response to an enteric infection (Figure 2). Human small intestinal organoids converted into a confluent monolayer were inoculated with bacteria on their apical surface to imitate the luminal portal of entry. It could be noted that phagocytic activity of macrophages led to an efficient

Table 1

Causative agent	Source and characteristics of infected organoid culture system	Findings/ objectives	References
Campylobacter jejuni	(i) Adult murine small intestinal organoids	(i) Infection-related genotoxicity (induction of DNA strand breaks)	(i) [86]
Clostridium difficile	(i) iPSC-derived human intestinal organoids (ii) iPSC-derived human intestinal organoids	(i) Attenuation of Cdt B-induced cytopathic effect by exposure to HSA (ii) Neutralization of TcdB-induced cytoskeletal disarray by bacitracin	(i) [83] (ii) [84]
Enterohemorrhagic Escherichia coli	(i) Adult human colon-derived organoids (ii) ESC-derived human intestinal organoids supplemented with PMN	(i) EspP-mediated proteolytic degradation of protocadherin 24, effacement of microvillar bridges of the enterocytic brush border; EspP equipped with enterotoxin-like properties (ii) *In vitro* modeling of EHEC enteric invasive infection	(i) [44] (ii) [49]
Enterotoxinogenic Escherichia coli	(i) Adult human small intestinal organoids (ii) Adult human small intestinal organoids supplemented with monocyte-derived macrophages	(i) Preferential adhesion to blood group A-glycosylated epithelial surfaces via lectin EtpA (ii) Mitigation of bacteria-stimulated inflammation and intestinal barrier dysfunction by resident macrophages	(i) [30] (ii) [31]
Listeria monocytogenes	(i) Fetal tissue-derived human intestinal organoids (ii) Adult human small intestinal "basal-out" organoids (iii) Adult murine intestinal organoids	(i) Goblet cells as preferred cell type of entry (ii) Enforced endocytotic uptake via basolateral cellular target structures E-cadherin and Met (iii) Accelerated epithelial renewal and reduction in goblet cell numbers mediated by STAT1 and STAT3	(i) [67] (ii) [59] (iii) [72]
Salmonella spcc.	(i) Adult human small intestinal organoids (ii) Adult murine small intestinal "apical-out" organoids (iii) Adult human ileum-derived organoids (iv) Adult murine small intestinal organoids (v) Adult murine small intestinal organoids supplemented with probiotic L. acidophilus	(i) Inducible transdifferentiation of enterocytes into M cells as favored portal of entry (ii) Preference of apical transmission route; luminal shedding of infected enterocytes (iii) Invasion and intracellular dispersion dependent on exploitation of host cell cytoskeleton (iv) Disintegration of epithelial Zonula occludens, enhanced NF-κB signaling; upregulation of goblet cell gene markers (v) Reversal of infection-induced upregulation of Wnt3 and Toll-like receptor 2 and 4	(i) [54] (ii) [59] (iii) [60] (iv) [61, 62] (v) [63]
Shigella flexneri	(i) Adult human colon-derived organoids (ii) Adult human ileum-derived organoids pretreated with TNF α to amplify M cell population (iii) Adult human colon-derived organoids (iv) Adult human intestinal organoids	(i) Production of adhesive biofilm dependent on luminal exposure to glucose and bile salts (ii) Preferred host cell invasion via M cell transcytosis, enhanced NF-κB signaling; upregulation of MUC2 expression (iii) Polymerization of actin fibers to facilitate intracellular trafficking; enhanced NF-κB signaling (iv) Anti-infective effectiveness of bacteriophages	(i) [37] (ii) [38] (iii) [39] (iv) [41]
Vibrio cholerae	(i) Swelling assay conducted in human adult rectum-derived organoids (ii) iPSC-derived human intestinal organoids	(i) Neutralization of CT by monovalent and multivalent metanitrophenyl α-galactoside-bound polymers (ii) *In vitro* modeling of cholera enteric infection	(i) [21] (ii) [23]

internalization of bacteria, while at the same time, infection-related impairment of the epithelial barrier function was partially restored in the presence of macrophages [37]. This constellation is consistent with previous findings that upon migration into intestinal lamina propria tissue, peripheral blood monocytes differentiate into resident macrophages with an anergic phenotype but preserve transepithelial antigen-scavenging and bactericidal properties [38].

2.3. Shigella flexneri. *Shigella* sp. rank among the most common causes of infectious diarrhea, especially in debilitated and immunocompromised persons in developing countries. The genome of *Shigella*, a gram-negative nonmotile enterobacterium, is known to harbor a set of virulence factors including *Shigella* enterotoxins 1 and 2 (ShET 1 and 2) and Shiga toxin (Stx), encoded by the chromosomal DNA and the virulence plasmid, respectively. ShET 1 ultimately results in an increased luminal secretion of ions and water by the enterocytes [39], while ShET 2 is involved in regulating secretion of the proinflammatory cytokine IL 8 by the intestinal epithelium [40]. By contrast, *Shigella dysenteriae*-exclusive *Stx* mediates the attachment of the bacterium to the endothelium of the intestinal vasculature. This results in occlusive ischemia, which is further exacerbated by inadequate activation of platelets [41, 42]. Prior to the actual event of invasion, *Shigella* sp. initiates the production of an adhesive biofilm induced by prolonged exposure to bile salts and glucose during small intestinal passage. Adherence analysis in human colon-derived organoids infected with *Shigella flexneri* (*S. flexneri*) revealed the emergence of adhesive structures contacting the host epithelial cell [43]. Basic characteristics of host cell infection with *S. flexneri* were captured in an organoid-derived monolayer model originating from different sections of the human intestine [44]. To gain access to the basolateral epithelial compartment, *S. flexneri* enforces its own transcytosis via M cells of the ileum- and colon-associated FAE. Human ileum organoids pretreated with the NF-κB-inducing ligand tumor necrosis factor (TNF) α to specifically expand the M cell population prior to infection with *S. flexneri* serotype 2a strain 2457T, yielded significantly higher numbers of intracellular bacteria than conventionally grown organoids [44]. Inoculation of either the apical or basolateral epithelial surface with pathogenic *S. flexneri* strain 2457T or mitigated plasmid-cured noninvasive derivative strain 4243A confirmed preferential access across the basolateral epithelial membrane. This invasion route proved to be far more frequented by the virulent *S. flexneri* strain 2457T [44]. The same mechanism of entry was observed in a similar experimental design with human colon-derived organoids using the identical *S. flexneri* strain [45]. Additionally, intracellular mobility of *S. flexneri* hijacks the cytoskeleton of the host cell to form long actin polymers [39]. This process has been reported to be essential for the further cytosolic dissemination of *S. flexneri* to neighboring epithelial cells [46]. Assessment of the epithelial immune response in *S. flexneri*-infected intestinal organoids revealed a transcriptional upregulation of IL 8, TNF α, interferon (IFN) β, and TNF α-induced protein 3 (TNFAIP3), which are largely associated with the NF-κB-mediated inflammation signaling pathways [44, 45]. Furthermore, the infected epithelium expressed higher levels of the intestine-specific mucin (MUC) 2 [44, 45]. According to the authors, this finding has so far remained equivocal as it may either signify a protective host response to reinforce the mucus' barrier function or mirror a subversive effect to modify mucus composition to accommodate the pathogen's requirements [44].

Another field of application of intestinal organoids has evolved with the experimental usage of bacteriophages to specifically fight *Shigella* infections. Frequent administration of antibiotics has given rise to the emergence of resistance plasmids, calling for an alternative therapeutic approach. Bacteriophages refer to viruses exclusively infecting and replicating in bacterial cells. A prominent feature refers to their property to target distinct bacterial species or even specific strains within a species, whereby the phages pursue either a lytic (exploitation of the host translation machinery with subsequent cell death and release of new phages) or a lysogenic replication strategy (mere incorporation of the phage DNA into the host genome, host cell remains unscathed). A therapeutic trial with bacteriophages to fight *Shigella* infection was conducted by Llanos-Chea et al. in both the human colorectal cancer cell line HT-29 and intestinal organoids [47]. Human intestinal organoids were inoculated with several *Shigella* sp. including *S. flexneri* serotype 2a strain 2457T. Subsequent co-incubation with the bacteriophage φ2457T demonstrated an efficient clearing of infection with *S. flexneri* serotype 2a strain 2457T, reflected by diminished bacterial recovery rates for both adherence and invasions assays [47].

2.4. Enterohemorrhagic Escherichia coli. Enterohemorrhagic *Escherichia coli* (EHEC), a gram-negative, rod-shaped enterobacterium, is a human pathogenic strain associated with food-borne colitis with occasional outbreaks of bloody diarrhea [48]. EHEC serotype O157:H7 is commonly responsible for a particularly aggressive disease course involving the hemolytic-uremic syndrome. It is precipitated by the Shiga-like toxins (Sltx) 1 and 2 and characterized by a non-immune hemolytic thrombotic microangiopathy of the kidneys, ultimately leading to acute renal impairment [49]. To probe the initial steps of epithelial invasion, human colon-derived organoids converted into an epithelial monolayer were apically infected with the Sltx-negative EHEC O157:H7 strain EDL933 and mutants deficient for the virulence factors StcE or EspP [50]. StcE refers to a zinc metalloprotease engaged in cleaving the protective layer of mucin glycoproteins to facilitate the attachment of bacteria to the intestinal epithelium [51]. However, infection with a StcE-deficient EHEC strain did not result in impaired destruction of the mucus layer previously reported for EHEC, suggesting an alternative mucus-depleting pathomechanism. EspP is a member of the family of high molecular weight serine protease autotransporters shared among several *Enterobacteriaceae* species and plays a critical role in the disruption of actin-bound cytoskeletal proteins in the host cell [52]. The authors of this study demonstrated that EspP promotes proteolytic reduction of the brush border resident protein protocadherin 24, leading to subsequent effacement of the

microvillar bridges which is considered to be a hallmark of EHEC infection [53]. Interestingly, human colon-derived organoid-based *in vitro* studies revealed that EspP can also functionally act as an enterotoxin by triggering aberrant ion currents independent of CFTR activity, potentially contributing to diarrheal symptoms [54].

Human intestinal organoids *in vitro* differentiated from the H1 human embryonic stem cell line were used by Karve et al. to emulate an enteric infection with the Sltx-producing EHEC strain O157:H7 (STEC) [55]. In accordance with the preceding study, gradual disruption of the epithelial lining in conjunction with a perturbed actin cytoskeleton occurred after luminal microinjection of STEC accompanied by the intimate apposition of pathogens to and eventually breaching of the intestinal epithelial barrier. Consistent with the natural course of the infection, culture conditions allowed detection of the host cell-derived burst of reactive oxygen species and reactive induction of Sltx by STEC. The host immune response was characterized by upregulated epithelial expression of the chemokines IL 1β and IL 18 and recruitment of co-cultured polymorphonuclear cells from the periphery into the organoids [55].

2.5. Salmonella enterica. Salmonella enterica, a gram-negative facultative anaerobe, rod-shaped, motile bacillus, which is ranked among the most common causative agents of foodborne diarrheal illnesses, is equipped with an ample armory of virulence factors to facilitate attachment, invasion, replication, and evasion of the host immune detection [56]. Preliminary data unveiled a predilection of π-class Std fimbriae encoded by the Std operon of *Salmonella enterica* serotype *typhimurium* (*S. typhimurium*) for binding terminal α 1,2-fucose residues [57]. This enzyme catalyzes the addition of fucose sugar to host membrane-bound glycans crucial to the expression of ABH and Lewis histo-blood group antigens on mucosal membranes and in body fluids [58]. This adherence strategy has been further corroborated by *in vitro* studies on intestinal organoids grown from α 1,2-fucosyl transferase 2 WT mice. Ileum- and colon-derived organoids were inoculated with a Std fimbriae-expressing apathogenic *Escherichia coli* strain which preferably bound to fucosylated cells [59]. Furthermore, Rouch et al. demonstrated that in human small intestinal organoids, *S. typhimurium* selects M cells as their preferred portal of entry [60]. Furthermore, if applied in highly infective doses, it induces an additional transdifferentiation of enterocytes into M cells [60]. To gain access to and travel inside the host cell, *Salmonella* sp. have been shown to exercise control over the intracellular signaling pathways involved in cytoskeletal rearrangement processes. One such mechanism deployed by *Salmonella* sp. aims at manipulating the host GTP-ases Cdc42, Rac1, and RhoG via secreting effector proteins into the host cell to activate the Arp2/3-complex. This central element steering the actin filament assembly is required for the formation of lamellipodia and membrane ruffles, providing intracellular mobility for and permitting the ingress of pathogens into the cell [61–63]. Invasion of the host cell by *Salmonella* sp. appears to be made through the apical transmission route. This finding was confirmed in human small intestinal organoids whose cell polarity had been reversed by depriving them of a matrix scaffold after maturation. *S. typhimurium* added to organoids with reversed polarity ("apical-out") and conventionally grown organoids ("basal-out") as well as organoids with a mixed phenotype preferentially penetrated the host cell from the apical surface. Upon intracellular replication of *S. typhimurium*, the infected host cell is usually shed into the luminal space, as has been previously reported in the human colon cancer cell line Caco-2 and murine primary intestinal cells [64]. This exit strategy was reproduced in "apical-out" small intestinal organoids that had been infected with *S. typhimurium*. Hereby, bacteria were detected both within actively extruding epithelial cells and fully extruded epithelial cells [65]. Further investigations centered on the prominent role of the host cell cytoskeleton for the intrusion and intracellular mobility of *Salmonella* sp. were conducted on human ileum-derived organoids inoculated with *Salmonella enterica* serotype *typhi* strain Ty2 (*S. typhi*). Transmission electron microscopy images confirmed the presence of cytoskeletal protrusions suggestive of microvilli dissolution and cytoplasmic reorganization as observed in whole tissue biopsy samples. It could be demonstrated that upon pre-incubation of the organoids with an actin or microtubule inhibitor, the cytoskeleton-dependent mechanism of invasion of *S. typhi* was efficiently disabled [66]. In line with that, intestinal organoids derived from murine ileum and jejunum displayed significant decomposition and downregulation of the tight junction-defining protein Zonula occludens protein 1 following colonization with *S. typhimurium* strain 14028S [67]. Furthermore, in this study, particular interest was vested in examining the epithelial immune response which was characterized by increased NF-κB signaling and consecutive upregulation of the downstream proinflammatory cytokines IL 2, IL 4, IL 6, TNF α, and IFN γ [67]. Similar results were obtained from an iPSC-based intestinal organoid model infected with *S. typhimurium* strain SL1344 [68]. Gene expression analysis of the host epithelium displayed a preponderance of proinflammatory cytokines such as IL 8, IL 1β, IL 23A, TNF α, and CXCL 2 but also of the goblet cell-associated genes encoding glucosaminyl-N-acetyl-transferase 3 and MUC 2, suggesting a reactive proliferation of the goblet cell population [68]. By contrast, commensal bacteria colonizing the gut lumen have been assigned an overall protective role by reducing mucosal inflammation and restoring intestinal homeostasis in invasive enteric infections. The integrity of small intestinal organoids challenged with *S. typhimurium* strain SL1344 rapidly deteriorated unless pretreated with the probiotic *Lactobacillus acidophilus* ATCC4356 (*L. acidophilus*). Furthermore, addition of *L. acidophilus* to the organoids caused a reversal of Wnt3 and Toll-like receptor 2 and 4 upregulation, which had been precipitated by *S. typhimurium* infection [69]. Based on the authors' opinion, these results implied an *L. acidophilus*-induced correction of crypt hyperproliferation towards physiological levels and reduced susceptibility towards inflammatory stimuli [69].

2.6. Listeria monocytogenes. Listeria monocytogenes (*L. monocytogenes*) is a gram-positive, motile, rod-shaped

bacterium causing food-borne diarrheal illness in immunocompetent persons but triggering septicemia and meningitis in immunocompromised patients and neonates [70]. Previous reports indicated that *L. monocytogenes* preferably traverses the intestinal epithelium both through goblet cells and M cells at the Peyer's patch level [71, 72]. Recently introduced by Roodsant et al. as an equivalent novel organoid culture model, human fetal tissue-derived intestinal organoids were plated as a monolayer and apically inoculated with *L. monocytogenes* which predominantly colocalized with MUC 2-positive goblet cells [73]. Furthermore, it was noted that the fluorescent staining signal for actin became weaker in the apical region of infected cells [73]. This finding might be linked to the property of *L. monocytogenes* to rearrange the host cell's actin into so-called "comet tails" to facilitate intracellular mobility, as previously reported by Co et al. in human small intestinal organoids [65]. *L. monocytogenes*' predilection sites of entry in enterocytes are not limited to specific cell types but also include areas with ubiquitously expressed adhesion protein E-cadherin and the hepatocyte growth factor receptor-associated tyrosine kinase Met. Both are exploited as target receptors by the two major invasion proteins In1A and In1B, respectively, to initiate the endocytotic uptake of *L. monocytogenes* into the host epithelium [74–76]. Under identical experimental conditions as previously described by Co et al., "apical-out," "basal-out," and mixed-polarity human small intestinal organoids were inoculated with *L. monocytogenes*. It was demonstrated that *L. monocytogenes* more frequently adhered to "basal-out" small intestinal organoids and spots of exposed basolateral space in "apical-out" intestinal organoids [65]. Such an uneven distribution pattern is attributed to the basolateral localization of E-cadherin and Met and particularly gains in importance at the villus tip, where the epithelial lining is occasionally interrupted by the expulsion of apoptotic enterocytes into the lumen. In the early phase of enteric infection with *L. monocytogenes*, the epithelial segment adjacent to the Peyer patches has been suggested to occupy a central position in initiating an efficacious host immune response [77]. Additionally, it has been implicated in modulating intestinal epithelial homeostasis by inducing acceleration of intestinal villus epithelium renewal and a decline in goblet cell numbers to lock down one potential portal of entry for *L. monocytogenes*. In an intestinal organoid-based model, it was demonstrated that for the induction of epithelial cell proliferation, phosphorylation of both signal transducer and activator of transcription (STAT) proteins STAT1 and STAT3 is mandatory [78]. Intriguingly, STAT1 and STAT3 appear to exert opposing cellular functions with regard to cell cycle regulation, survival signaling, and tumor immunity [79]. *In vitro* activation of the respective STAT proteins could be elicited by incubation with IL 22 or IL 11, originally derived from the pericryptal subset of gp38+ stromal cells and IFN γ supplied by natural killer cells [78].

2.7. Clostridium difficile. Similarly to *Salmonella enterica* sp., *Clostridium difficile* (*C. difficile*), a gram-positive, anaerobic, sporulating bacterium which accounts for a significant proportion of cases of antibiotics-associated diarrhea and pseudomembranous colitis, provokes cytoskeletal disarray by targeted inactivation of host cell Rho/Ras GTP-ases through its single-chain toxins *C. difficile* toxins (Cdt) A and B. Both toxins are equipped with a N-terminal glucosyltransferase and autoprotease domain which, after internalization and endosomal acidification, diffuse into the cytosol. Following autoproteolytic cleavage and release of glucosyltransferase, the Rho/Ras GTP-ase family members RhoA, Rac, and Cdc42 become mono-O-glycosylated and thereby inactivated, preventing them from interaction with their effectors possibly via steric hindrance [80]. Due to extensive involvement of the Rho/Ras GTP-ases in most actin-dependent processes, including stabilization of cell-cell contacts and cell shape-retaining stress fibers, any perturbation of this delicate switching element results in cell shrinkage, dissociation, and hence break-down of the intestinal barrier function. Furthermore, both toxins are able to induce apoptosis and pyrin inflammasome-induced pyroptosis [81–84]. A basic *in vitro* model of *C. difficile* infection was established using induced human intestinal organoids (iHIO) microinjected with toxigenic *C. difficile* strain VPI 10463 or nontoxigenic clinical isolate F200, respectively [85]. As expected, while infection with the latter did not result in a noticeable compromise of epithelial barrier function, inoculation with the toxin-producing strain caused apoptosis and severe disruption of the epithelium. Strikingly, separate microinjection of purified Cdt A into the iHIOs resulted in a profound redistribution of adherens and tight junction proteins as well as decomposition of actin filaments, exceeding the impact of Cdt B microinjection [85]. This observation contradicts former *in vitro* studies with intestinal cell lines, reporting an altogether higher potency for Cdt B [86–88]. However, in a mouse model of *C. difficile* colitis, rectal instillation of Cdt A alone triggered severe mucosal tissue damage and increased granulocyte infiltration as compared to Cdt B alone [89]. Arguably, these differences are related to the experimental conditions, with intestinal organoids being more likely to behave biologically like *in situ* tissue.

As far as incidence is concerned, the clinical severity and mortality rates of C. difficile infection seem to be inversely correlated to level of human serum albumin (HSA), which is considered a potential protective factor. Mechanistically, HSA is thought to bind Cdt A and Cdt B and therefore enhance auto-proteolytic cleavage, preventing toxin entry into the intestinal epithelial cell. Preliminary studies conducted by Di Masi et al. had resulted in a rapid decrease in serial transepithelial resistance measurements and cell viability of a Caco-2 monolayer culture exposed to a Cdt A-Cdt B mixture and CdtB alone, respectively [90]. By contrast, pretreatment with HSA was able to partially reverse the aforementioned effects and decrease the cellular uptake of Cdt B [90]. The same group was able to corroborate these findings using iPSC-derived human intestinal organoids generated by cellular reprogramming of keratinocytes from the plucked hair of a healthy human donor. After exposure to identical experimental conditions, intestinal organoids were assessed for macroscopic signs of structural disarray. These were reflected by the number of intact crypts as well as the distribution pattern of adherens junctions, which altogether

pointed to a significantly diminished toxic effect associated with HSA [90]. Using the same *in vitro* model based on iPSC-derived intestinal organoids, Zhu et al. showed that the antibiotic bacitracin possesses TcdB-neutralizing properties translating into a reduction of TcdB-related glucosylation of Rac1 as well as reduced destruction of the filamentous actin cytoskeleton [91].

2.8. Campylobacter jejuni. Enteric microbial pathogens may not only cause infectious diseases of the intestinal tract but also have been linked with an increased risk of developing colorectal cancer. Malignant transformation can be achieved by promotion of an inflammatory environment, production of molecules affecting DNA stability, and alteration of proliferative responses [92]. Among others, *Campylobacter* sp., a common causative agent of food-borne infectious enteritis in industrial countries, is capable of synthesizing a genotoxin referred to as cytolethal distending toxin (CDT). This toxin is a ternary protein complex consisting of three subunits CDT A, B, and C, whereby CDT B acts as a DNase, inducing host DNA strand breaks. This critical role of CDT B was illustrated by the *in vitro* exposure of murine small intestinal organoids to bacterial lysates either from *Campylobacter jejuni* (*C. jejuni*) WT strain or *C. jejuni* containing a mutant CDT B allele [93]. In line with previous results derived from intestinal cell lines, incubation of intestinal organoids with lysates from the *C. jejuni* WT strain resulted in increased phosphorylation of histone H2AX, a marker for DNA damage, thus indicating elevated levels of DNA strand breaks [93].

3. Chances and Drawbacks of Intestinal Organoids

With the advent of the organoid technology, intestinal organoids have gained widespread acceptance as a validated and powerful platform to faithfully reflect the environmental conditions in the gut epithelium. A variety of source materials are suitable for efficiently generating intestinal organoids, ranging from adult multipotent to embryonic or reprogrammed pluripotent stem cells, all of which share the ability of self-renewal, directional expansion, and lineage commitment to differentiate into the principal cell types of the intestinal epithelium. Beyond that, pluripotent stem cells are competent to develop into any of the three germ layers (i.e., endoderm, mesoderm, and ectoderm) after exposure to spatially and temporally varying combinations and concentrations of growth factors. Therefore, intestinal organoids originating from pluripotent stem cells may additionally include mesodermal residues providing fibroblasts and smooth muscle cells, which have been shown to encase the organoids and support their morphogenesis via intimate epithelial-mesenchymal interactions [94, 95]. Undisputedly, organoids generated in such way will have a greater potential to resemble the complex cellular composition of the original tissue, allowing the role of the subepithelial stroma to be studied in the context of enteric invasive infections. In general, the use of intestinal organoids instead of a clonal cell line may prove advantageous in the context of scrutinizing transmission routes in which a specific cell type serves as the preferred site of invasion or provides a potential target structure for individual pathogens and their toxins, respectively. In this respect, intestinal organoids are also an inviting option for investigations of the gut epithelium-owned defense system mainly represented by the Paneth cell population. Paneth cells not only deliver antimicrobial substances for instantaneous neutralization of pathogens but also dynamically respond to infectious or inflammatory stimuli by undergoing hyperproliferation or de-differentiation into stem cells to replenish the Lgr5+ stem cell compartment and preserve epithelial integrity [96, 97]. We think that, owing to their unique genomic signature, intestinal organoids may theoretically be utilized for personalized studies to determine the individual susceptibility to certain toxins or toxin-producing pathogens. In several studies, individuals with non-blood group 0 have been predicted to be at higher risk of contracting diarrheal diseases caused by ETEC LT and CT, both of which rely on the basic sugar residue N-acetylgalactosamine for stable binding to the host cell membrane [98, 99]. In addition, pathogen- or toxin-treated intestinal organoids may be used to directly explore the efficacy of antitoxin agents by assessing and quantifying the residual cytotoxic impact on a naturally behaving population of primary intestinal cells. Planar arrays of human colon-derived organoids fused with automated imaging and analysis tools have already yielded promising results which may in future enable large-scale screening of toxic compounds and drugs, respectively [100].

However, intestinal organoids do not come without shortcomings. The host's defensive capacities are not confined to the intestinal epithelium itself but are equally dependent on the resident microbial community of the gut. In a homeostatic ecosystem, the highly diversified commensal microbiome hedges enteropathogenic colonization of the mucosal surface through a mechanism termed "colonization resistance." Mainly due to a limited nutrient supply, resident microbiota constantly compete with invading pathogens to prevail against the occupation of available nutrient niches and thus prevent their uncontrolled spreading. Not only by inhabitation of the gut lumen itself but also by excretion of metabolic waste products does the commensal microbiome efficiently contribute to containing enteropathogens and at the same time fortifying the intestinal epithelial barrier. Under the influence of *Bifidobacteria*, *Lactobacilli*, and *Firmicutes* sp., complex carbohydrates are broken down into short chain fatty acids (SFA) which have been reported to promote colonization resistance [101, 102]. Exposure of intestinal organoids to the SFA butyrate, propionate, and acetate was significantly associated with a promotion of epithelial proliferation and cell turnover for each single agent, with an additive effect being observed for a mixture of all three SFA [103]. So far, the microbiome as a critical protective factor has only been inadequately reflected by organoid-based enteric infection models for a number of reasons. Given that the intestinal luminal content comprises trillions of commensal microbes accounting for an estimated 500-1,000 bacteria species [104], a selection of a manageable number (usually 1-2 according to the literature) of microbial

species to be incorporated into the organoids is rather insufficient. Another hurdle is imposed by the standard aerobic culturing conditions of organoids precluding the propagation of obligate anaerobic commensal microbiota. Therefore, only oxygen-tolerant commensal bacterial species such as *Akkermansia muciniphila*, *Faecalibacterium prausnitzii* [105], *Escherichia coli* [55], and *Bacteroides thetaiotaomicron* [106] have been successfully used to colonize intestinal organoids. This essential limitation has been recognized and addressed by modification of the microfluidic gut-on-a-chip technology to create an anoxic-oxic interface resembling the colonic mucosa. This permits a stable cultivation of the obligate anaerobic commensal bacteria *Bifidobacterium adolescentis* and *Eubacterium hallii*, respectively, in direct contact with an intestinal epithelial monolayer [107]. As opposed to establishing optimal growth conditions for anaerobes, bacterial growth within inoculated intestinal organoids is to be restricted to the luminal space by the utilization of antibiotic-containing media and the selection of microbial strains according to their individual resistance. Besides the microbiota, the intestinal luminal content carries an abundance of nutrients provided by dietary ingredients and endogenous metabolites, mucus, and bile acids, all of which have been shown to affect host defense response to a variable extent but are only poorly recapitulated by intestinal organoids.

Intestinal organoids have impressively demonstrated their ability to serve as a resource for the advanced *in vitro* modeling of enteric infections. Although this review is primarily dedicated to outlining current bacteriological knowledge acquired from infected intestinal organoids, it is noteworthy that analogous disease models exist for various parasitic [108, 109] and viral pathogens [110, 111] of the gastrointestinal tract. In fact, in light of the ongoing COVID-19 pandemic, attention has been shifted to employing organoid technology to help reveal fundamental mechanisms of viral entry and intracellular replication. In particular, human intestinal organoids play a pivotal role in supporting a robust replication of formerly unculturable viral agents such as the human norovirus [111], extending their utility for future SARS-CoV-2-related pathogenetic studies and high-throughput therapeutic drug screening. Considering the presumable zoonotic background of SARS-CoV-2, researchers have for the first time established intestinal organoids from Chinese horseshoe bats suspected to be one of the natural reservoirs [112]. With the intestinal organoid culturing protocol also being applicable to other non-human mammalian species such as the cow [113], pig [113, 114], dog [115], and cat [116], important strides have been made to consolidate our current pathophysiological understanding of zoonotic diseases. Combining the findings derived from intestinal organoids spanning different species will certainly be of added value for characterizing a broad spectrum of common zoonotic bacterial pathogens affecting the intestinal tract. Prospectively, among the numerous advantages related to intestinal organoids, preterm recognition of potentially human relevant microorganisms and expeditious *in vitro* screening of promising drug candidates might become a key application in opposing zoonotic bacterial diseases with life-threatening potential.

Conflicts of Interest

The author declares that there is no conflict of interest regarding the publication of this paper.

Authors' Contributions

V. H. and M. M. conceived the thematic focus of the review. V. H. wrote the text of the manuscript. Professional supervision and content suggestions were provided by A. K., M. M., N. A., and F. A. Accompanying graphics were drafted by F. A. All authors contributed to the final manuscript.

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Review Article

Adipose-Derived Stem Cells: Current Applications and Future Directions in the Regeneration of Multiple Tissues

Jiaxin Zhang,[1] **Yuzhe Liu**,[2] **Yutong Chen**,[1] **Lei Yuan**,[3] **He Liu**,[2] **Jincheng Wang**,[2] **Qiran Liu**,[1] **and Yan Zhang**[1]

[1]*Department of Breast Surgery, The Second Hospital of Jilin University, Changchun 130041, China*
[2]*Orthopaedic Medical Center, The Second Hospital of Jilin University, Changchun 130041, China*
[3]*Department of Cardiovascular Surgery, The Second Hospital of Jilin University, Changchun 130041, China*

Correspondence should be addressed to Yan Zhang; zhangy01@jlu.edu.cn

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Adipose-derived stem cells (ADSCs) can maintain self-renewal and enhanced multidifferentiation potential through the release of a variety of paracrine factors and extracellular vesicles, allowing them to repair damaged organs and tissues. Consequently, considerable attention has increasingly been paid to their application in tissue engineering and organ regeneration. Here, we provide a comprehensive overview of the current status of ADSC preparation, including harvesting, isolation, and identification. The advances in preclinical and clinical evidence-based ADSC therapy for bone, cartilage, myocardium, liver, and nervous system regeneration as well as skin wound healing are also summarized. Notably, the perspectives, potential challenges, and future directions for ADSC-related researches are discussed. We hope that this review can provide comprehensive and standardized guidelines for the safe and effective application of ADSCs to achieve predictable and desired therapeutic effects.

1. Introduction

Organ or tissue transplantation is a preferred treatment option for patients with terminal organ or tissue failure. In a retrospective study of data for a 25-year period using the United Network for Organ Sharing database, organ transplantation was associated with a significant survival benefit, saving over 2,270,859 life-years [1]. However, the World Health Organization estimates that only 10% of the global need for organ and tissue transplantation can be satisfied [2]. Inadequate tissue and organ supply remains a major public health challenge. Stem cells are particularly useful in the area of organ and tissue reconstruction, as they are abundant, can be harvested through minimally invasive methods, can be induced to differentiate into multiple cell lineages, and can be manufactured based on good manufacturing practice guidelines [3]. The application of stem cells has emerged as a means of compensating for the lack of tissue or organ availability and has resulted in a quantum leap in regenerative medicine [4].

Stem cell candidates include embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), and adult stem cells, such as mesenchymal stromal cells (MSCs) [5]. The use of ESCs creates ethical concerns and can also evoke immune responses. Besides, iPSCs avoid ethical concerns and immune responses, but the cell preparation method is relatively complex and time-consuming [6]. Although MSCs can be found in diverse tissues, bone marrow-derived mesenchymal stem cells (BM-MSCs) and adipose-derived stem cells (ADSCs) have been the subject of more comprehensive, in-depth research [7]. ADSCs have several advantages over BM-MSCs. On the one hand, higher yields of ADSCs can easily be obtained from subcutaneous regions through a minimally invasive and painless procedure; furthermore, ADSCs can maintain their phenotype longer in culture, present a greater proliferative capacity [8], and may also be more suitable for allogenic transplantation than BM-MSCs [9]. Besides, ADSCs can differentiate into cell types of the three developmental germ layers (endoderm, mesoderm, and ectoderm), including adipocytes, osteoblasts, chondrocytes,

neurocytes, and hepatocyte [10, 11]. These advantages render ADSCs to be the most attractive source of MSCs for regenerative medicine. Meanwhile, currently, the clinical approaches involving ADSCs gradually increased.

For the effective use of ADSCs in the regeneration of different types of tissue, the recent research progress regarding the use of these stem cells in the field of tissue engineering must be evaluated. Above all, the different ADSC harvesting methods can affect the basic properties of the cells, such as their ability to proliferate and their antiapoptotic capacity [12]. The gene expression patterns and the tendency toward specific germ layer differentiation will also be affected by the harvesting method [13]. Furthermore, because monolayers cannot mimic the interactions between cells and the extracellular matrix (ECM), ADSCs expanded as a 2-dimensional (2D) monolayer lose their ability to proliferate, differentiate, and form colonies after several passages [14]. Compared with the 2D environment, 3D culture enhances ADSC osteogenic differentiation, increases matrix mineralization, and enhances ADSC viability during proliferation [15].

A main point of concern in the field of tissue engineering is the maintenance of homeostasis in the ADSC microenvironment. A 3D scaffold architecture typically comprises porous, biocompatible, and biodegradable materials that provide a suitable microenvironment for stimulating cell growth and function [16]. The porosity and pore size of scaffolds can have direct effects on their function, including the mass transport of oxygen and vital nutrients necessary for stem cell proliferation, differentiation, and migration [17, 18]. In addition to the factors mentioned above, various cytokines can also enhance the proliferative and migratory ability of ADSCs, as well as promote their differentiation [19]. Under an ischemic environment, cytokines play a vital role in ADSC-mediated promotion of the recovery of blood supply and wound healing through the induction of angiogenesis [20]. In an inflammatory environment, those additional effects of cytokines can enhance the secretion of angiogenic and anti-inflammatory factors by ADSCs [21].

Recent reviews in related areas have not highlighted or detailed the optimal methods for the preparation of ADSCs, the latest progress in the application of ADSCs in various organs, or the potential risk for tumor invasiveness associated with ADSC-cancer cell interactions [22]. The following sections shed light on the methods for harvesting, isolating, preserving, and identifying ADSCs. Furthermore, we discuss the novel clinical uses for ADSCs as regenerative therapies, including bone regeneration, cartilage repair, nerve system rebuilding, liver regeneration, myocardium restoration, and skin regeneration (Scheme 1). The current challenges for the use of ADSCs in the field of regenerative medicine are summarized to provide directions for their clinical application.

2. Preparation of ADSCs

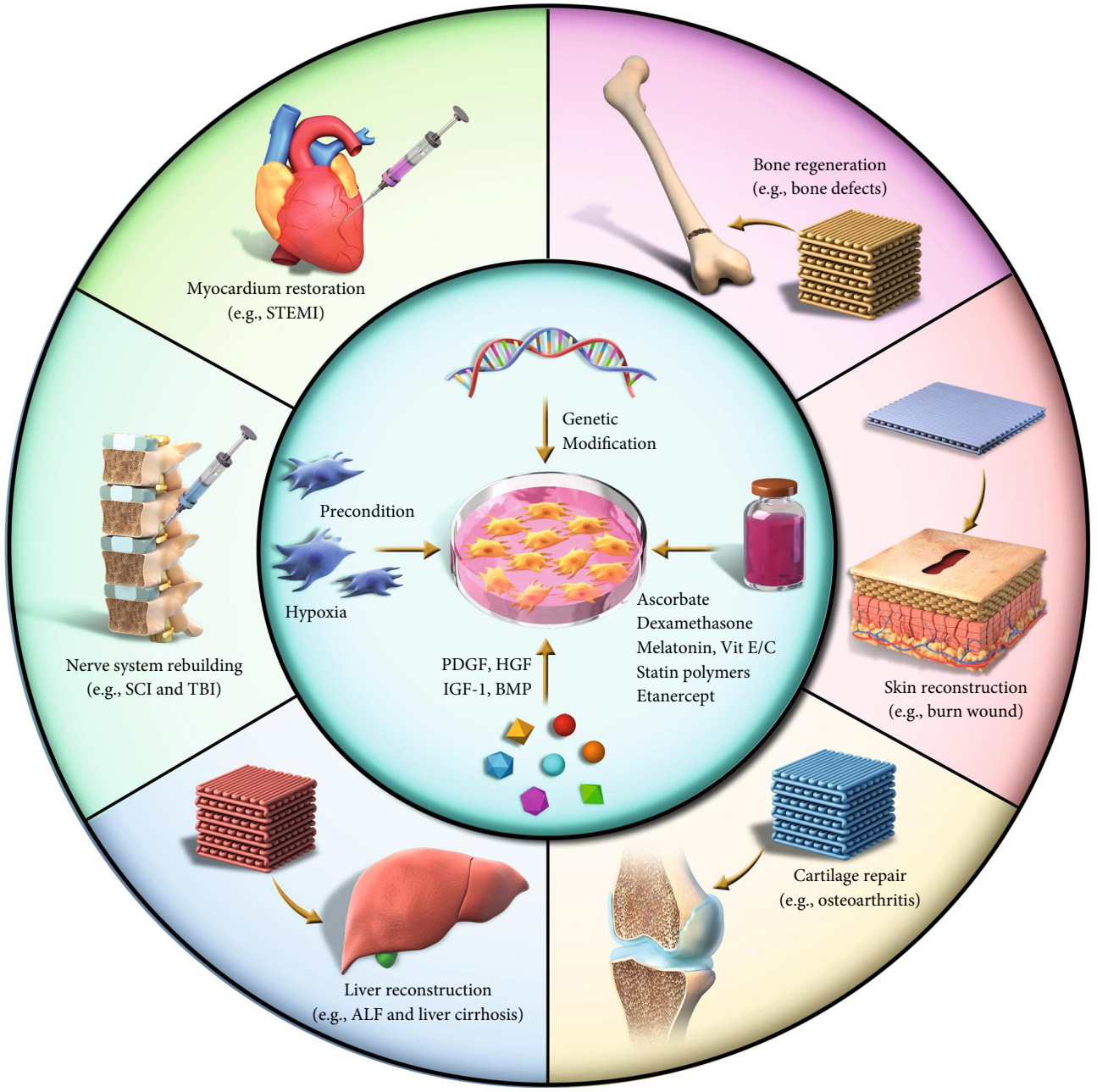
A rich source of ADSCs is an essential foundation for the extensive investigation and application of regenerative medicine. ADSC applications sourced these cells from subcutaneous adipose tissues obtained by aspiration, liposuction, or excision. Then, the most widely utilized approach to isolate ADSCs from the obtained fragments relies on collagenase digestion, followed by centrifugation. Finally, the isolated ADSCs are proliferated in conventional culturing conditions and distinguished from other cells by flow cytometry (Figure 1).

2.1. Harvesting of ADSCs. Current methods for harvesting ADSCs include aspiration, liposuction, and direct excision. Coleman's aspiration technique is currently the most commonly used method for the collection of adipose tissue, which relies on the slight negative pressure with a syringe. Furthermore, the negative pressure (<250 mmHg) of liposuction-related methods by motor could harvest a large volumetric adipose tissue. Liposuction-related methods include conventional, ultrasound-assisted liposuction, power-assisted liposuction, and laser-assisted. And direct excision could harvest a piece of adipose tissue, and the obtained fragments require mincing into tiny particles with the use of surgical blades [23].

The yield and properties of ADSCs may differ according to multiple variables, such as the harvesting method, the adipose tissue depot, medical comorbidities of the patient, body mass index (BMI), and age. There is evidence that harvesting adipose tissue by aspiration halves the concentration of ADSCs compared with harvesting by excision [24]. The yield and biological characteristics of viable ADSCs obtained by excision are significantly improved when compared with those obtained through liposuction [25]. The gene expression pattern and the tendency toward differentiation into a specific germ layer can also be affected by the harvesting method. ADSCs collected through direct excision tend toward mesodermal and ectodermal differentiation, whereas those obtained by liposuction are more likely to differentiate into endoderm [13].

The collection location also affects the yield and differentiation capability of ADSCs. There is some evidence showing that the thigh provides a better yield of ADSCs than the abdomen, waist, and inner knee [26]. In contrast, there is no significant difference in cell viability among the donor areas. ADSC yields and differentiation potential are also reported to be higher in subcutaneous tissue than in visceral depots [27]. Additionally, the differentiation capability of ADSCs also depends on the characteristics of the donor, such as age, gender, and metabolic index. Older age, high BMI (>30 kg/m^2), suffering from diabetes mellitus, or exposure to radiotherapy and endocrine therapy will decrease the proliferative and differentiation potential of ADSCs [28]. However, further research is needed to determine whether the *in vitro* and *in vivo* findings translate into clinically significant differences.

2.2. Isolation and Culture of ADSCs. The most widely utilized method for isolating ADSCs was first proposed by Zuk and colleagues [29]. This method involves extensive washing with phosphate-buffered saline (PBS) and digestion of lipoaspirate with 0.075% collagenase to release the stromal vascular fraction (SVF) of cells. The SVF is incubated in the medium overnight at 37°C in an atmosphere with 5% CO_2 after a series of washes and centrifugation steps. Following incubation, the plates are extensively washed with PBS to remove residual,



Scheme 1: Schematic representation of the applications for ADSC-based therapies in regenerative medicine. PDGF: platelet-derived growth factor; HGF: hepatocyte growth factor; IGF-1: insulin-like growth factor 1; BMP: bone morphogenetic protein; SCI: spinal cord injury; TBI: traumatic brain injury; ALF: acute liver failure; STEMI: ST-elevation acute myocardial infarction.

nonadherent red blood cells. The resulting cells are considered to be ADSCs.

Collagenase digestion remains the gold standard among the currently used methods for isolating ADSCs, although other enzymes, such as trypsin, clostripain, and dispase, can also be used [30]. A recent study suggested that, even though trypsin-digested and collagenase-digested ADSCs present similar adipogenic differentiation and proliferative ability, the osteogenic differentiation potential of the trypsin-treated cells is up to sevenfold higher [31]. Despite the widespread use of the above-mentioned methods for isolating ADSCs, enzymatic digestion-based methods have many disadvantages. The use of enzymes may alter or disrupt cell viability and surface antigens, which may reduce ADSC regenerative potential [32, 33], while question marks also remain regarding whether residual enzyme activity can affect safety. Consequently, an increasing number of studies have explored economical enzyme-free methods for ADSC isolation, including new mechanical methods [34, 35] or techniques that do not rely on enzymatic activity or centrifugation [36].

Although ADSC culture methods can vary across laboratories, a typical culturing condition comprises a monolayer of cells cultured with 10% fetal bovine serum (FBS) and 1%

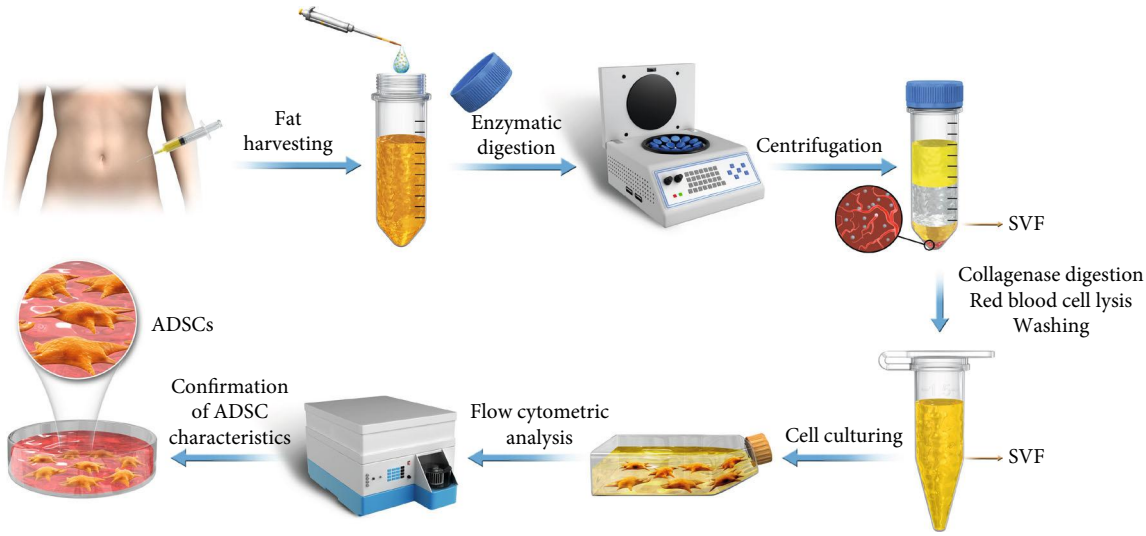


Figure 1: The typical process for the preparation of ADSCs from human adipose tissue. SVF: stromal vascular fraction.

antibiotics at 37°C and 5% CO_2 [37]. Although effective, the use of FBS in cell culturing processes is highly discouraged by regulatory agencies due to the potential risk of the transmission of xenogeneic infectious agents and immunization [38]. Many researchers propose alternative protocols, such as the use of human platelet lysate [39], which shows equivalent results in relation to the typical FBS-related methodologies. Additionally, platelet-rich plasma (PRP) also presents an efficient alternative supplement for ADSC proliferation [40]. Atashi et al. studied the capacity of autologous nonactivated PRP (nPRP) or thrombin-activated PRP (tPRP) on ADSC proliferation compared with 10% FBS. The final results revealed that nPRP possessed stronger proliferation-promoting effects than FBS or tPRP without changing the ADSC phenotype and chromosome status.

2.3. Identification of ADSCs. The presence of ADSC characteristics is commonly evaluated by flow cytometric analysis of cell surface markers [37], and the International Society for Cellular Therapy (ISCT) and the International Federation for Adipose Therapeutics and Science (IFATS) specify three minimal criteria for defining ADSCs: (1) cells must be plastic-adherent; (2) they must express CD73, CD90, and CD105 and lack the expression of CD14, CD11b, CD45, CD19, CD79, and human leukocyte antigen-DR (HLA-DR); and (3) they must have the potential to differentiate into preadipocytes, chondrocytes, and osteoblasts [37]. The ISCT also proposed that MSCs should lack the expression of CD117, CD14, CD11b, CD34, CD45, CD19, and CD79; nevertheless, the definitive markers that can effectively discriminate ADSCs remain controversial [41] (Table 1). Numerous studies have confirmed that ADSCs can express CD34 [42]. Compared with late passage ADSCs, early passage cells express higher levels of CD117, HLA-DR, and CD34 [43]. Although there are several differences among isolation and culture procedures, the immunophenotype remains consistent across laboratories. The immunophenotype of ADSCs is >90% identical to that of BM-MSCs [44]. Similar to BM-MSCs, ADSCs show uniformly positive expression of the surface antigen markers CD90, CD73, CD105, and CD44 but are negative for CD45 and CD31 [45]. Flow cytometric analysis has shown that ADSCs express CD13, CD29, CD34, CD36, CD49d, CD73, and CD133 [46]. More specifically, BM-MSCs lack the expression of CD34 and CD49d, and only ADSCs express these markers [47].

Furthermore, the detection and identification of the multiple differentiation of ADSCs are necessary. The osteogenic, chondrogenic, and adipogenic differentiation in ADSCs could be detected by the ALP assay, oil red staining, and GAG analysis [48, 49]. The real-time PCR assay may also be useful in the detection of neuron-like cells, hepatocytes, and myocytes, which are derived by differentiation of ADSCs [50]. The extraordinary characteristics of ADSCs endow them with considerable potential for use in tissue engineering and regenerative medicine. However, a standard definition of harvesting and processing techniques has yet to be established. More extensive studies are required to set a standard protocol, which would contribute significantly to the development of adipose tissue engineering.

2.4. Paracrine Secretion by ADSCs. Many studies have summarized the secretory profiles of ADSCs, which were assessed using enzyme-linked immunosorbent assays or related techniques. The proangiogenic and cardioprotective effects of ADSCs have been attributed to the production of growth factors, including fibroblast growth factor 2 (FGF-2), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), and insulin-like growth factor 1 (IGF-1) [51]. Matrix metalloproteinase- (MMP-) 3 and MMP-9 are expressed by ADSCs and are vital for the higher proangiogenic activity observed in ADSCs when compared with that of BM-MSCs [52]. Therefore, if ADSCs are exposed to a focus of inflammation or ischemic injury, they will secrete growth factors and cytokines to promote healing and tissue regeneration. ADSCs also secrete high levels of factors that have a significant role in neuroprotection and differentiation, such as

Table 1: Potential surface markers for the identification of ADSCs.

Surface markers	Name	Category	Positive/negative
CD11b	α_b integrin	Adhesion molecule	Negative
CD104	β_4 integrin	Adhesion molecule	Negative
CD14	Lipopolysaccharide	Receptor molecule	Negative
CD45	Leukocyte common antigen	Receptor molecule	Negative
CD79	MB-1	Receptor molecule	Negative
CD16	Fc receptor	Receptor molecule	Negative
HLA-DR	Human leukocyte antigen DR	Histocompatibility antigen	Negative
CD73	Ecto-5′-nucleotidase	Surface enzyme	Positive
CD13	Aminopeptidase	Surface enzyme	Positive
CD10	Endopeptidase	Surface enzyme	Positive
CD105	Endoglin	Adhesion molecule	Positive
CD49d	α_4 integrin	Adhesion molecule	Positive
CD29	β_1 integrin	Adhesion molecule	Positive
CD44	Hyaluronate	Receptor molecule	Positive
CD36	Thrombospondin	Receptor molecule	Positive
CD117	c-Kit	Receptor molecule	Positive
CD90	Thy-1	Extracellular matrix	Positive
CD146	Muc-18	Extracellular matrix	Positive
CD34	Hematopoietic progenitor cell antigen	Stem cell	Positive
CD133	Prominin-1	Stem cell	Positive

brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), and glial-derived neurotrophic factor (GDNF) [53]. At the level of the immune system, there is substantial evidence that prostaglandin E2 (PGE2) partially regulates some of the immunomodulatory properties of ADSCs. In response to inflammatory stimuli, ADSCs can increase the production of angiogenic factors such as VEGF, HGF, and IGF-1 as well as that of hematopoietic/inflammatory factors such as macrophage-colony stimulating factor (M-CSF), granulocyte-colony stimulating factor (G-CSF), interleukin- (IL-) 6, and tumor necrosis factor (TNF) [54]. These findings demonstrate that both ADSCs and BM-MSCs can suppress the immune response by suppressing peripheral blood mononuclear cell proliferation and the differentiation of immature monocyte-derived dendritic cells. However, higher levels of cytokine secretion by ADSCs induce stem cells to increase the release of immunomodulatory factors [55] such as IL-10, PGE2, galectin-1, and galectin-3. Studies have shown that PGE2 and IL-10 [56] can suppress the maturation of dendritic cells and helper T cells following their activation, thereby limiting inflammation, while transforming growth factor-beta (TGF-β) can accelerate the premature differentiation of T helper cells into T regulatory cells [57].

3. Regenerative Medicine Based on ADSCs

3.1. Bone Regeneration. Bone tissue engineering (BTE) is an optimal therapeutic approach for reconstructive surgery to repair critical-size bone defects and improve patient quality of life following high-energy trauma, malformations, osteomyelitis, and tumor resection. Osteoprogenitor seeding cells, combined with an appropriate scaffold and bioactive factors, are crucial for BTE. ADSC-based strategies for bone regeneration are widely used as ADSCs can differentiate into osteoblasts. In this study, the immunohistochemical analyses presented the new immune-positive bone tissue and bone trabeculae in the hydroxyapatite (HAP) group and HAP +ADSC group (Figure 2(a)). Meanwhile, the residual indents caused by nanoindentation testing at the maximum force of 50 mN were clearly visible (Figure 2(b)). Moreover, the ADSC-seeded scaffold construct was found to be much stiffer and harder than the unseeded scaffold (Figure 2(c)) [58].

To ensure the efficacy of ADSC-based therapeutic for bone regeneration, the related factors should be highly valued. Firstly, the subpopulations of ADSCs could affect osteogenic performance, such as pericytes and adventitial cells, which could improve angiogenic and osteogenic differentiation ability. Some authors defined CD146+ CD34- CD45- as pericytes and CD146- CD34+ CD45- as adventitial cells; these cells are isolated from multiple organs, including adipose tissue, possessing the capacity to differentiate into osteoblasts and displaying a synergistic function to promote bone healing [59]. In their trials, the sorted pericytes formed significantly more bone in comparison with unsorted cells.

Bone regeneration also involves a complex interaction between ADSCs and biological factors. The concentration of bioactive factors and the degree of tropism associated with the differentiation medium will affect the osteogenic potential of ADSCs [60]. ADSCs release growth factors that promote angiogenesis and enhance bone formation, including PDGF, VEGF, FGF-2, MMP, and bone morphogenic protein- (BMP-) 2 [61]. Recently, Yanai et al. showed that the

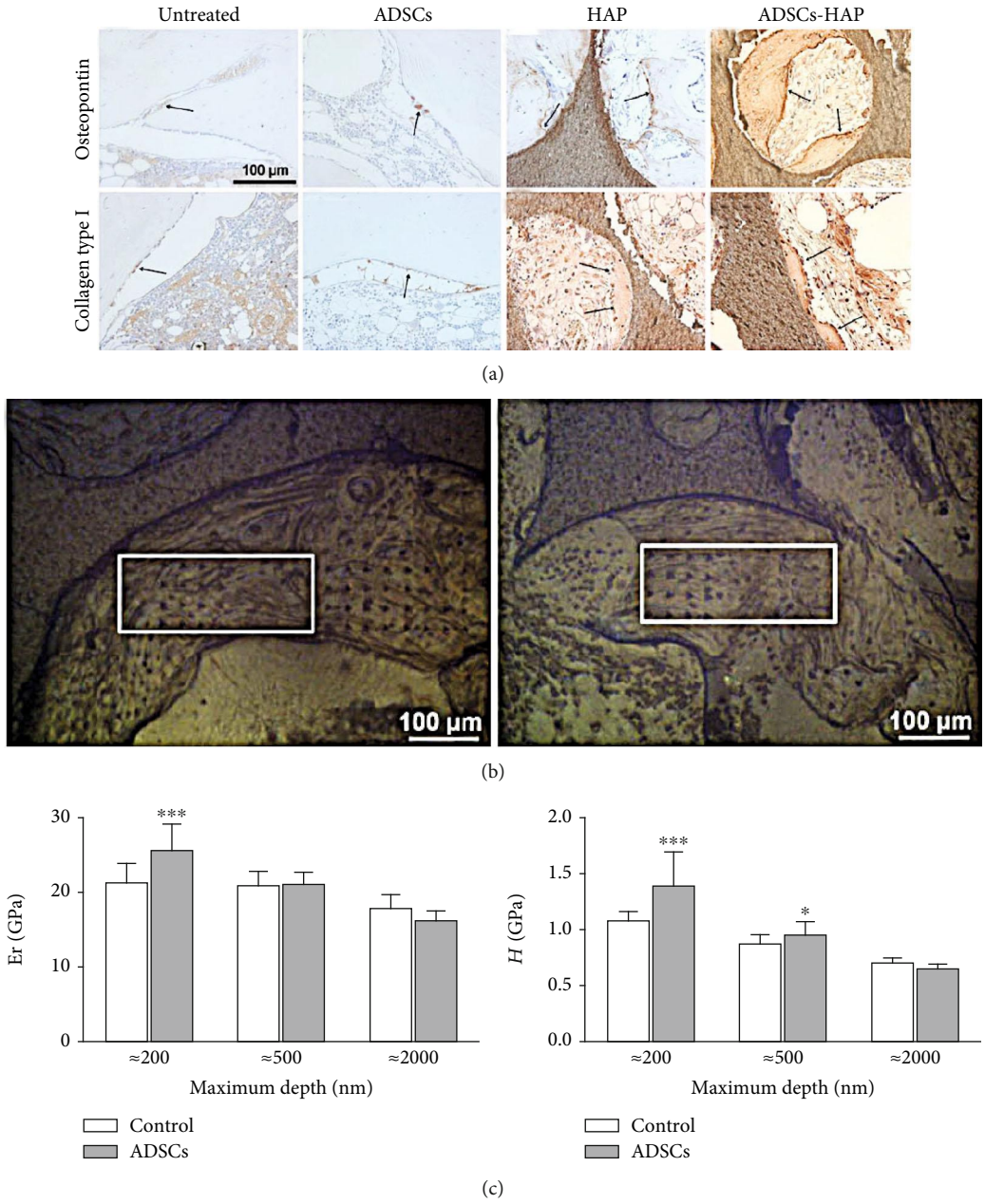


Figure 2: Results of the expression of bone markers and mechanical properties of scaffold construct (a). Expression of osteopontin and collagen type I in sections of decalcified tibial samples (b). Locations of the indentation experiments on the empty hydroxyapatite disk (control) and the ADSC-hydroxyapatite disk (ADSCs) (c). The reduced modulus Er and hardness H values of the two groups at the three loads were investigated. Adapted from a previous study [58], with permission.

expression level of BMP-2 can be enhanced *via* augmenting extracellular calcium concentrations; this increase activates the calcium-sensing receptor (CaSR), leading to a transient increase in intracellular calcium concentration and the stimulation of the calcium/calmodulin-dependent nuclear factor of activated T cell signaling pathway [62]. Another study reported that miRNA-375 promotes ADSC osteogenic differentiation through the Yes-associated protein 1/DEP domain containing mTOR interacting protein/protein kinase B (YAP1/DEPTOR/AKT) regulatory network [48]. Additionally, the inductive medium (ascorbic acid and dexamethasone) also affected the production of both osteogenic and angiogenic factors [63].

To be ideal bone graft substitutes, scaffolds must be biodegradable and biocompatible and exhibit strong osteoinductive properties. To date, ADSCs have been employed for BTE using several types of organic or inorganic scaffolds, including decellularized matrices, ceramics (e.g., HAP, tricalcium phosphate, coralline-derived HAP, calcium sulfates, glass ceramics, calcium phosphate-based cement, and bioglass), synthetic polymers and hybrid scaffolds (e.g., polylactic acid (PLA), polyglycolic acid (PGA), copolymer poly(lactic acid-co-

glycolic acid) (PLGA), and polycaprolactone (PCL)), and natural polymers (e.g., fibrin, collagen, gelatin, and silk) [64]. Different scaffolds have different advantages and disadvantages. The composition of synthetic polymers can be controlled, thereby reducing the risk of toxicity, immunogenicity, and the favoring of infection. However, the hydrophobicity of synthetic scaffolds can lead to problems in cell adhesion and infiltration [65, 66]. Ceramics exhibit osteoconductive properties and can bind directly to the bone under certain conditions [67]. However, owing to their slow degradation rate and low mechanical strength, they are not suitable for use as a loading scaffold alone [68, 69]. Hybrid scaffolds are composed of organic and inorganic materials that can gradually degrade without generating toxic byproducts. The type of structure acquires multiple functionalities with appropriate mechanical and thermal properties as well as structural stability [70]. Mazzoni et al. reported that porous hydroxylapatite/collagen composite biomaterials have excellent osteoinductive properties and show good biocompatibility [71].

The type of mechanical support also will affect osteogenic potential. Optimal porosity plays an essential role in directing the cells to grow into the desired physical form and to support the vascularization of the ingrown tissue [72]. Notably, total porosity and bone surface area are the main factors that must be controlled. Pore diameters of 150 mm were shown to improve endothelial cell function, as evidenced by the promotion of cell adhesion and migration, increased cell proliferation, and the enhanced expression of platelet-endothelial cell adhesion molecules (PECAMs) and VEGF [73]. A typical porosity of 90% and a pore size of at least 100 mm are known to be necessary for cell penetration and the proper vascularization of bone tissue. The mechanical properties of the scaffold are also affected by stiffness. Nii et al. used a poly(ethylene glycol) diacrylate platform to culture ADSCs and examine mineralization and osteocalcin gene expression and found that intermediate stiffness and a low concentration of fibronectin could increase osteocalcin gene expression by over 130-fold [74].

Based on *in vitro* experiments and preclinical studies, the capability of ADSCs to promote bone regeneration has been verified in clinical studies. A case report describing the repair of a posttraumatic calvarial defect using autologous ADSCs in a seven-year-old child was the first clinical study to be published on ADSC-repaired bone defects. Owing to the limited amount of autologous cancellous bone available from the iliac crest, the ADSCs were engrafted onto the calvarial defect. The postoperative course was uneventful, and computed tomography scans showed new bone formation and near-complete calvarial continuity 3 months after the reconstruction [75]. Current clinical ADSC therapies for bone regeneration have demonstrated promising results for craniofacial [76, 77] and lone bone defects [78]. Although *in vitro* experiments, preclinical trials, and clinical studies have confirmed the osteogenic differentiation ability of ADSCs, further investigations are still needed to standardize the procedures for the use of ADSCs in bone regeneration.

3.2. Cartilage Regeneration. Cartilage injury is a major cause of disability worldwide owing to the weak self-healing ability of cartilage tissue [79]. Currently, the clinically applied cartilage repair approaches include microfracture [80], subchondral drilling [81], and autologous chondrocyte implantation [82]; however, the limited availability and substantial associated donor site morbidity restrict their application [83]. The emergence of ADSC-based cartilage tissue engineering has received particular attention. CD146 is not a specific osteogenic marker in ADSC subpopulations; indeed, there is evidence that a CD146+ subset of ADSCs also has chondrogenic differentiation potential, as well as inflammation-modulating properties (Figures 3(a)–3(c)) [84]. Interestingly, CD146-negative subsets also have a similar cartilage differentiation ability [85]. The cell-biomaterial correlative structure established between surface receptor and adhesion molecules on the surface of materials enhanced the chondrogenic differentiation of ADSCs into articular chondrocytes [86]. On the other hand, the expression of the CD73, CD90, CD105, and CD106 markers is also necessary for ADSC differentiation into cartilage [87].

In addition to the use of specific subpopulations of ADSCs, biological factors are also indispensable for enhancing cartilage formation. *In vitro* studies have demonstrated that ADSCs can differentiate into chondrocytes when they are cultured with IGF-1, TGF-β, or BMP, and these chondrocytes express the same type II collagen as mature chondrocytes [49, 88, 89]. Several TGF-β members such as TGF-β1, 2, and 3 are known to possess good chondrogenic differentiation potential [90]. An induction medium containing a combination of TGF-β3 and BMP-6 has shown better chondrogenic potential than that containing TGF-β3 alone [91]. Moreover, L-ascorbic acid and PRP can maintain the survival of ADSCs and improve their expected chondrogenic function when delivered at an appropriate concentration [92]. Current studies have focused on the efficacy of PRP in cell differentiation and proliferation as it contains high concentrations of PDGF, TGF-β, IGF, VEGF, and EGF [93]. TGF-β positively regulates the transcription of chondrogenesis-related genes, including SRY-box transcription factor 9 (*SOX9*), through SMAD phosphorylation [94]. The SOX9 protein, one of the earliest chondrogenic markers, is essential for the expression of collagen type II [95]. Liao et al. discovered that the overexpression of SOX9 enhanced BMP2-induced chondrogenic differentiation and inhibited the osteogenic differentiation of MSCs [96].

Osteoarthritis (OA) is a progressive degenerative joint disease characterized by the deterioration of articular cartilage and pathological changes in the adjacent subchondral bone [97]. Current conventional treatments (physical therapy, glucosamine, chondroitin sulfate supplementation, and arthroscopic surgery) or surgical therapies (abrasion arthroplasty, subchondral drilling, and microfracture) are aimed at alleviating pain and enhancing joint function; however, they are limited by their low efficacy [98], and intra-articular (IA) injection of ADSCs to repair damaged cartilage has potential as a suitable alternative. Recently, Spasovski et al. suggested that the IA injection of a proposed dose of ADSCs may be a safe and efficient method for use in the treatment of osteoarthritis. During a 6-month follow-up, they found that the clinical symptoms had improved following an IA injection of ADSCs [99].

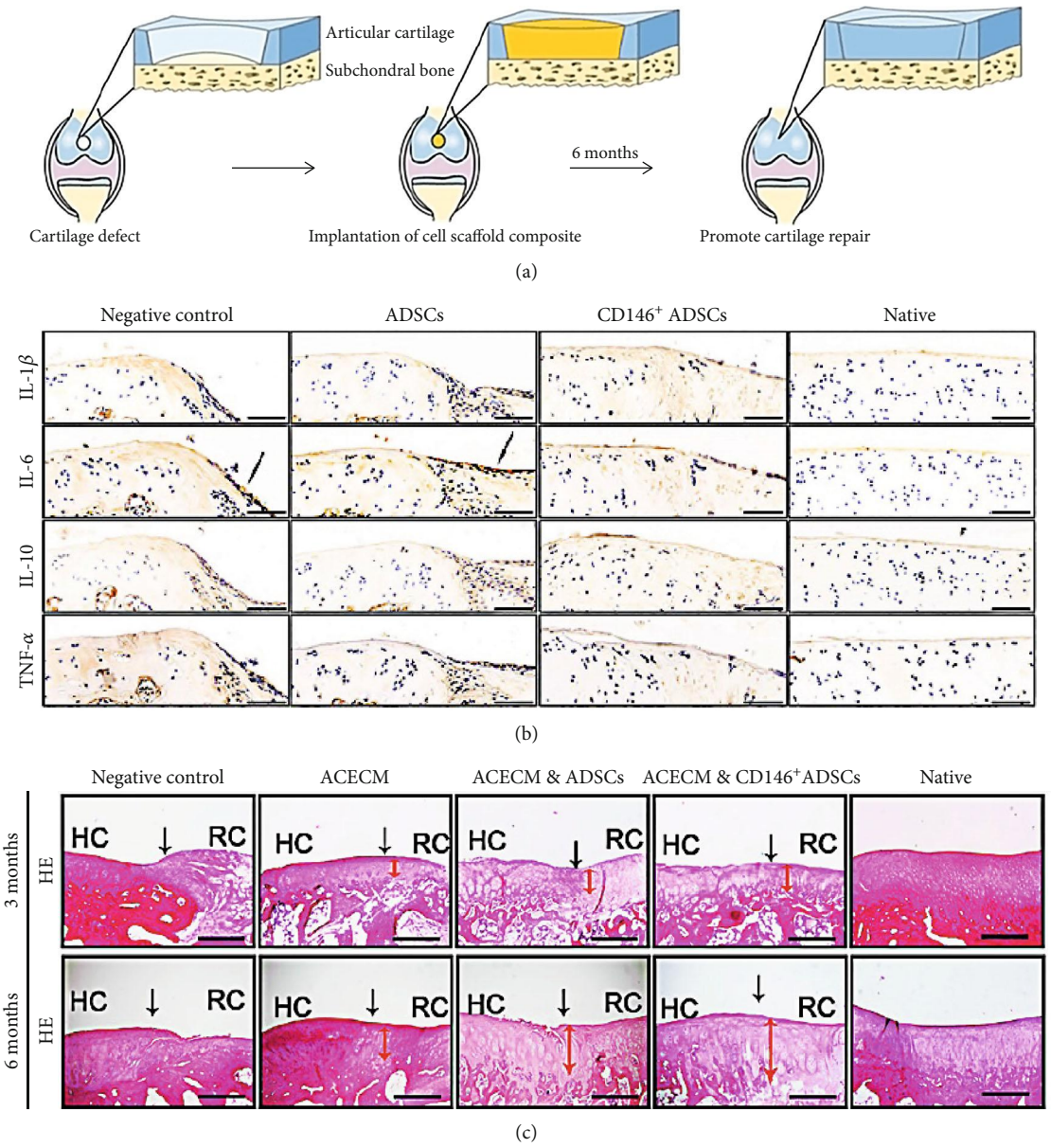


Figure 3: Flow chart of the experimental steps for long-term cartilage repair in rabbits (a). Immunohistochemical staining of interleukin- (IL-) 1β, IL-6, IL-10, and tumor necrosis factor (TNF). Black solid arrows denote the positive expression of IL-6 in the repair interface (b). Histological analysis of the cartilage defect after 3 and 6 months by hematoxylin and eosin (H&E) staining. Black solid arrows denote the repair interface. Red solid arrows denote the depth of the repaired cartilage (c). HC: host cartilage; RC: repaired cartilage. Adapted from a previous study [84], with permission.

Despite the marked clinical efficacy of IA, the dose and timing of ADSC injection are important. In a study aimed at evaluating the safety and therapeutic potential of autologous human adipose-derived mesenchymal stem cells in patients with osteoarthritis, 18 patients with knee osteoarthritis were enrolled and divided into three dose groups: low dose (1.0×10^7 cells), middose (2.0×10^7), and high dose (5.0×10^7); clinical, radiological, and histological parameters were evaluated with 96 weeks of follow-up. The high-dose group exhibited better pain relief and greater improvement in knee function than the other two groups [100]. Several studies have indicated that the inhibitory effect of ADSCs is affected by the stage of OA. In a mouse model, a single injection of ADSCs into the knee during the early stage of OA can inhibit synovial thickening, the formation of enthesophytes associated with ligaments, and cartilage destruction. However, no effect was observed in the late stage of the disease [101]. In addition, swelling of the injected joints is frequently observed and is thought to be associated with the survival rate of the ADSCs [102]. Directly injected cells usually have limited cell retention and survival rates, especially in large cartilage lesions. Koh and colleagues reported that ADSCs seeded in scaffolds may have better viability, preservation, and aggregation [103]. To improve the efficacy of this

procedure, as well as the comfort of the patients, appropriate cell-loaded scaffolds should be developed for treating patients with large cartilage defects.

The 3D structure of loaded ADSCs is a key for promoting the recovery of joint cartilage, and the materials, pore size, and rigidity of the scaffold must all be taken into consideration. Natural materials should favor cell adhesion and exhibit enhanced mechanical support and biodegradability [104]. Type I collagen is an appropriate scaffold as it induces low inflammatory responses and also has excellent cell compatibility. Recent findings have underlined that 3D collagen scaffold culture combined with PDGF and insulin promotes the chondrogenic differentiation of ADSCs [105]. Studies have confirmed that hydrogel-based scaffolding systems also allow for the creation of high-quality engineered cartilage but may exhibit inferior mechanical properties [106]. The replacement of a natural scaffold with a synthetic material allows the artificial adjustment of the pore size and stiffness of the structure. Based on the above characteristics, Yin et al. concluded that a TGF-β1-immobilized PLGA-gelatin scaffold seeded with ADSCs considerably enhanced the quality of the tissue-engineered cartilage [107]. The effect of scaffold pore size on chondrogenesis should also be taken into account. The proliferation and chondrogenic differentiation of stem cells are affected by scaffold porosity [108]. Scaffolds with smaller pore sizes (90–250 μm) are better for preserving cell adhesion and proliferation and also allow for higher expression levels of collagen, aggrecan, and type II collagen [109].

Sometimes, the cause of a knee injury may be a defect in the meniscus, and a degenerating meniscus leads to instability and a low level of nutrient supply to the cartilage. Intra-articular injection of stem cells can promote meniscus regeneration, and the immature meniscus will protect cartilage [110]. In conclusion, it is important to establish a therapeutic specification and provide suitable, patient-specific solutions.

3.3. Nervous System Regeneration. Studies have shown that ADSCs can differentiate into neurons, endothelial cells, and Schwann cells [111] and exhibit higher levels of neural marker expression and a faster proliferation rate than other stem cells [112]. The neural differentiation of ADSCs involves a complex regulatory network. ADSCs are known to release a range of neurotrophic factors, including NGF, BDNF, GDNF, FGF, and IGF-1, which are vital for the healing and regeneration of damaged nerves [113, 114]. Vascularization also plays a pivotal role in nerve healing by sustaining cell survival and promoting cell proliferation [115]. Furthermore, ADSCs also regulate antiapoptotic functions [116].

Controlling the inflammatory response could be thought of as another element in neural repair. TNFα-stimulated gene-6 (TSG-6) is a component of the negative feedback loop secreted by ADSCs [117]. It can reduce signaling in the resident macrophages and thereby modulates the cascade of pro-inflammatory cytokines. A growing body of evidence has confirmed the therapeutic potential of ADSCs in rebuilding the central nervous system and peripheral nervous system.

3.4. Central Nervous System (CNS) Regeneration. Several animal models of SCI and TBI have been developed to evaluate the efficacy and safety of ADSC-based therapy. Primary acute injury results mainly from the immediate external force exerted on the brain, whereas secondary injury occurs over time through a cascade of biochemical activation that leads to neuroinflammation and neurodegeneration; the latter is also the primary mechanism associated with subacute and chronic phases [118]. Current therapies for TBI focus primarily on suppressing the secondary insult. Xu et al. found that ADSCs can modulate TBI-induced neuroinflammation and subsequent secondary injury by increasing the ratio of M2 (anti-inflammatory) to M1 (proinflammatory) microglia (Figure 4(a)). The M1-related proinflammatory cytokines IL-6 and TNF and the M2-related anti-inflammatory cytokines TGF-β and TSG-6 have changed accordingly (Figures 4(b) and 4(c)) [119]. Additionally, ADSC-derived exosomes can inhibit the activation of microglia by downregulating nuclear factor kappa-B and the mitogen-activated protein kinase (MAPK) pathway and can also reduce the cytotoxicity associated with activated microglia [120]. Neuronal degeneration and blood vessel damage following a traumatic wound can induce inflammation, followed by the loss of neurons and oligodendrocytes. Therefore, controlling the inflammatory response after injury may have potential as a therapeutic option [121]. Yin and colleagues seeded ADSCs on acellular spinal cord scaffolds and demonstrated that this model enhanced functional recovery in spinal cord-injured rats by promoting axon regeneration and reducing reactive gliosis [122]. An ongoing multidisciplinary clinical trial also presents positive results [123]. In this trial, ADSCs are intrathecally injected at the L3–4 level. The subjective (physical therapy and occupational therapy reports) and objective (International Standards for Neurological Classification of Spinal Cord Injury scores) measures showed different degrees of improvement. For neurological disorders, such as amyotrophic lateral sclerosis [124], Alzheimer's disease (AD) [125], Huntington's disease [126], and Parkinson's disease (PD) [127], the treatment efficacy of ADSCs was confirmed in some animal and cell models. Many clinical trials are underway to test the efficacy and safety of ADSC-based treatment in AD and PD patients (ClinicalTrials.gov Identifier: NCT03117738 and NCT02184546).

3.5. Peripheral Nervous System (PNS) Regeneration. Peripheral nerve injury (PNI) is a complicated, multifactorial disorder with varying degrees of severity. During peripheral nerve repair, Schwann cells are the main factors promoting axonal regeneration in distal nerve stumps [128]. Recent studies have reported that ADSCs can differentiate into Schwann cells and facilitate native Schwann cell activity [129]. To bridge nerve defects, scientists have focused on nerve conduits and acellular nerve grafts combined with ADSCs. For conduit scaffolding, the tube was initially composed of silicon; however, highly biocompatible materials, such as autogenous vein nerve conduits, allografts, PGA, PCL, and collagen, are now used in tubes [130]. PGA-collagen conduits have been tested in a 15 mm gap model to compare the regenerative nerve effects of conduits combined with or without ADSCs and resected nerve [131]. PLA conduits and cell therapy with ADSCs lead to a better functional and

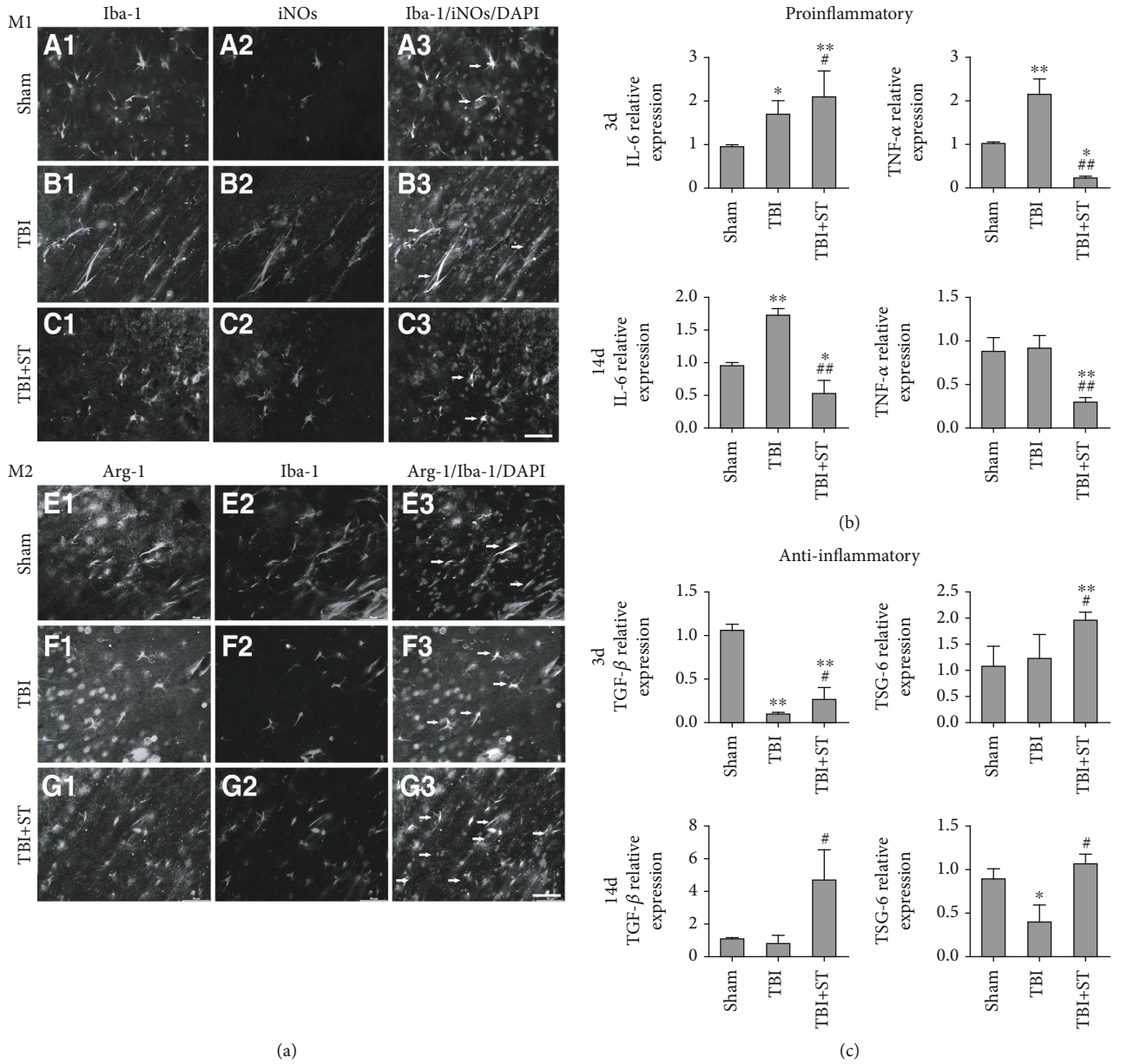


Figure 4: Double immunostaining with anti-iNOS and anti-Iba-1 antibodies to identify M1 and M2 microglia in the cortex within 1 mm of the lesion in the sham, TBI, and TBI+secretome of ADSCs (TBI+ST) groups 7 days after traumatic brain injury (TBI) (a). Cytokine expression levels at 3 and 14 days after TBI were evaluated by qPCR (b, c). iNOS: inducible nitric oxide synthase; Iba-1: ionized calcium-binding adaptor molecule 1; Arg-1: arginase 1. Adapted from a previous study [119], with permission.

morphological recovery after sciatic nerve transection. Nerves in the ADSC experimental group showed a greater number of myelinated fibers and better tissue organization with well-defined fascicles compared with the Dulbecco's modified Eagle's medium (DMEM) experimental group (Figures 5(a)–5(f)). The total number of myelinated fibers was significantly greater in the ADSCs and normal group compared with that in the DMEM group (Figure 5(g)). Meanwhile, a quantitative morphological analysis of the axon area, fiber area, myelin area, and G-ratio in the regenerating sciatic nerve did not show statistically significant differences among the experimental groups (Figures 5(h)–5(j)) [132]. However, to date, experiments have been conducted using small animals, and future evaluations will inevitably have to include larger animals to allow the progression toward clinical applications.

3.6. Myocardium Regeneration. Cardiovascular disease (CVD) is the leading cause of death globally and can lead to ischemia in critical regions, as well as myocardial necrosis. Ischemic heart disease, particularly myocardial infarction (MI), is a typical type of CVD that can cause heart failure [133]. ADSC therapy has been widely investigated as a prospective treatment for MI in preclinical and clinical trials. The mechanics of the therapeutic application of ADSCs in CVD can be classified into three categories: the differentiation

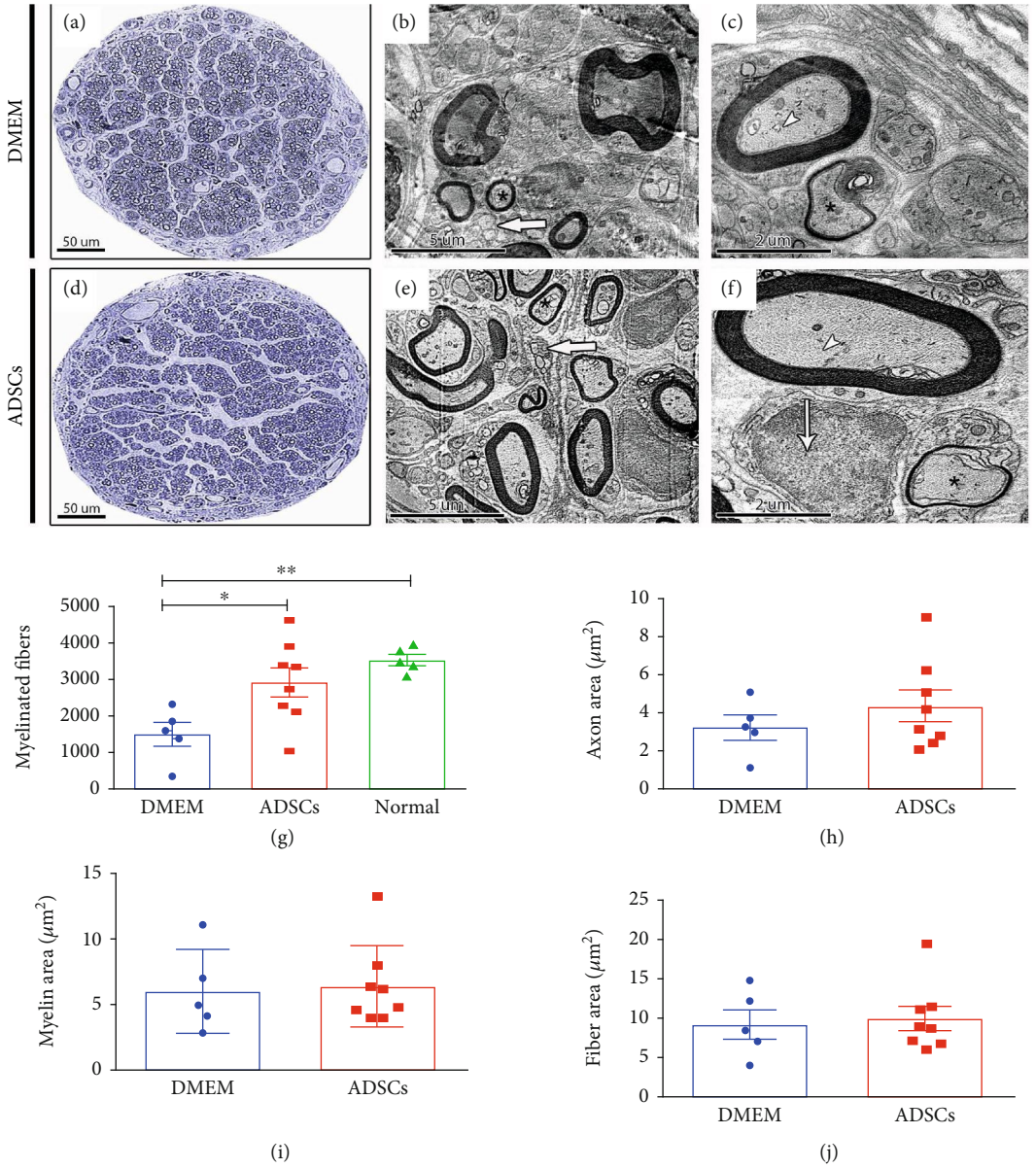


Figure 5: Representative images of semithin cross-sections of the regenerating sciatic nerve in the Dulbecco's modified Eagle's medium (DMEM) and ADSCs (a–c). Electron micrographs of a regenerating sciatic nerve in the transverse plane (b, c, e, f). Graph showing the total number of myelinated fibers in the sciatic nerve for all the groups (g). Quantitative morphological analyses of the axon area, fiber area, and myelin area in the regenerating sciatic nerve (h–j). Adapted from a previous study [132], with permission.

of ADSCs into cardiomyocytes [134]; supplying a large amount of antiapoptotic, angiogenic, and anti-inflammatory factors [135, 136]; and preventing adverse cardiac remodeling by inhibiting myocardial fibrosis [137]. To date, four different transplant methods—intramyocardial injection, intravenous injection, intracoronary injection, and cell spray transplantation—have been intensively investigated. Although the effect of the intravenous injection is affected by a pulmonary first-pass effect, this method showed a beneficial influence on reducing infarct size and enhancing cardiac function and blood vessel formation [138]. For left ventricular (LV) systolic function, both intramuscular injection and intracoronary injection show a promising ability to improve the left ventricular ejection fraction (LVEF) [139]. Bobi et al. reported that intracoronary injection suppressed the apoptosis of infarcted myocardium but did not significantly change the LVEF [140]. Stem cell spray transplantation markedly attenuated left ventricular remodeling and enhanced vascular density in the infarct border area [141].

The efficacy of ADSC injection into the infarcted myocardium remains limited by low survival and retention rates. Numerous attempts have been made using preconditioning and engineering strategies to overcome these hurdles. Guo et al. found that resistin-treated ADSCs intravenously injected into mice with myocardial ischemia significantly improved the LVEF, mitigated fibrosis, and reduced cardiomyocyte apoptosis [142]. The same effect was found with melatonin pretreatment [143]. The engineered ADSCs

enhanced retention, increased angiogenesis, reduced the degree of fibrosis, and decreased infarct size. When compared with ADSCs alone, transglutaminase cross-linked gelatin (Col-T gel) combined with ADSCs markedly reduced the size of the myocardial fibrotic area (Figures 6(a) and 6(b)). T gel-ADSCs significantly increased the LVEF at 4 weeks after MI. Additionally, T gel-ADSCs significantly decreased the left ventricular end-systolic diameter (LVESD), but not the left ventricular end-diastolic diameter (LVEDD), at 4 weeks after MI when compared with PBS treatment (Figure 6(c)) [144]. Furthermore, the decellularized extracellular matrix created a favorable microenvironment for ADSCs in the infarct area, reducing fibrosis and increasing the LVEF [145]. Genetic modification, which can be used to enhance the secretion of stromal cell-derived factor 1 (SDF-1), IGF-1, VEGF, HGF, and FGF-2, has been extensively investigated in heart regeneration. This approach is correlated with reduced cardiomyocyte apoptosis and enhanced angiogenesis [146, 147].

Convincing evidence obtained in preclinical ADSC transplantation studies on MI has prompted several clinical trials. The APOLLO trial was a randomized, double-blind, placebo-controlled, phase I/II study (NCT00442806) to test the feasibility of using ADSC transplantation for the treatment of STEMI [148]. The results showed that ADSC infusion could improve cardiac function and perfusion defects, accompanied by a 50% reduction in myocardial scar formation. ATHENA trials I (NCT01556022) and II (NCT02052427) focused on assessing intramyocardial ADSC transplantation. In this trial, ADSC treatment promoted a marked increment in Minnesota Living with Heart Failure Questionnaire (MLHFQ) and SF-36 scores, while heart failure and angina symptoms also improved. However, no significant changes were found in the LVEF or LV volumes by echocardiography. Further detailed and comprehensive clinical trials are needed to achieve more precise and accurate benefits in delaying ventricular remodeling and heart failure development.

3.7. Liver Regeneration. Acute liver failure (ALF) and chronic liver disease are mainly caused by exposure to factors such as viral infection, toxins, and genetic disorders. ADSC-based therapy is a promising alternative for the treatment of these disorders. ADSCs can differentiate into several types of liver cells and secrete antiapoptotic or anti-inflammatory factors, thereby promoting the healing of liver injury [149, 150].

Ischemia-reperfusion injury (IRI) is a universal complication of liver surgery, often leading to postoperative complications and liver dysfunction. Ge et al. injected ADSCs into the liver parenchyma following partial laparoscopic hepatectomy [151]. ADSC treatment increased the activity of superoxide dismutase and suppressed the generation of both myeloperoxidase and malondialdehyde, thereby reducing oxidative stress. Additionally, ADSC treatment led to a marked decline in the levels of adverse hematological indicators, such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin (T-BIL), and lactate dehydrogenase (LDH). A different study reported that ADSCs suppressed the level of inflammatory cytokines such as IL-1β, IL-6, and TNF, while enhancing the secretion of the anti-inflammatory factor IL-10 and the regenerative factors HGF and cyclin D1, thereby ameliorating the IRI-induced damage [152]. Similar hepatoprotective effects were also found in other trials [153]. In a carbon tetrachloride-(CCl_4-) induced acute liver injury model, Yan et al. intravenously injected ADSCs to assess their effects on acute liver injury [154]. ADSC treatment reduced the serum concentrations of ALT, AST, and T-BIL and restored the liver structure and glycogen synthesis ability in the canine model animals.

Liver fibrosis is a frequent outcome of chronic liver disease and is characterized by hepatocyte death, hepatic inflammation, and activation of hepatic stellate cells (HSCs) [155]. Studies to date have shown that ADSCs suppress the expression of inflammatory cytokines and the proliferation of alpha-smooth muscle actin-positive activated HSCs [156]. Hao and colleagues showed that ADSC transplantation markedly attenuated liver fibrosis by inhibiting HSC proliferation and promoting HSC apoptosis in animals with CCl_4-induced liver fibrosis [157]. For the treatment of liver diseases, the ADSCs that are functionally reinforced through pretreatment have greater therapeutic efficacy. Forkhead box transcription factor 2- (FOXA2-) overexpressing ADSCs loaded in a PLGA scaffold markedly reduced the size of the necrotic area and improved liver function in an acute liver injury model (Figures 7(a)–7(c)). The FOXA2-overexpressing experimental group showed greater glycogen storage ability (Figure 7(c), i and ii). The necrotic area was significantly lower in the FOXA2-overexpressing ADSC/scaffold group than in the other groups (Figure 7(c), iii) [158]. ADSCs cultured in hypoxia-conditioned media induced higher expression of antioxidant enzymes and nuclear factor erythroid 2-like 2 (Nrf2), thereby protecting against reactive oxygen species-related toxicity in the injured liver [159]. Many clinical trials have been designed to confirm the efficacy and safety of ADSCs in patients with liver cirrhosis or ALF. In these trials, ADSC transplantation did not raise any safety concerns. Besides, tests that measure liver function, such as the ^{13}C methacetin breath test, METAVIR score, Child-Pugh score, and MELD score, have yielded positive results [160, 161]. Combined, these findings suggest that ADSC transplantation is a promising therapeutic option for the treatment of liver injury. However, additional clinical trials with large sample size are needed to convincingly show the benefits of using ADSCs.

3.8. Skin Wound Healing. Preclinical and clinical trials have recently greatly improved the use of ADSC therapy for the treatment of severe burn injuries and intractable ulcers [162], which involves the interaction of many soluble factors and the activation of multiple biological pathways. Angiogenesis-related cytokines released from ADSCs, such as G-CSF, PDGF, SDF-1, VEGF, b-FGF, HGF, MMP, IL-6, and IL-8, promote the recovery of wound blood supply [163]. ADSCs can not only enhance the migration and proliferation of fibroblasts but also inhibit collagen deposition and the expression of α-smooth muscle actin in hypertrophic scar fibroblasts [164]. ADSCs differentiate into skin stem cells and promote the accumulation of autologous skin stem cells via the epithelial growth factor receptor/methyl ethyl ketone/extracellular regulated protein kinase (EGFR/MEK/ERK) pathway to accelerate wound

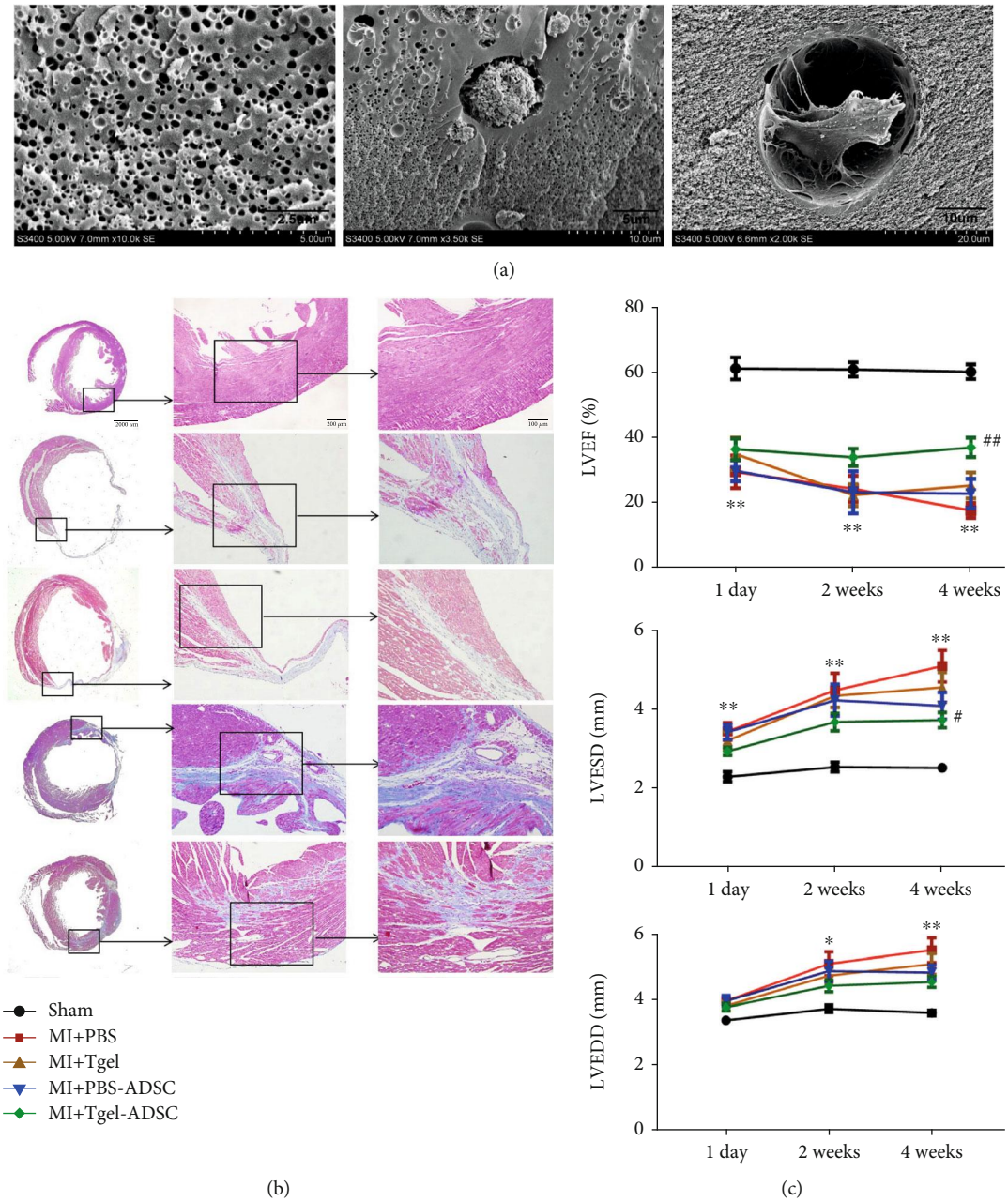


Figure 6: Scanning electron micrographs of Col-T gel-encapsulated ADSCs 3 days after encapsulation (a). Representative images of Masson trichrome staining of the transverse planes of heart sections (b). LVEF, LVESD, and LVEDD at 1 day, 2 weeks, and 4 weeks after myocardial infarction (c). LVEF: left ventricular ejection fraction; LVESD: left ventricular end-systolic diameter; LVEDD: left ventricular end-diastolic diameter. Adapted from a previous study [144], with permission.

healing (Figures 8(a)–8(c)). Xiong et al. established a wound model of seawater (SW) immersion and compared it with normal wound healing. The results showed that the protein expression level of EGF was significantly higher in the control and the SW+ADSC groups than in the SW group or the SW +DMEM group (Figure 8(b)). Microscopic observations of wound sections showed that the skin in the SW and the SW +DMEM groups was significantly thinner than that in the control and SW+ADSC groups (Figure 8(c)) [165]. During wound healing, a reduction in wound inflammation is associated with a switch in macrophage polarization from a proinflammatory (M1) to a prorepair (M2) phenotype [166]. However, the examination of local ADSC injection always revealed reduced cell viability, which ensued from shear stress during the treatment.

ADSCs combined with a scaffold substantially improve the proliferative, differentiation, and paracrine signaling abilities of ADSCs. Li et al. discovered that ADSCs seeded on a collagen 3D scaffold could better differentiate into keratinocytes and epithelial cells than those seeded on a two-dimensional niche [167]. An *in situ* formed hydrogel system that could easily cover irregularly contoured burn wounds

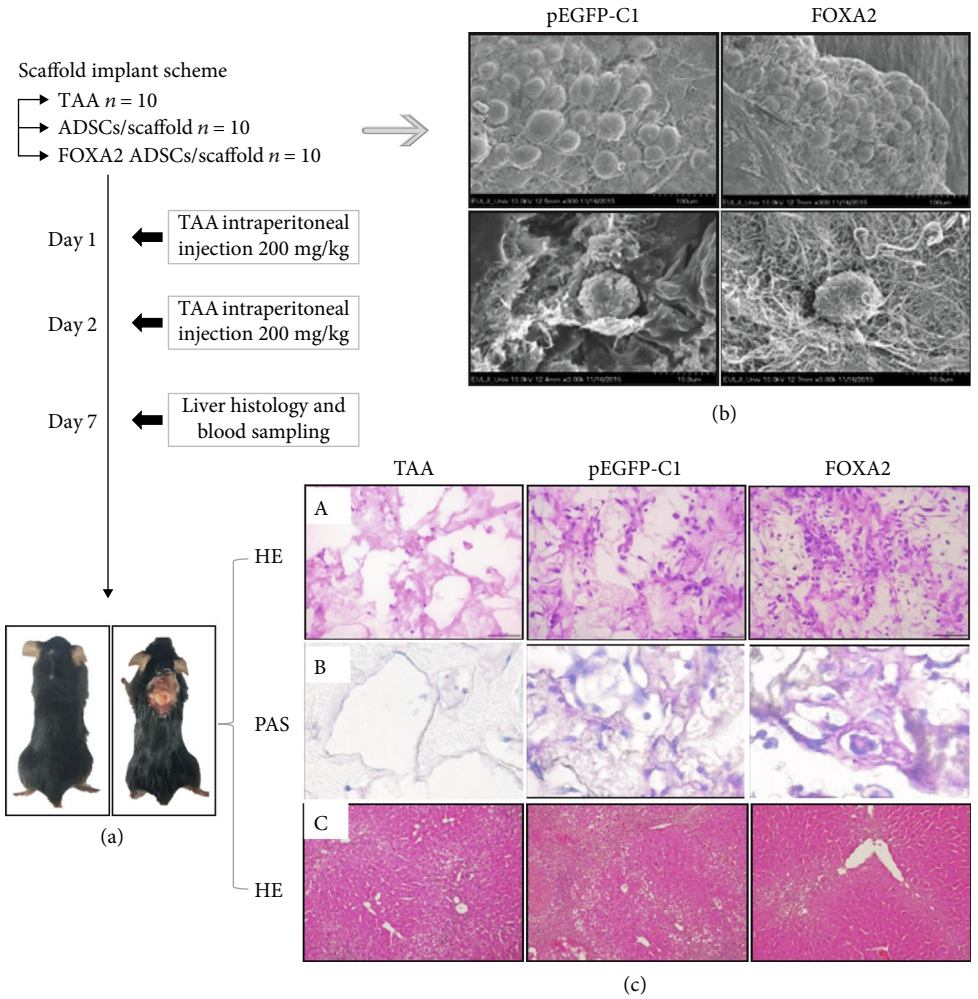


Figure 7: A schematic representation of the experimental design (a). Scanning electron micrographs of ADSCs in a pEGFP-C1-transfected ADSCs/scaffolds and FOXA2-transfected ADSCs/scaffolds (b). Hematoxylin and eosin (H&E) staining of the necrotic area and retrieved scaffolds (c). TAA: thioacetamide. Adapted from a previous study [158], with permission.

significantly enhanced neovascularization, accelerated wound closure, and reduced scar formation [168]. An acellular dermal matrix (ADM) combined with ADSCs attenuated inflammation in diabetic wounds and promoted wound healing. Meanwhile, immunohistochemical staining following ADM-ADSC treatment showed increased expression of EGF, Ki-67, and prolyl 4-hydroxylase and reduced expression of CD45 [169]. Ding et al. utilized Bcl-2-modified ADSCs embedded within collagen scaffolds in the treatment of diabetic wounds. This frame significantly improved wound healing, promoted neovascularization, and shortened healing time compared with the control group [170]. ADSC-based cell-free therapy and scaffold-free culture systems for repairing wounds have attracted a great deal of attention. ADSC-derived supernatants stimulate wound healing by increasing the proliferation of fibroblasts, endothelial cells, keratinocytes, and cells of human skin origin [171]. A scaffold-free culture system, called adipose-derived stem cell sheets, could inhibit CCL2 release and macrophage recruitment *via* secreting greater amounts of C1q and TNF-related 3 (C1QTNF3) in the wound area. Moreover, no transplanted ADSCs were found in the fourth week, thereby reducing the undesirable long-term side effects associated with ADSC transplantation [172]. ADSCs have also proved beneficial for chronic radiation skin injuries and ischemia-reperfusion injuries of the flap [173–175].

Compared with animal experiments, comparatively few clinical trials have been performed to evaluate ADSC treatments. Jo et al. used ADSC transplantation to repair facial skin defects in four patients and reported that the defects were rapidly covered over by the patients' regenerated tissue [176]. ADSC therapy was also effective and safe when used for the treatment of 10 cases of decade-long radiation injuries [175]. In contrast, a clinical study [177] reported that a single treatment with autologous fat grafts was insufficient to ameliorate mature pediatric burn scars, although this may have been due to the small sample size. This indicates that more accurate and rigorous trials are needed to assess the therapeutic effects of ADSCs on wound healing.

3.9. Other ADSC-Based Treatment Modalities. In the past few years, a wide variety of methods, particularly drug therapies, have been proposed as treatments for eye disorders.

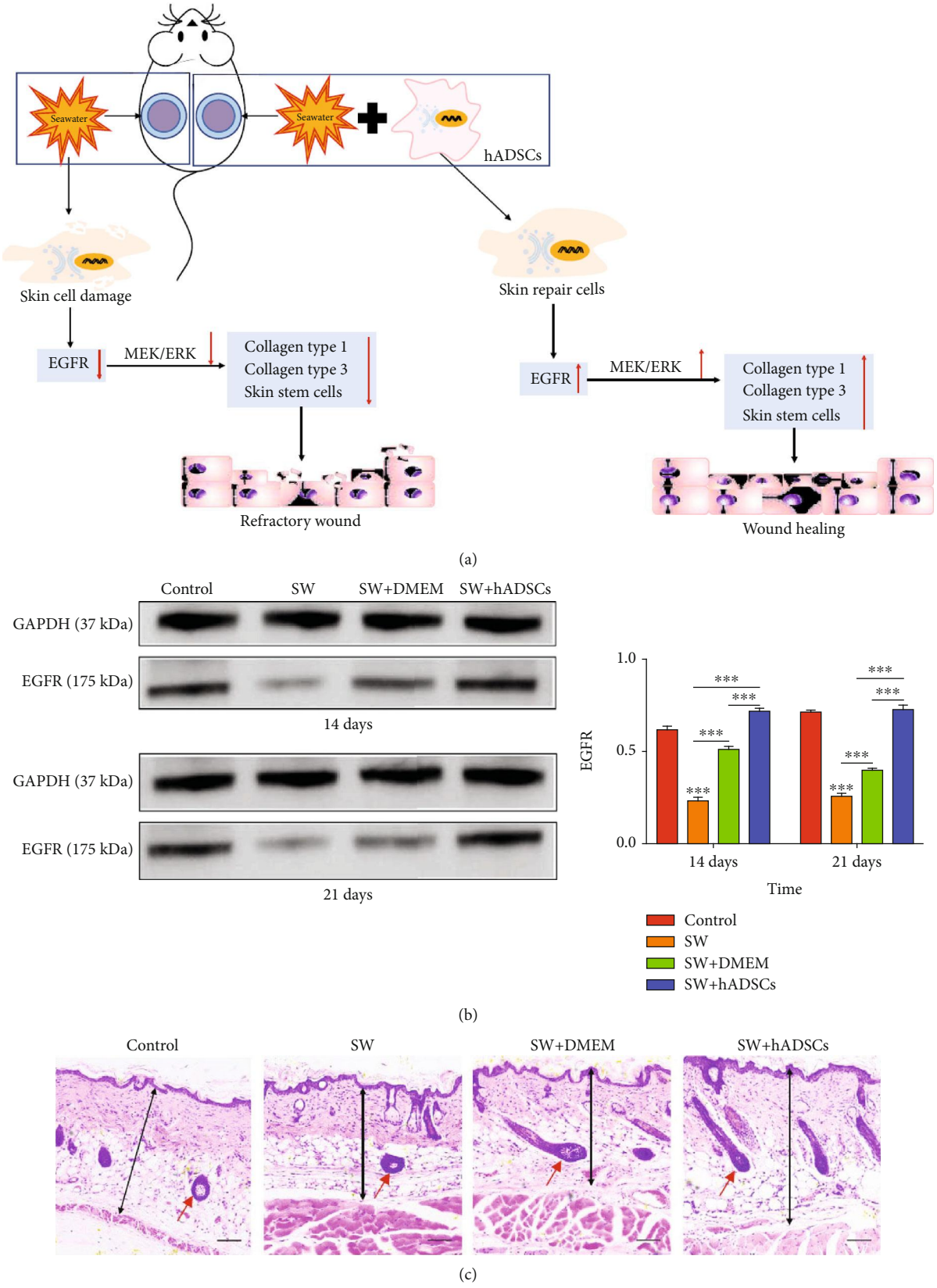


Figure 8: Schematic showing how seawater (SW) and adipose-derived stem cells (ADSCs) regulate wound healing through the EGFR/MEK/ERK signaling pathway (a). The EGF protein expression levels were significantly higher in the control and SW+ADSC groups than in the SW and SW+DMEM groups (b). Hematoxylin and eosin (H&E) staining for wound repair, skin thickness, and a number of subcutaneous appendages (c). The red arrow denotes a hair follicle. Adapted from a previous study [165], with permission.

Table 2: Summary of *in vivo* application of ADSCs in experimental disease animals.

	Application	ADSC source	Administration route	Animal model	Results
Arrigoni et al. [58]	Bone	Rabbit	Surgical implantation	Rabbit	Bone formation with the ADSC was demonstrated by a significant increase in bone content
Chen et al. [48]	Bone	Human	Surgical implantation	Mice	Overexpression of miR-375 significantly enhanced ADSC osteogenesis both *in vitro* and *in vivo*
Li et al. [84]	Cartilage	Human	(i) Injection	Rat	ADSCs showed a better inflammation-modulating property
			(ii) Surgical implantation	Rabbit	ADSCs with scaffold promoted cartilage regeneration in the long term
Cho et al. [49]	Cartilage	—	Surgical implantation	Rabbit	The quality of regenerative cartilage significantly improved in the ADSC group
Huurne et al. [101]	Cartilage	Mouse	Injection	Mice	The ADSC-based treatment could inhibit synovial thickening, the formation of enthesophytes associated with ligaments, and cartilage destruction
Yin et al. [107]	Cartilage	Rabbit	Surgical implantation	Rabbit	ADSCs containing the TGF immobilized scaffold better-promoted cartilage regeneration in defective articular cartilage
Hu et al. [113]	Nerve	Rat	Surgical implantation	Rat	Improved nerve regenerative ability for ADSC group compared to control
Kingham et al. [114]	Nerve	Human	Surgical implantation	Rat	Both ADSCs and stimulated-ADSCs could promote axon regeneration
Li et al. [116]	Nerve	Rat	Injection	Rat	ADSCs alleviated neurological deficits and reduced brain water content in rats
Durco et al. [132]	Nerve	Human	Surgical implantation	Mice	The number of nerve fibers and motor plates was higher in the ADSC group
Nagata et al. [134]	Myocardium	Mice	Transfusion	Mice	The transfusion of ADSCs exhibited the highest cardiac functional recovery and the high frequency of the recruitment to ischemic myocardium
Bobi et al. [140]	Myocardium	Pig	Injection	Pig	Myocardial perfusion at the anterior infarct border increased in ADSC-treated animals
Mori et al. [141]	Myocardium	Human	Surgical implantation	Porcine	Left ventricular remodeling attenuated and vascular density increased in the infarct border area in the ADSC group
Qiao et al. [145]	Myocardium	Rat	Injection	Rat	ADSC and dECM groups could increase angiogenesis, reduce the degree of fibrosis, and decrease infarct size
Ge et al. [151]	Liver	Pig	Injection	Pig	AST, ALT, T-BIL, and LDH were significantly decreased in ADSC treatment
Jiao et al. [152]	Liver	Pig	Injection	Pig	ADSC transplantation ameliorated the IRI-induced histopathological damage
Zhang et al. [153]	Liver	Pig	Injection	Pig	ADSC group promoted liver function recovery, reduced oxidative stress, and promoted liver regeneration
Yan et al. [154]	Liver	Canine	Injection	Canine	AST and ALT were rapidly decreased in ADSC treatment
Nishiwaki et al. [162]	Skin	Mice	Surgical implantation	Mice	ADSCs contributed to wound healing in a dorsal skin defect model in diabetic mice
Xiong et al. [165]	Skin	Human	Injection	Mice	ADSCs significantly accelerated the healing of skin wounds by promoting cell proliferation
Chou et al. [169]	Skin	Rat	Injection	Rat	The wound treated with ADM-ADSCs showed a significantly higher wound healing rate than other groups
Yu et al. [172]	Skin	Human	Surgical implantation	Mice	The neoskin formed in the presence of ADSC exhibited a thickness comparable to normal skin and possessed a highly organized collagen structure
Nakamura et al. [180]	Trachea	Rat	Surgical implantation	Rat	The mucociliary transport function was improved by ADSC transplantation
Jin et al. [181]	Bladder	Rat	Surgical implantation	Rat	The rat bladder repair effect was better in the ADSC group

Table 3: Summary of clinical studies on treatments with ADSCs.

	Study type	Application	Cell source	Administration route	Patients	Follow-up time (month)	Results
Lendeckel et al. [75]	Case	Bone	Human	Implantation	1	3	The CT scans showed new bone formation and near-complete calvarial continuity
Sándor et al. [76]	Case	Bone	Human	Implantation	13	12-52	Successful integration of the surrounding skeleton; the construct was noted in 10 of the 13 cases
Thesleff et al. [77]	Case	Bone	Human	Implantation	5	79.2	The clinical results are not superior to results achieved by conventional cranial repair methods
Vériter et al. [78]	Case	Bone	Human	Implantation	17	1-54	ADSC therapy is safe and feasible for clinical indications
Spasovski et al. [99]	Case	Cartilage	Human	Injection	9	18	MOCART score showed significant cartilage restoration
Song et al. [100]	Comparative	Cartilage	Human	Injection	18	24	The high-dose group of ADSCs exhibited the highest improvement
Pak et al. [102]	Case	Cartilage	Human	Injection	91	30	VAS improved 50–60% No major complications
Koh et al. [103]	Case	Cartilage	Human	Injection	44	24	94% patients excellent or good satisfaction; 76% abnormal repair
Bydon et al. [123]	Case	Nerve	Human	Injection	1	18	The subjective and objective measures showed different degrees of improvement
Konstanty-Kalandyk et al. [136]	Case	Myocardium	Human	Injection	15	1	No major complications
Houtgraaf et al. [148]	Comparative	Myocardium	Human	Injection	10	6	ADSC infusion could improve cardiac function and perfusion defects, accompanied by a 50% reduction in myocardial scar formation
Huang et al. [160]	Case	Liver	Human	Injection	6	6	The METAVIR score, Child-Pugh score, and MELD score showed positive results
Gotze et al. [161]	Case	Liver	Human	Injection	3	1-2	The reduction of liver stiffness and increase of ^{13}C methacetin breath test outcome were observed
Jo et al. [176]	Case	Liver	Human	Injection	4	—	In these cases, they observed rapid coverage of the wound with the patient's regenerated tissue

Nevertheless, there is still a lack of effective treatments for corneal injury and retina or optic nerve degeneration, and ADSC transplantation has increasingly been used for this purpose. ADSC transplantation accelerated recovery from corneal epithelial damage, as evidenced by the proliferation of corneal epithelial cells, reduced levels of inflammation-related cytokine levels, and increased numbers of M2 macrophages [178]. To date, the feasibility of using ADSCs for stabilizing the retinal microvasculature has been conclusively established in the diabetic retinopathy model [179].

Additionally, the ADSC-loaded collagen sponge is an effective strategy to repair the tracheal defect and recover the motility function of cilia [180]. Similarly, studies have found that ADSCs seeded onto an RNA-bladder acellular matrix graft scaffold could promote bladder regeneration [181]. Current evidence supports the possibility that ADSC-based therapeutic is an important site of tissue regeneration.

The details of the aforementioned studies on animals and clinical studies on humans are summarized in Tables 2 and 3.

4. The Potential Risk for Tumor Invasiveness Associated with ADSC-Cancer Cell Interactions

Despite the large number of preclinical and clinical studies reporting the potential of stem cells to act as an "off-the-shelf" therapy for the repair and regeneration of damaged tissues, the clinical application of ADSCs remains limited. Several studies have demonstrated that the proliferative and

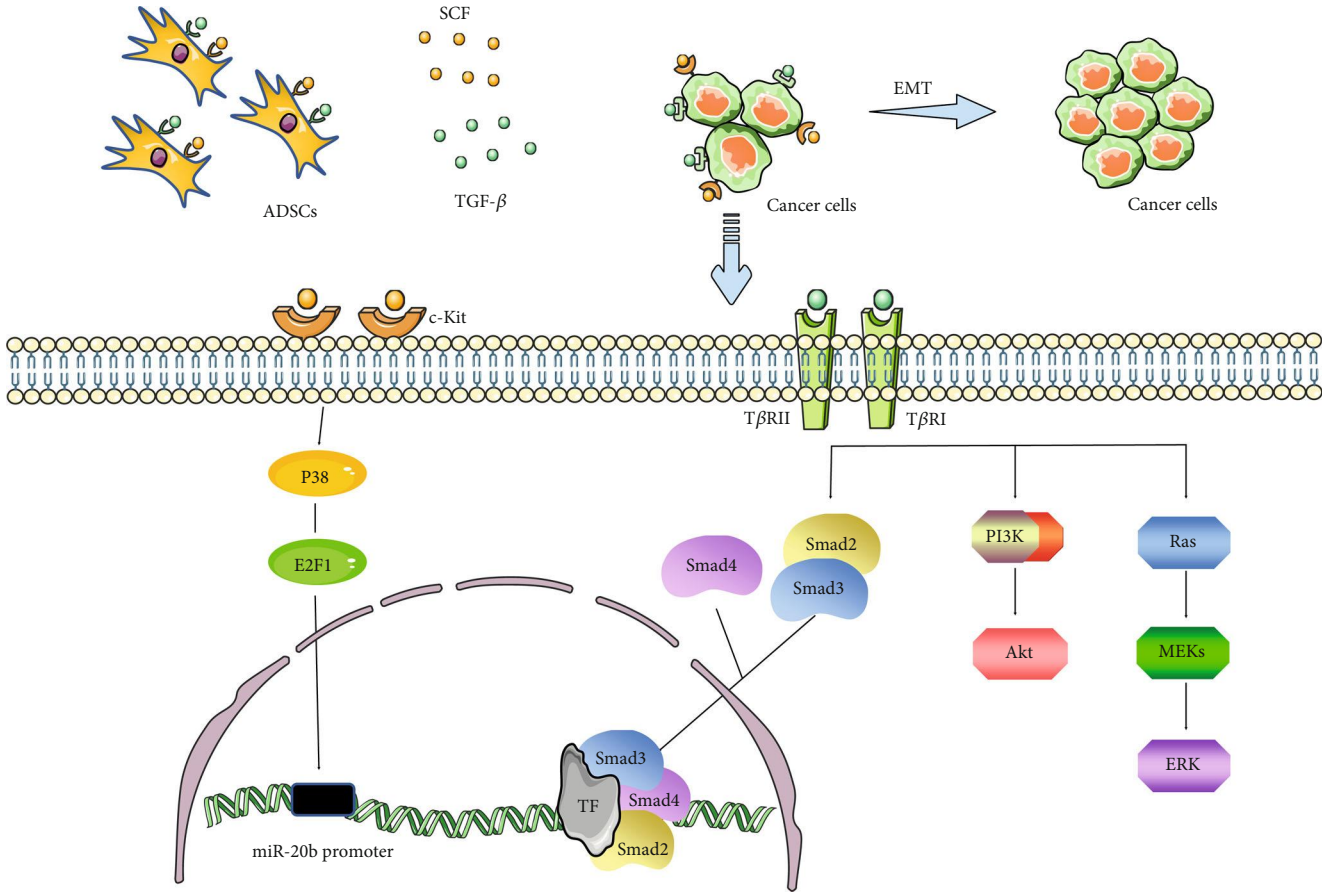


Figure 9: ADSCs promote the migration, invasion, and mesenchymal-epithelial transformation of cancer cells by secreting TGF-β and SCF. TF: transcription factor; TβR: TGF-β receptor.

invasive ability of breast cancer cells is increased following interaction with ADSCs. Cancer stem cells, also called tumor-initiating cells, represent a subpopulation of cancer cells displaying long-living, drug-expelling, and antiapoptotic properties [182]. Chan et al. found that hybrids produced through the spontaneous fusion of ADSCs and breast cancer cells express markers characteristic of breast cancer stem cells [183]. Additionally, the expression of HIF-1α/VEGF and the metastasis of breast cancer cells were induced *via* the downregulation of miR20b by ADSC-released stem cell factor (SCF), and this process was dependent on the activation of the c-Kit/p38-MAPK/E2F1 signaling pathway [184] (Figure 9). ADSCs are associated with the activation of epithelial to mesenchymal transition (EMT), another crucial step in the switch toward a more invasive phenotype. ADSCs can stimulate the expression of EMT-associated transcription factors, likely through TGF-β/SMAD-dependent and SMAD-independent phosphatidylinositol 3 kinase (PI3K)/AKT signaling pathways [185] (Figure 9).

The findings of the procarcinogenic role of ADSCs in laboratory studies appear to be contradictory to those of clinical reports. In the RESTORE-2 trial, the 67 enrolled patients were treated with ADSCs for the reconstruction of postoperative breast defects [186]. No treatment-related serious adverse events or local cancer recurrences were reported during the follow-up. Clinical data on the oncological safety of ADSCs are predominantly derived from female breast cancer patients, while follow-up times have been sufficiently long. Thus, further clinical studies are needed to determine whether ADSC-based regenerative therapy can be safely used for the treatment of other disorders.

5. Current Challenges and Future Directions

The application and development of ADSC therapy present more systematic and professional theoretical support for tissue engineering and regenerative medicine: (1) abundance and easy access, (2) immunomodulatory and anti-inflammatory effects, (3) autocrine and paracrine functions through the generation of chemokines and growth factors, and (4) the ability to differentiate into damaged tissue- and organ-specific cell types. However, ADSCs are not available as a ready-to-use product, and some key challenges remain.

Immunoreactivity is one of the greatest challenges. During ADSC culture, 10–20% FBS or calf serum is commonly used; however, the risk inherent to animal-derived products remains a concern. Contamination with viruses, prions, mycoplasmas, or unidentified xenogeneic proteins from animal-derived serum has the potential to cause immunological reactions in patients. In addition, xenobiotic growth factors may disturb ADSC differentiation and proliferation.

Therefore, to avoid these risks, serum-free or xeno-free culture media without animal serum should be developed.

Genetic modification is a widely used tool for enhancing repair efficiency. Virus-associated gene transfection has been the mainstay for gene therapy to extend the functions of ADSCs. However, this procedure is inevitably associated with safety concerns, including immune reactions and vector-mediated genotoxicity. The latter may manifest as inflammation, insertional mutagenesis, and activation of protooncogenes [187]. Oncogenesis primarily occurs due to promoter insertion, promoter activation, or truncation of gene transcripts. Despite years of research and numerous clinical trials, only two gene therapy treatments, Glybera and Strimvelis, have been approved for clinical use, indicating that the choice of preclinical and clinical trial populations is important to ensure efficacy and safety.

Hladik et al. indicated that the ability of MSCs to recognize DNA double-strand breaks is gradually lost after long-term culture [188]. Additionally, the frequency of cytogenetic alterations increases in aged cells, resulting in chromosomal instability. Notably, impaired DNA damage responses and chromosomal instability may increase the risk of tumorigenesis [189]. However, ADSCs are typically considered to be stable in long-term culture *in vitro* compared with cells derived from other sources. Li et al. found that chromosomal aberrations can be detected after 20 culture passages, while the gene expression levels of p53 and telomerase reverse transcriptase remain stable at all passages [190]. This is still a controversial issue with ADSC transformation, and further experiments are needed to clarify this concern.

Taking account of the expense and complexity of the regulatory problems associated with ADSCs, it is evident that a large part of physicians are hesitant to perform any stem cell supplemented transfer operation procedures [27]. Meanwhile, automated devices for isolating ADSCs are classified into class III medical devices by the Food and Drug Administration (FDA), which cannot be approved for clinic application. Besides, the FDA stipulates that ADSC transplantation must be minimally manipulated, enzyme-free, and used in the same surgical procedure. Thus, an enzyme-free, cost-effective, and reproducible manufacturing of high-quality ADSCs for clinical use is desperately needed.

In addition to the aforementioned challenges, the biomaterials and their impact on ADSC in tissue engineering also needed more long-term *in vivo* experiments. Although biomaterials are biocompatible, most parts of them are extracted from animals and may prompt an immune response in the long term [10]. Moreover, with the prolongation of the culture time in the body, the biomaterials will be degraded and the fraction may serve as host antibodies eliciting the robust immune response. In this sense, further prospective studies investigating the safety of the biomaterials should be carried out, before application in human patients.

In summary, further preclinical and clinical studies are needed to determine whether ADSC-based therapies can fulfill expectations and be used to reconstruct damaged organs or tissues to treat diseases for which current treatments are ineffective.

6. Conclusion

The emergence of ADSC therapy provides a novel means for tissue regeneration. Numerous clinical and preclinical studies have demonstrated the vital role of ADSCs in reconstructing and repairing target organs, such as bone, cartilage, myocardium, liver, nervous system, and skin. However, many safety issues need to be urgently addressed, from the preparation of ADSCs to their application. Furthermore, additional researches are required to identify appropriate scaffolds and potent inducing bioactive factors to provide an optimal microenvironment for ADSC proliferation and differentiation, and long-term studies are needed to ensure the implant-tissue interactions, resorption, and hierarchical structure and finally to turn them into a clinically viable method. Due to the significant differences between preclinical studies and clinical trials, the oncogenicity of ADSC differentiation warrants further research. Despite current challenges, the great pace of progress in this field suggests that ADSC-based approaches will play increasingly important roles in regenerative medicine.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

Acknowledgments

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