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CONTENTS

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EDITORIAL

Materiobiology-driven engineering for next-generation organoids

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1. Introduction

The rapid advancement of organoid technology has significantly transformed biomedical research, offering sophisticated *in vitro* platforms for disease modeling, precision medicine, and regenerative therapies.[1] These three-dimensional (3D) structures, derived from stem cells, intricately recapitulate tissue-specific microenvironments and physiological complexities, bridging gaps previously unfillable by traditional cell cultures or animal models. Central to the efficacy and potential of these engineered tissues is the foundational role played by biomaterials, which actively govern the cellular self-organization, functional maturation, and translational viability of organoids.[2] The emergence and advancement of a new field often called materiobiology has provided systematic theoretical guidance for the design of organoid matrix materials. Innovations in materiobiology science are not simply incremental improvements but represent transformative shifts enabling precise, reproducible, and clinically relevant organoid systems.

2. Historical insights: Natural biomaterials and emerging constraints

Initial organoid models were primarily leveraged by the use of naturally derived biomaterials, such as collagen, laminin, Matrigel, and other natural origin materials due to their inherent biocompatibility and biological fidelity to the native extracellular matrix.[3] These materials offer essential biochemical cues critical for early organoid development. However, their limitations-including variability between batches, unpredictable degradation kinetics, potential immunogenicity, and limited mechanical tunability have increasingly impeded scalability, reproducibility, and translational potential.[4] The inherent heterogeneity in these natural matrices underscores an unmet need for also looking for synthetic alternatives that combine biological compatibility with precise material controllability or for combinations of synthetic and natural materials.

3. Present progress: Synthetic biomaterials and alternative hydrogels innovations

Recent advances in synthetic polymers (e.g., poly(lactic-co-glycolic acid), polyethylene glycol) and alternative hydrogels (e.g., gelatin methacryloyl, gellan gum, chitosan, and marine origin materials) have enabled more precise control over organoid microenvironments. Across photopolymerization, 3D bioprinting, and other fabrication techniques, scientists now design scaffolds with tunable stiffness, porosity, and topography to mimic organ-specific niches.[5,6] For example, vascularization of liver organoids requires dynamically responsive materials, where

elastomers (e.g., polydimethylsiloxane) or thermosensitive polymers (e.g., poly(N-isopropylacrylamide)) can guide directional growth of vascular networks. Conductive polymers (e.g., polypyrrole, melanin-based materials) are also being explored to replicate electrical signaling in neural organoids[7] and being used in other alternative approaches. Researchers highlight that the present focus lies in coupling the physicochemical properties of different types of materials with biological functions, such as cell adhesion and metabolic regulation, to achieve "functionalized" organoids.

4. Future directions: Smart, multifunctional materials and integrated systems

Looking ahead, intelligent biomaterials and functional integration will drive the next leap in organoid technology. Stimuli-responsive materials (e.g., pH-sensitive, temperature or enzymatic sensible hydrogels, light-degradable polymers, and others) could enable spatiotemporal control over organoid development. Meanwhile, nanomaterials (e.g., quantum dots, carbon nanotubes, natural nanoparticles) may equip organoids with real-time monitoring or drug-delivery capabilities.[8] For instance, embedding magnetic nanoparticles into scaffolds could allow precise morphogenesis manipulation through external fields. Cutting-edge innovations include DNA origami-based molecular scaffolds and microfluidic-organoid chip systems that synergize biomaterials with fluid dynamics.[9,10] Several other biomaterials experts predict that within the next decade, tailor-made multifunctional materials will overcome present complexity barriers, empowering organoids to advance personalized medicine and organ replacement therapies.

5. Addressing translational barriers: Standardization and clinical readiness

Despite the versatility of biomaterials, challenges persist in standardization and clinical translation. Challenges to achieving long-term biosafety of synthetic or natural biomaterials, matching degradation rates to tissue regeneration timelines, and ensuring cost-effective scalability demand urgent solutions. To address these issues, the global biomaterials community is advocating for unified characterization protocols and performance benchmarks. Collaborative efforts with clinicians are also intensifying to ensure material designs align with real-world medical needs.

Organoid research is dedicated to highlighting pioneering research at the biomaterial-organoid interface, fostering a global dialogue that will propel the next generation of biomedical breakthroughs. In this issue, we feature a collection of timely and innovative articles that reflect both the rapid evolution and expanding scope of organoid science.

We begin with "Organoid research breakthroughs in 2024: A review,"[11] which offers a comprehensive overview of the field's most recent advances, spanning disease modeling, bioengineering innovations, and clinical translation. In the realm of disease modeling, "Parkinson's disease in a dish: The emerging role of organoids in research and therapy"[12] illustrates how brain organoids are enabling mechanistic insights into neurodegeneration and offering platforms for drug discovery. Complementing this, "Generation of vascularized brain organoids: Technology, applications, and prospects"[13] explores the challenges and progress in engineering vascular networks within cerebral organoids, a key step toward physiologically relevant neural models. Cancer research also takes center stage in "Application of cancer organoids: The forefront of personalized oncology and pre-clinical testing,"[14] where patient-derived tumor organoids are showcased as powerful tools for individualized therapy selection and drug screening. Expanding the organoid paradigm into musculoskeletal repair, "A trabeculae-like biomimetic bone-filling material as a potential organoid for bone defect treatment"[15] proposes a novel strategy for bone regeneration, blurring the lines between bioactive scaffolds and organoid systems. Finally, "Organoids: Applications and challenges of advanced hydrogels in tissue systems"[16] provides a materials science perspective, emphasizing the role of hydrogels in mimicking the native extracellular matrix and supporting organoid development across diverse tissues.

Together, these contributions highlight the interdisciplinary nature of organoid research and underscore the vital role of biomaterials in shaping its future.

Conflict of interest

The author declares no conflict of interest.

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REVIEW ARTICLE

Parkinson's disease in a dish: The emerging role of organoids in research and therapy

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Abstract

Parkinson's disease (PD) is a progressive neurodegenerative disorder marked by the degeneration of dopaminergic (DA) neurons in the substantia nigra and the accumulation of α-synuclein aggregates, leading to motor and non-motor dysfunctions. The pathogenesis of PD involves a complex interplay of genetic mutations, environmental factors, and cellular mechanisms, including mitochondrial dysfunction, impaired proteostasis, neuroinflammation, and gut-brain axis dysregulation. Traditional research models, such as animal models and two-dimensional cell cultures, have provided valuable insights but often fall short in replicating the multifaceted and progressive nature of PD, especially in sporadic cases. The emergence of organoid technology offers a transformative approach to PD research. This technology enables the generation of three-dimensional structures that closely mimic the architecture, cellular composition, and functionality of the human midbrain. Midbrain organoids have become pivotal models for investigating disease mechanisms, including DA neuron degeneration, α-synuclein aggregation, and neuroinflammatory responses. Moreover, organoids enable high-throughput drug screening and the identification of potential therapeutic targets. Beyond modeling, recent advancements have demonstrated the feasibility of organoid transplantation as a therapeutic strategy. This review summarizes the current progression of organoid technology in PD research, focusing on its application in modeling pathomechanisms, drug discovery, and therapeutic applications. Despite being in its early stages, organoid technology holds significant promise for advancing our understanding of PD pathogenesis and developing translational therapies.

Keywords: Parkinson's disease; Organoids; α-synuclein; Midbrain dopaminergic neurons; Drug screen; Organoid transplantation; Neurodegeneration

1. Introduction

Parkinson's disease (PD), first detailed by James Parkinson in 1817,[1] is a progressive neurodegenerative disorder characterized by the degeneration of dopaminergic (DA) neurons in the substantia nigra and the accumulation of α-synuclein aggregates, known as Lewy bodies. These pathological changes disrupt motor control and contribute to non-motor symptoms. Genetic mutations, including those in the leucine-rich repeat kinase 2 (*LRRK2*), *SNCA*, *PARK7*, PTEN-induced kinase 1 (*PINK1*), and *PRKN* genes, play significant roles in familial PD, while environmental factors, such as pesticide exposure and aging, contribute to sporadic cases.[2] Clinically, PD presents with motor symptoms such as tremors, rigidity, bradykinesia, and postural instability, alongside non-motor issues such as cognitive decline, sleep disturbances, and autonomic dysfunction.[3]

The pathogenesis of PD is driven by a complex network of cellular and molecular dysfunctions. A hallmark feature of PD is the progressive degeneration of DA neurons in the substantia nigra, leading to dopamine depletion in the striatum and subsequent motor deficits.[4] This neurodegeneration is accompanied by the accumulation of α-synuclein aggregates in the form of Lewy bodies and Lewy

neurites, which are implicated in neurotoxicity through mechanisms such as disrupted proteostasis, mitochondrial dysfunction, and synaptic impairments.[5] These pathological changes not only compromise neuronal survival but also contribute to the widespread neurodegenerative processes observed in PD. Mitochondrial dysfunction plays a critical role in PD pathogenesis, particularly in cases associated with mutations in PINK1 and Parkin.[6] These mutations impair mitochondrial quality control mechanisms, leading to the accumulation of damaged mitochondria, increased oxidative stress, and heightened susceptibility to neurodegeneration.[6] Neuroinflammation is another key contributor to PD pathology. Chronic neuroinflammation, characterized by the sustained activation of microglia and astrocytes, leads to the release of proinflammatory cytokines and reactive oxygen species, exacerbating neuronal damage and promoting the progression of PD.[7] Disruptions in lysosomal function and autophagy pathways further contribute to PD pathology. Mutations in genes such as *GBA1* and *LRRK2* impair lysosomal degradation and autophagy, leading to the accumulation of toxic protein aggregates and increased cellular stress.[8] The inability to clear misfolded α-synuclein exacerbates neurotoxicity, reinforcing a vicious cycle of protein accumulation and neuronal dysfunction. Emerging evidence suggests that gut-brain axis dysfunction also plays a role in PD pathogenesis. The bidirectional communication between the gut and the central nervous system may influence neuroinflammatory and neurodegenerative processes, with some studies suggesting that α-synuclein pathology may originate in the gut and propagate to the brain through the vagus nerve.[9] While significant progress has been made in identifying individual genetic mutations and environmental risk factors, the precise mechanisms by which these elements interact to drive PD pathogenesis remain incompletely understood, highlighting the necessity for continued research to elucidate these interactions.

Research in PD relies on various models, each with unique advantages and limitations. Human brain tissue from post-mortem donations, biopsies, or patient-derived induced pluripotent stem cells (iPSCs) provides valuable insights into disease mechanisms within DA neurons, offering greater relevance than traditional animal models or cell cultures. However, challenges such as sample variability, ethical concerns, and difficulty in accessing early disease stages limit their utility. Animal models, including toxin-induced and genetic models, are widely used but have inherent limitations. Toxin-based models like 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-hydroxydopamine (6-OHDA) replicate DA neuron loss and motor deficits but fail to mimic the progressive and multifactorial nature of PD.[10] Genetic models incorporating mutations in *SNCA*, *LRRK2*, *PINK1*, and *Parkin* help study familial PD but often do not fully represent sporadic cases.[11] In addition, transgenic mouse models generally lack the gradual onset and α-synuclein aggregation seen in human PD, making them less ideal for studying disease progression. They do not fully replicate the complexity of human PD, especially in terms of the gradual onset and progression of the disease. For example, PINK1 and Parkin mutant mice often exhibit early-onset neurodegeneration, which does not mirror the gradual progression observed in human PD.[12] In addition, these mouse models often lack the same degree of α-synuclein aggregation, a hallmark of PD pathology, limiting their ability to model this key aspect of the disease.[13] Similarly, *LRRK2*-related animal models provide insights into genetic factors but fail to encompass environmental influences and age-related degeneration typical of human PD.[14] Moreover, these models do not effectively reproduce the involvement of non-DA systems and broader factors, such as neuroinflammation, that contribute to the full spectrum of PD pathology.[15] Consequently, while valuable for basic research, these animal models have significant limitations in accurately modeling the human form of PD.

The rapid advancement of stem cell technology has led to the emergence of organoids as a transformative tool in biomedical research. Organoids are three-dimensional (3D), miniaturized versions of organs that are created from stem cells and exhibit some of the key functions and structures of the original organ. They are derived by differentiating stem cells into specific tissue types in a laboratory setting, and they can replicate the architecture and functionality of organs such as the brain, liver, and kidneys. The first brain organoids were generated in 2009,[16] which shows that adult intestinal stem cells can form 3D intestinal organoids in Matrigel. Since then, the field of organoid research has expanded rapidly, particularly in the study of neurodegenerative diseases, like PD and Alzheimer's disease, cancer, and organ development, providing valuable insights for disease modeling and drug testing. In PD research, human midbrain organoids (hMOs) are frequently used because they closely mimic the structure and cellular composition of the midbrain, which is the region most affected by the disease.[17] Unlike conventional two-dimensional (2D) cell cultures, which often have a limited range of cell types and fail to capture the complex interactions within the tissue, hMOs feature a 3D structure that simulates the brain's natural environment, containing a variety of cell types, such as DA neurons and glial cells, allowing the examination of cell-cell interactions and neuronal networks. In addition, 3D organoids can be maintained for extended periods, enabling researchers to study key pathological mechanisms in the context of both aging and early-onset PD. In this review, we first provide an overview of organoid models in

Parkinson's research, focusing on their role in elucidating disease pathomechanisms (Table 1). We then explore their applications in drug discovery and high-throughput screening. Finally, we discuss the potential of organoid transplantation as a therapeutic strategy for PD.

2. Organoid models for PD

2.1. *SNCA*

α-Synuclein, a 140-amino-acid presynaptic neuronal protein encoded by the *SNCA* gene, plays a critical role in synaptic function, but its pathological aggregation into Lewy bodies is a hallmark of PD.[18] *SNCA* mutations, particularly missense mutations and gene amplifications, are associated with autosomal dominant PD. Among these, *SNCA* triplications lead to a substantial increase in α-synuclein expression, accelerating pathological processes and neurodegeneration.[19] Studies using hMOs derived from iPSCs carrying *SNCA* triplication mutations have successfully replicated key features of α-synuclein pathology seen in patients with synucleinopathies. hMOs with *SNCA* triplications exhibit elevated α-synuclein levels and a time-dependent increase in α-synuclein aggregation.[20-22] This aggregation includes both oligomeric and phosphorylated forms of α-synuclein, which are detected in both neurons and glial cells.[20] The progressive accumulation of pathological α-synuclein in these organoids is accompanied by DA neuron loss and increased apoptosis, mirroring neurodegenerative processes observed in PD brains.[20-22] Single-cell RNA sequencing of *SNCA* triplication hMOs has provided insights into molecular dysfunctions affecting DA neurons, including impaired oxidative phosphorylation, dysregulated protein translation, and endoplasmic reticulum stress.[23] Furthermore, recent studies suggest that α-synuclein pathology is linked to cellular senescence, particularly in astrocytes, leading to a phenomenon termed astrosenescence, supporting that pathological α-synuclein may contribute to the induction of neuroinflammation, which ultimately increases the susceptibility of DA neurons to degeneration.[22]

In addition to using hMOs to investigate the potential cellular mechanisms of *SNCA*-induced neuronal toxicity, organoid models have also been employed to study the propagation of α-synuclein pathology. In a recent study, researchers used mouse intestinal organoids expressing human α-synuclein to examine the transfer of α-synuclein from epithelial cells within the organoids to co-cultured nodose neurons lacking α-synuclein expression. This study highlights a potential non-neuronal source of fibrillar α-synuclein, suggesting that gut mucosal cells may contribute to the initiation or spread of α-synuclein pathology.[24] In addition to the genetic manipulation of *SNCA*, hMOs are also utilized to study the propagation of

Table 1. Genetic and phenotypic characteristics of human midbrain organoids in Parkinson's disease research

Genetic characteristic	Mutation	Key features	References
SNCA	*SNCA* triplication	Elevated α-synuclein levels, age-dependent aggregation, selective dopaminergic neuron loss, detergent-resistant β-sheet-rich α-synuclein aggregates, Lewy body-like inclusions with eosinophilic cores, astrosenescence with nuclear and chromatin alterations, and morphological features resembling various stages of Lewy body formation.	20-22,56
LRRK2	G2019S	Increased α-synuclein aggregation, impaired clearance, altered gene expression, reduced midbrain dopaminergic neuron complexity, increased *FOXA2* expression, and incomplete differentiation with decreased cellular variability.	17,28-31
PINK1/Parkin	Knockout	Reduced growth rate, impaired dopaminergic neuronal differentiation, increased oxidative stress levels, and mitochondrial dysfunction.	38-42
DNAJC6	Knockout	Pathological α-synuclein aggregation, heightened neuronal firing, mitochondrial and lysosomal dysfunction, and neurodevelopmental abnormalities.	45,46
DJ1	Knockout	Elevated oxidative stress levels, mitochondrial dysfunction, and impaired protein quality control in astrocytes, resulting in the accumulation of advanced glycation end products and α-synuclein aggregates	48,49
GBA1	L444P/N370S	Reduced GCase activity, impaired autophagy, mitochondrial dysfunction, altered lipid metabolism, decreased TH^+ neurons, increased insoluble and phosphorylated α-synuclein, and fewer complex dopaminergic neurons.	52-59
Toxin-induced PD organoids	N/A	Treated with neurotoxins (e.g., MPP^+, rotenone) to induce PD-like pathology. Increased phosphorylated and detergent-insoluble α-synuclein.	60
Idiopathic PD organoids	N/A	Generated from PBMCs of idiopathic PD patients, models non-genetic PD. Dysregulation of *TH*, *PTX3*, *LMX1A*, and *FOXA2* expression may increase neuronal vulnerability to damage and degeneration	61

Abbreviations: DJI: Parkinson's disease protein 7; GCase: Glucocerebrosidase; MPP^+: 1-methyl-4-phenylpyridinium; N/A: Not available; PBMCs: Peripheral blood mononuclear cells; PD: Parkinson's disease; TH: Tyrosine hydroxylase; LMX1A: LIM homeobox transcription factor 1 alpha.

α-synuclein proto-fibrils (PFFs). Using PFF-treated HMOs, researchers discovered that Tilorone effectively inhibits α-synuclein PFF-induced neuronal toxicity.[25] Overall, hMOs carrying *SNCA* triplications provide a valuable platform for studying α-synuclein aggregation, DA neuron degeneration, and the mechanisms driving PD progression. These models not only enable the exploration of disease mechanisms but also offer a robust system for evaluating potential therapeutic interventions targeting α-synuclein pathology and its downstream neurotoxic effects.

2.2. *LRRK2*

The *LRRK2* gene encodes a kinase that regulates protein trafficking and inflammatory pathways, playing a significant role in PD pathogenesis. Since its identification in 2004 as a key genetic factor for PD, several pathogenic mutations – including Asn1437His, Arg1441Cys/Gly/His, Tyr1699Cys, Ile2020Thr, and Gly2019Ser – have been associated with disease development.[26] Among these, the G2019S missense mutation is the most common genetic contributor to both familial and sporadic PD, leading to overactive kinase activity that disrupts normal cellular processes.[27] This dysfunction is linked to various pathological characteristics of PD, such as mitochondrial impairment, compromised autophagy, and abnormal protein aggregation, including α-synuclein. Studies confirm that hMOs derived from PD patients carrying the *LRRK2* G2019S mutation exhibit PD-related phenotypes, including reduced complexity, increased α-synuclein aggregation, and its impaired clearance, as well as a decrease in the number of DA neurons.[17,28] Zagare *et al.*[29] used single-cell transcriptome datasets to compare the shared cellular identities between healthy hMOs and human embryonic midbrain tissue, as well as between healthy midbrain organoids and LRRK2-G2019S isogenic hMOs. Their analysis demonstrates that hMOs accurately replicate human midbrain development and capture a gene expression profile associated with LRRK2-G2019S mutations, which may underline the phenotypes related to LRRK2 mutations.[29] Further investigations into the molecular mechanisms of *LRRK2* G2019S mutations have revealed their impact on dopamine metabolism and neuronal survival. Zhou *et al.*[30] demonstrated that *LRRK2* mutations upregulate tyrosine hydroxylase (TH) expression and dopamine levels in the early stages of PD, ultimately leading to DA neuron degeneration.[30] In addition, Kim *et al.*[28] identified thioredoxin-interacting protein (TXNIP) as a key mediator that regulates *LRRK2* G2019S pathological phenotypes in hMOs.[28] TXNIP was previously found to be a risk factor for PD that significantly accelerates the accumulation of α-synuclein.[31] Dysregulation of TXNIP may exacerbate PD pathogenesis in *LRRK2* G2019S sporadic PD within a three-dimensional cellular environment.[28] Moreover, using cerebral organoids (COs) derived from fibroblasts of individuals carrying the *LRRK2* G2019S mutation, Zhou *et al.*[32] observed significant electrophysiological alterations in disease-associated COs compared to healthy controls.[32] These changes included reduced neuronal network communication, slowed neuronal oscillations, and increased coupling of delta and theta phases to gamma oscillation amplitudes.[32] In addition to neuronal cells in organoid models, human iPSC-derived microglia carrying the *LRRK2* G2019S mutation also replicate key aspects of the transcriptional signature observed in midbrain microglia from individuals with idiopathic PD.[33] These studies highlight that organoid models for *LRRK2* effectively replicate the pathological features of PD and serve as valuable models for exploring the disease mechanisms underlying *LRRK2*-induced neuronal toxicity.

2.3. *PINK1* and *Parkin*

PINK1 and *Parkin* are crucial genes implicated in autosomal recessive PD, playing fundamental roles in mitochondrial quality control and mitophagy.[34,35] Mutations in *PINK1*, such as E283A and R342H, and in *Parkin*, such as C418Y, result in mitochondrial dysfunction and impaired mitophagy, ultimately leading to the degeneration of DA neurons. PINK1, a mitochondrial kinase, accumulates on the outer membrane (TOM) of damaged mitochondria and recruits the E3 ligase Parkin.[34,35] Parkin ubiquitinates mitochondrial proteins, targeting them for autophagy, which removes defective mitochondria to maintain quality, prevent dysfunction, and protect DA neurons from PD-related damage.[36,37] A human isogenic organoid model with *PINK1* deficiency revealed that while overall neuronal differentiation remained comparable between *PINK1*-deficient organoids and their isogenic controls, the deficient organoids exhibited specific impairments in DA neurogenesis.[38] This finding underscores *PINK1*'s essential role in DA neuron development and suggests that its loss may contribute to early neurodevelopmental vulnerabilities in PD. In addition, studies using hMOs have highlighted *PINK1*'s role in mitochondrial stress responses. Eldeeb *et al.*[39] demonstrated that *PINK1* is necessary for stabilizing translocase of TOM and translocase of the inner membrane 23 complexes under mitochondrial stress conditions, such as carbonyl cyanide m-chlorophenylhydrazone and ammonium chloride exposure.[39] PD-associated *PINK1* mutations disrupt the interaction between TOM20 and *PINK1*, preventing the formation of the super complex, which links mitochondrial stressors to PINK1 accumulation in midbrain organoids.[39] Beyond *PINK1*'s role, organoid models derived from iPSCs of PD patients with *Parkin* mutations have provided additional insights into disease pathology. These hMOs display abnormal astrocytic reactivity compared to age- and sex-matched controls, suggesting that *Parkin* mutations may influence glial function in PD.[40] Taken together, these models reveal critical defects in DA neurogenesis, astrocytic reactivity,

and mitochondrial stress responses, which are central to the pathogenesis of PD. Notably, since the *PINK1/Parkin* mouse models do not exhibit predominant DA neuron loss or severe mitochondrial dysfunction, hMOs further demonstrate their valuable role in exploring the underlying pathomechanisms of *PINK1/Parkin* in PD. In line with this, knocking out *PINK1* and *Parkin* in human iPSC-derived midbrain-specific DA neurons significantly impairs ionophore-induced mitophagy and reduces mitochondrial membrane potential, though it does not affect neuronal differentiation.[41,42] This further underscores the importance of human iPSC-derived cells and organoid models in studying the pathomechanisms of *PINK1*-related dysfunction.

2.4. *DNAJC6*

DNAJC6 encodes auxilin, a key protein involved in the recycling of clathrin-coated vesicles during endocytosis, a process essential for synaptic vesicle recycling and the proper functioning of neurons. Loss-of-function mutations in *DNAJC6* lead to impaired synaptic vesicle recycling, disrupting neurotransmitter release and contributing to DA neuronal dysfunction and degeneration, a characteristic of early-onset or autosomal recessive juvenile parkinsonism (AR-JP).[43,44] hMLOs with *DNAJC6* loss-of-function mutations have been proven to effectively recapitulate several key disease phenotypes, including DA neuron degeneration, pathological α-synuclein aggregation, and increased intrinsic neuronal firing frequency. Furthermore, the disruption of *DNAJC6*-mediated endocytosis in these models leads to significant defects in cellular processes critical for neuronal health, including impaired cellular trafficking and organelle maintenance. Loss of *DNAJC6* impairs the function of the WNT-LIM homeobox transcription factor 1 alpha (LMX1A) signaling cascade, resulting in reduced expression of LMX1A, a key transcription factor essential for DA differentiation. The reduced LMX1A expression ultimately leads to the generation of vulnerable DA neurons that exhibit pathological characteristics, such as α-synuclein aggregation and altered cellular functions.[45] Furthermore, one group generated an iPSC-derived midbrain DA neuronal model using fibroblasts from patients with pathogenic loss-of-function *DNAJC6* mutations. Using this model, they demonstrated that lentiviral *DNAJC6* gene transfer successfully restored *DNAJC6* expression and rescued clathrin-mediated endocytosis deficiency in midbrain DA neurons.[46] These findings further underscore the complex molecular mechanisms underlying AR-JP and highlight the utility of hMOs as a valuable model for studying the disease.

2.5. PD protein 7

From PD protein 7 (DJ1), encoded by the *PARK7* gene, is a multifunctional protein that plays a crucial role in protecting cells oxidative stress and maintaining mitochondrial function, both of which are essential for neuronal health, particularly in DA neurons.[47] Mutations in *PARK7*, which are primarily associated with autosomal recessive early-onset PD, result in a loss of DJ1 function, leading to increased oxidative damage, mitochondrial dysfunction, and DA neurodegeneration.[47] Parfitt *et al.*[48] found that in a PD-associated hMO model with DJ1 deficiency, impaired protein quality control pathways in astrocytes lead to the accumulation of advanced glycation end products and α-synuclein aggregation, both of which are hallmark features of PD pathology. The accumulation of these toxic proteins disrupts cellular homeostasis, contributing to DA neuron degeneration. In addition, the deficiency suppressed the clearance of misfolded proteins in astrocytes, further exacerbating neuronal toxicity. These findings suggest that DJ1 is not only critical for oxidative stress and mitochondrial function but also plays a significant role in maintaining cellular protein integrity.[48] Thus, defects in DJ1 function can initiate a cascade of molecular events leading to the degeneration of DA neurons, reinforcing its pivotal role in the pathogenesis of PD. Consistent with findings from organoid models, one group also utilized iPSC-derived neurons carrying the homozygous c.192G>C mutation in DJ1 and identified a U1-dependent splicing defect that led to a drastic reduction in DJ1 protein levels and subsequent mitochondrial dysfunction. Targeting this defective exon skipping with genetically engineered U1 small nuclear RNA successfully restored DJ1 protein expression and mitochondrial function in both neuronal precursor cells and differentiated neurons.[49] Organoid models have been instrumental in elucidating DJ1's role in PD pathogenesis, providing valuable platforms for testing therapeutic interventions aimed at restoring DJ1 function and mitigating neurodegeneration in PD.

2.6. *GBA*

GBA is a gene encoding the lysosomal enzyme β-glucocerebrosidase (GCase), which is essential for breaking down glucocerebroside into glucose and ceramide. *GBA* was initially identified for its association with Gaucher disease; mutations in *GBA* were later linked to PD through genetic studies.[50,51] As one of the most common genetic risk factors for PD, *GBA* mutations (e.g., N370S and L444P) reduce enzyme activity, leading to lysosomal dysfunction, the accumulation of glucocerebroside, and impaired autophagy. These defects promote the aggregation of α-synuclein. In patient-specific *GBA* N370S hMOs, researchers found *GBA*-PD-related phenotypes, including reduced GCase activity, impaired autophagy, mitochondrial dysfunction, and a decline in both the number and complexity of DA neurons.[52] Moreover, *GBA*-N370S-PD hMOs exhibit DA neuron loss and an altered lipid profile, along with reduced sensitivity to changes in insulin

signaling activity compared to healthy midbrain organoids. Dysregulated insulin signaling in *GBA*-PD exacerbates DA neuron loss through *FOXO1* overexpression, while elevated insulin levels disrupt lipid metabolism and trigger cellular death. These findings emphasize the potential of targeting insulin signaling to combat PD-related neurodegeneration.[53] Frattini *et al.*[54] generated another hMOs model from *GBA1*-L444P-associated PD patients' iPSCs. These hMOs display a reduced GCase activity, recapitulating DA neuron loss and fundamental features of Lewy body pathology observed in human brains, including the generation of α-synuclein fibrillary aggregates. Notably, ambroxol and GZ667161, two GCase modulators in clinical development, effectively reduced α-synuclein pathology, highlighting midbrain organoids as a valuable platform for preclinical drug screening.[54,55] Recent studies using hMOs derived from genetically modified human embryonic stem cells have provided significant insights into the synergistic effects of *GBA1* loss and *SNCA* overexpression on α-synuclein aggregation.[56] Specifically, the concurrent loss of *GBA1* function and overexpression of wild-type α-synuclein leads to the substantial accumulation of detergent-resistant, β-sheet-rich α-synuclein aggregates and the formation of Lewy body-like inclusions within these organoids.[56] Notably, these pathological features do not emerge when either genetic alteration occurs independently. In addition, impaired GCase function has been shown to promote α-synuclein aggregation in hMOs derived from PD patients carrying *SNCA* triplication mutations.[56]

Along this line, beyond α-synuclein accumulation, *GBA1* loss in human iPSC-derived neurons indeed disrupts lysosomal protein degradation, leading to neurotoxicity through aggregation-dependent mechanisms.[57] α-synuclein itself inhibits the lysosomal trafficking of GCase, creating a bidirectional feedback loop that perpetuates disease progression.[57] This self-reinforcing cycle suggests that targeting both α-synuclein accumulation and lysosomal dysfunction may be necessary for effective PD therapies.[57] Additionally, the interaction between *GBA* and *LRRK2* has been well established. DA neurons carrying *LRRK2* R1441C, R1441G, or G2019S mutations exhibit reduced GCase activity, which can be restored by *LRRK2* kinase inhibition, accompanied by decreased α-synuclein phosphorylation.[58,59] These findings strongly suggest that the interplay between *GBA* and other PD-related proteins may have synergistic effects on neuronal toxicity, further driving disease progression. Understanding these interactions provides critical insights into PD pathogenesis and highlights the potential of targeting multiple pathways for therapeutic intervention.

2.7. Other organoid models

In addition to hMOs incorporating PD-related genetic factors, other 3D culture models utilizing Matrigel and commonly used cell lines, such as SH-SY5Y cells, have also been developed.[60] In these 3D-cultured cells, phosphorylated α-synuclein and a detergent-insoluble α-synuclein fraction were observed following 1-methyl-4-phenylpyridinium and rotenone treatment. This study presents a cost-efficient and accessible 3D PD model that effectively recapitulates key α-synuclein pathologies, providing a valuable platform for PD-related research and therapeutic applications.[60] Alternatively, it has also been reported that organoids derived from peripheral blood mononuclear cells (PBMCs) of patients with the idiopathic form of PD have been developed[61] (Table 1). This approach offers a less invasive method for obtaining patient-specific cells, facilitating the study of PD pathology and the development of personalized therapeutic strategies.

3. Organoid models in drug screening for PD

The challenges in drug screening for PD are multifaceted, stemming from the disease's complexity, limitations of current models, and difficulties in translating preclinical findings into clinical outcomes. Traditional 2D cell cultures and animal models often fail to replicate the intricate pathophysiology of PD due to limitations in capturing key aspects such as α-synuclein aggregation, neuroinflammation, mitochondrial dysfunction, and progressive neuronal loss. While 2D cultures of DA neurons provide valuable insights, they lack cellular diversity, extracellular matrix interactions, and long-term maturation, limiting their ability to model chronic neurodegeneration.[62,63] Similarly, animal models, including MPTP-treated mice, rotenone-exposed rats, and α-synuclein transgenic models, only partially mimic human PD pathology, as differences in species affect dopamine metabolism, immune response, and protein aggregation dynamics. Moreover, these models often fail to recapitulate the prion-like spread of misfolded α-synuclein and the full spectrum of motor and non-motor symptoms seen in PD patients.[64-67]

To overcome these challenges, emerging alternatives such as 3D midbrain organoids, microfluidic organ-on-a-chip systems, and humanized animal models are being developed. iPSC-derived midbrain organoids, for instance, recreate nigrostriatal connectivity, glial-neuronal interactions, and progressive α-synuclein pathology, offering a more physiologically relevant platform for disease modeling and drug discovery. A group of researchers developed a novel organoid model based on optogenetic proteins, termed the optogenetics-assisted α-synuclein aggregation induction system (OASIS). This system enables rapid induction of α-synuclein aggregates and associated toxicity in PD-associated hMOs. By utilizing optogenetic proteins, OASIS allows precise light-induced spatiotemporal control of protein interactions, effectively

modeling the protein aggregation and toxicity characteristic of late-onset PD. They identified a small molecule, BAG956, capable of mitigating α-synuclein aggregation-induced toxicity in hiPSC-derived midbrain DA neurons and hMOs. OASIS offers a promising platform to investigate disease pathology and therapeutic interventions in a controlled and physiologically relevant context.[68]

Zhu *et al.*[69] developed an innovative drug screening platform utilizing electrochemical cytometry with nano-tip microelectrodes to analyze vesicular storage in hMOs. This platform allows for precise measurement of neurotransmitter storage in a high-throughput manner, providing valuable insights into the underlying cellular mechanisms of PD. Their study revealed a significant reduction in vesicular storage of neurotransmitters in young-onset PD iPSC-derived hMOs, which correlated with the upregulation of α-synuclein. Importantly, treatment with amantadine, a clinically used drug for PD, was found to alleviate this vesicular storage defect, demonstrating the potential of this model for assessing therapeutic efficacy. Additionally, phorbol 12-myristate 13-acetate, a promising candidate for PD treatment, also showed potential in restoring vesicular function, further validating this electrochemical cytometry-based platform as an effective tool for screening PD drugs.[69] These findings highlight the capability of this innovative platform to replicate key features of PD and its utility in identifying novel therapeutic candidates with the potential to target early disease mechanisms.

Several studies, not originally designed for high-throughput drug screening, have utilized hMOs PD models and identified promising therapeutic compounds. For instance, in patient-derived brain organoids, 2-hydroxypropyl-β-cyclodextrin was found to not only enhance the proportion of DA neurons but also boost neuronal autophagy and mitophagy capacity.[70] Similarly, in *LRRK2* G2019S mutant hMOs, reduced expression of nuclear receptor-related 1 protein (Nurr1) led to lower *Nurr1* and *TH* mRNA levels compared to isogenic controls. However, treatment with a newly designed Nurr1 agonist effectively restored these expression levels, underscoring the therapeutic potential of Nurr1 activation in PD.[71] Additionally, one study utilizing an α-synuclein aggregation assay identified four candidates—entacapone, tolcapone, phenazopyridine hydrochloride, and zalcitabine—that inhibited α-synuclein seeding activity in real-time quaking-induced conversion assays. These findings were further validated in *SNCA* triplication organoids, where the compounds significantly reduced α-synuclein aggregation and alleviated mitochondrial dysfunction.[72]

Organoid models hold great promise for drug discovery, offering human-derived, physiologically relevant platforms for studying disease mechanisms and therapeutic responses. However, several challenges hinder their widespread application, including batch-to-batch variability, limited scalability, and high-throughput screening constraints due to heterogeneity in organoid size and structure.[73,74] Additionally, organoids often exhibit developmental immaturity, lacking key aging-related features necessary for modeling late-onset diseases such as PD and AD. The absence of a functional microenvironment, including vasculature, immune cells, and the blood-brain barrier, further limits their ability to predict drug penetration, metabolism, and toxicity accurately.[75] Furthermore, the dense 3D structure of organoids can hinder drug diffusion, leading to inconsistent exposure across models.[76] High costs, technical expertise requirements, and a lack of standardized protocols also pose significant barriers to their integration into preclinical drug development.[77] Despite these limitations, advances in bioprinting, microfluidic platforms, artificial intelligence-driven analysis, and clustered regularly interspaced short palindromic repeats-based disease modeling continue to enhance organoid technology, making them increasingly valuable for precision medicine and next-generation drug discovery.

4. Organoid transplantation: A promising approach for PD treatment

Organoids show the ability to replicate key aspects of tissue architecture, cell-type composition, and organ functionality while retaining the benefits of simplified and accessible cell culture models. As a result, organoid technology holds significant potential as a promising alternative to traditional cell and tissue transplantation. Moreover, recent studies have demonstrated that these *in vitro* cultured organoids can be successfully transplanted into multiple animal models, including dogs, mice, and others.[78-80] In PD studies, several research groups have also reported preliminary findings using hMOs in mouse models.

To evaluate the feasibility of transplanting hMOs for PD treatment, Zheng *et al.*[81] generated organoids from hiPSCs and transplanted them into the striatum of 6-OHDA-lesioned immunodeficient mice to assess the safety and efficacy of the graft. Twelve weeks post-transplantation, they observed that hMOs survived and matured into midbrain DA neurons in the striatum of PD mice. This transplantation resulted in a reversal of motor function and the establishment of bidirectional connections with native brain target regions, with no signs of tumor formation or graft overgrowth.[81] Moreover, another study using a similar approach also demonstrated that the transplanted cell survived, differentiated efficiently into DA neurons, and integrated into the neural network of the PD mice. The differentiated human DA neurons were capable of releasing dopamine, and 4 weeks post-transplantation, the motor function of the mice showed significant improvement. These findings suggest that cell therapy using iPSC-derived

midbrain organoids could be a promising strategy for the clinical treatment of PD.[82,83]

In addition to organoid transplantation, several groups have investigated the feasibility of transplanting midbrain astrocytes or neural stem/precursor cells (NSCs) isolated from hMOs (Og-NSCs).[84,85] Astrocytes derived from hMOs have been shown to exhibit remarkable neurotrophic and regenerative capacities. Similarly, Og-NSC-derived midbrain DA neurons demonstrated enhanced synaptic maturity, functionality, resistance to toxic insults, and stable expression of midbrain-specific factors *in vivo*, even long after transplantation.

Organoid transplantation is a promising approach for PD treatment, offering the potential to replace lost DA neurons and restore brain function. However, several challenges must be addressed, including poor graft survival, lack of vascularization, immune rejection, and risks of uncontrolled proliferation or tumorigenicity.[86-88] Ensuring functional integration of transplanted organoids into host neural circuits remains a key hurdle, as differences in neuronal maturation and synaptic plasticity may affect their efficacy.[78,89,90] Additionally, ethical and regulatory concerns regarding the safety and long-term monitoring of organoid-based therapies present further barriers to clinical translation. Advancements in vascularized organoids, immune modulation strategies, and bioengineering are crucial to overcoming these challenges and realizing the full therapeutic potential of organoid transplantation for PD.

5. Conclusion and perspectives

Organoid technology has emerged as a powerful tool in PD research, bridging the gap between traditional models and the complexity of human pathology. Midbrain organoids accurately mimic the cellular and structural features of the human midbrain, enabling in-depth studies of PD mechanisms, including α-synuclein aggregation, DA neuron loss, neuroinflammation, and mitochondrial dysfunction. They have proven invaluable for drug discovery, high-throughput screening, and regenerative medicine, with promising preclinical results in organoid transplantation.

While challenges like scalability and consistency remain, progress in organoid technology and bioengineering is helping to address these issues. Further efforts are needed to refine organoid technology, including enhancing vascularization, incorporating microglia and astrocytes, and improving long-term culture conditions to better mimic *in vivo* PD pathology. From a drug screening perspective, while organoids offer a promising alternative to traditional 2D cultures and animal models, challenges remain in scalability, cost, and throughput efficiency. Integrating high-throughput screening technologies, along with omics-based analysis and artificial intelligence, may improve drug discovery efforts. Additionally, combining organoid models with patient-derived iPSCs could further personalize PD research and precision medicine approaches.

In conclusion, while current PD organoid models have significantly advanced our understanding of disease mechanisms, continued refinement and integration of cutting-edge technologies will be essential to overcoming existing limitations. These improvements will enhance their utility in mechanistic studies, biomarker discovery, and therapeutic development, ultimately contributing to better-targeted treatments for PD.

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OR

REVIEW ARTICLE

Organoids: Applications and challenges of advanced hydrogels in tissue systems

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Abstract

Repair and regeneration of tissues and organs remain central to medical research. Organoids, advanced models created through *in vitro* tissue engineering, enable stem cells to form three-dimensional structures that replicate organ tissues and function spontaneously. The advancement of organoid technology has greatly enhanced our understanding of disease progression and organ development. A crucial component of this technology is the development of appropriate scaffolds to support organoid growth. Recently, hydrogels have emerged as promising materials due to their excellent biocompatibility, tunability, and degradability, facilitating the *in vitro* culture of stem cells and their differentiation into various organoids. However, more complex organ models, which involve extensive intercellular and extracellular communication, present significant challenges for present research. Moreover, advancements in biomaterial fabrication and their integration with organoid technology remain underexplored. This review explores the pivotal role of hydrogels in organoid preparation, comparing the advantages and limitations of different hydrogel fabrication methods. It also highlights recent advancements in the application of organoid hydrogels across various biological systems and discusses future challenges and directions in this field.

Keywords: Organoids; Stem cells; Tissue regeneration; Hydrogels

1. Introduction

Traditional two-dimensional (2D) cell culture models have greatly enhanced our understanding of diseases involving simple cellular structures and contributed to research in signal transduction pathways and drug delivery.[1] Clinical drug design and treatment guidelines are typically based on experimental efficacy. However, 2D models possess limitations for studying complex organs such as the heart and brain due to their simplistic cellular layers and lack of structural complexity, which oftentimes are the factors keeping cells isolated from their native contexts, disrupting intercellular communication

crucial for drug responses and treatments.[2] Furthermore, these models do not account for interactions between matrix cells, microenvironmental factors, and cell-secreted cytokines, which are essential for accurate disease modeling.[3] Complex organ models involving extensive intercellular and extracellular communication present significant challenges for present research. Therefore, the demand for organoids has emerged to address these limitations.

Organoids are three-dimensional (3D) tissue-like structures composed of stem cells, capable of unlimited *in vitro* expansion, and exhibit defined spatial organization.[4] They are formed through stem cell differentiation, originating from embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), or adult stem cells (AdSCs), and can simulate and exhibit specific functions of real organs.[5] During differentiation, these cells establish downstream populations, allowing for the aggregation of organ-specific AdSCs into functional units, as seen in models for the intestine, lung, heart, bone, brain, retina, and other tissues.[6-8] Early organoid culture models expressed limited organ functionalities, primarily those created through cell separation and reaggregation techniques, while later models with a broader range of organ functions were created, making them more feasible in accurately recapitulating physiological conditions.[9]

Present research emphasizes the connectivity of various systems and multi-organ functions, studying bidirectional communication patterns, such as the bone-intestine axis, bone-brain axis, and gut-brain axis.[10] Inter-organ cell factor interactions and signal transduction are often invisible and challenging to monitor *in vivo*. However, organoid cultivation facilitates the study of bidirectional organ connections. By utilizing the diverse cell composition of intestinal organoids, which secrete neurotransmitters and mediate the connection between gut microbiome signals to host changes, the limitations of the traditional organ models can be addressed.[11,12]

In addition, the complexity of human organ systems differs significantly from rabbit and rodent models, often leading to failed translations of successful drug treatments from animals to humans. Organoids, by mimicking the spatial morphology of normal tissues, provide an alternative for simulating drug resistance, drug screening, and the tumor microenvironment.[13] In the context of cancer, the individual heterogeneity among tumor cells is a key reason for the failure of treatments. Patient-derived cancer organoid models have shown the ability to differentiate into various subcellular types.[14] For example, gastric cancer organoids have been used to construct models with Ras and Wnt signalling pathway activation, and diffuse models, which enable the assessment of drug sensitivity variations.[15] Similarly, organoids have shown success across various systems and organs, including cardiovascular, respiratory, digestive, urinary, and musculoskeletal systems, establishing a reliable platform for developing novel therapeutic approaches.

Hydrogels provide an environment that supports 3D cell structures, mimicking the extracellular matrix (ECM) in the body and allowing cells to grow and differentiate under conditions that resemble their natural physiology.[16,17] By adjusting their composition and structure, hydrogels can alter their mechanical properties and degradation rates, making them suitable for different types of organoid research. Compared to traditional 2D culture methods, hydrogels support complex tissue structures and simulate intercellular communication and signal transduction.[18,19] This characteristic gives them tremendous potential for drug screening, disease modelling, and regenerative medicine applications.

This review summarizes recent advancements in organoid development, highlighting biological development models and organoid-promoting experimental techniques. In addition, it also discusses the advantages of various hydrogels for culturing organoids across different human body systems. The review explores how these hydrogel-cultured organoids can mimic organ development, facilitate drug screening, and contribute to translational research, with the potential for future commercialization in clinical applications. In addition, it addresses the present limitations and challenges in organoid research, while emphasizing the progress in biomaterial fabrication and the convergence of bioengineering with organoid studies. These developments offer expansive opportunities for simulating the development of various systems and organs.

2. Organoids

2.1. Origin and development

The earliest research on organoids dates back to 1907 when H. V. Wilson developed an artificial cultivation method for sponges, laying the foundation for 3D cell growth.[20] In the 1980s, studies began utilizing Matrigel to simulate external environments for cell growth.[21] The 1990s saw significant advancements in stem cell research, particularly with the discovery and cultivation of ESCs and AdSCs, which provided essential cell sources for organoid research.[22] A significant breakthrough occurred by identifying leucine-rich repeat-containing G protein-coupled receptor 5 (*Lgr5*)-positive stem cells, which were shown to form intestinal villi structures without mesenchymal cell support.[23] In 2013, the first brain organoids derived from human pluripotent stem cells (hPSCs) were cultivated, demonstrating the potential for studying brain-related diseases, such as microcephaly.[24]

Present research focuses on combining organoids with gene editing technologies to engineer organs that express specific genes and functions, enhancing their utility in targeted cancer therapies.[25]

2.2. Cultivation and formation

2.2.1. Traditional techniques for organoid generation

Inducing intestinal organoids from human ESCs (hESCs) requires the stages of definitive endoderm (DE) and hindgut endoderm (HE). Crespo *et al.*[13] initiated DE formation using 3 μm *CHIR99021* (a glycogen synthase kinase-3 inhibitor) and 100 ng/mL *Activin A* (a transforming growth factor [TGF]-β family cytokine). For colon differentiation, *CDX2* expression was induced using a B27 medium (Neurobasal medium/B27 supplement) supplemented with *CHIR99021* and Fibrinogen growth factor 4 (*FGF4*). From day 8 onward, *CHIR99021* was used to inhibit Glycogen synthase kinase-3β (*GSK-3*β), while LDN193189 was employed to inhibit *TGF*-β. This was followed by a 12-day treatment with an epidermal growth factor (EGF)-containing colon culture medium, and finally, the organoid cell clusters were embedded in Matrigel beads.[13] Drakhlis *et al.*[26] described a novel approach for constructing cardiac organoids, which recapitulates early cardiac development using hPSCs. They embedded hPSC aggregates into the Matrigel and sequentially applied *CHIR99021* (a Wnt pathway activator) and *IWP2* (a Wnt pathway inhibitor) to promote differentiation.[26] The initial activation conditions of Wnt are crucial for cardiac organoid development. Subsequently, Lewis-Israeli *et al.*[27] continuously exposed hPSCs in embryoid bodies to *CHIR99021* (a Wnt activator that inhibits *GSK3*) and *Wnt-C59* (a Wnt inhibitor that inhibits PORCN). In addition, they proposed that the optimal induction of cardiac mesoderm is achieved at a concentration of 1 – 4 μm *CHIR99021*.[27] In the induction of bone marrow organoids, Khan *et al.*[28] initially used a combination of StemFlex and RevitaCell to form iPSC aggregates. From days 0 to 3, they induced mesodermal formation under 5% oxygen using *bone morphogenetic protein 4* (*BMP4*), *FGF2*, and *vascular endothelial growth factor A (VEGFA)*. From days 3 to 5, they introduced *BMP4*, *FGF2*, *VEGFA*, *stem cell factor (SCF)*, and *FMS-like tyrosine kinase 3 (FLT3)* as factors in the co-culture system. From days 5 to 12, the cells were embedded in a mixed-matrix hydrogel to facilitate angiogenesis[28] (Figure 1).

2.2.2. Emerging techniques for organoid generation

2.2.2.1. Suspension culture

Suspension culture mode is a cultivation method where organoids are suspended and grown within a culture medium without a supporting matrix.[29] Compared to traditional culture methods based on Petri dishes or microplates with supporting matrices, the suspension culture mode enables large-scale and high-throughput cultivation of organoids. This approach is particularly suitable for experiments that require a substantial number of samples, such as drug screening and high-throughput gene editing.[30] In addition, in traditional culturing methods, organoids must adhere to a fixed supporting matrix, which can lead to mechanical damage and uneven growth. Furthermore, cells within the fixed matrix suffer from a prolonged inadequate supply of oxygen and nutrients, necessitating frequent manual interventions to maintain a suitable environment. In contrast, suspension culture mitigates these issues, promoting more natural growth and differentiation of organoids.[31] Moreover, suspension culture better mimics the *in vivo* microenvironment, including the diffusion of nutrients and the exchange of oxygen and carbon dioxide, thus aiding in maintaining physiological functions and the 3D structure of organoids. Some studies developed a low-viscosity matrix suspension culture method, which uses a medium supplemented with Matrigel to provide initial microenvironmental support for the cells. During the passage process, the TrypLE Express enzyme effectively disperses cells and tissues, ensuring they can be more easily re-cultured. Using different matrix types (such as *BME-*

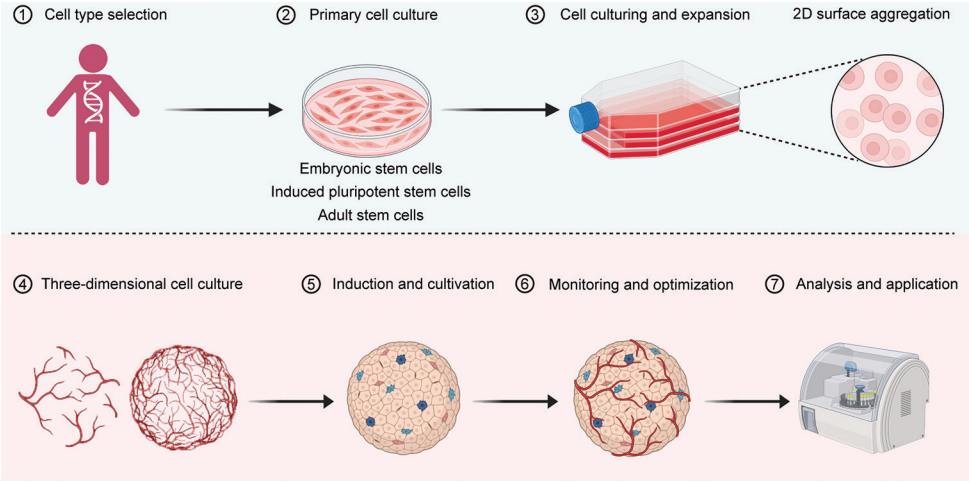

Figure 1. Cultivation techniques and morphogenetic patterns in organoid formation

1, *BME-2*, or collagen) for subsequent culture, enables continuous optimization of growth conditions.[31]

2.2.2.2. Microfluidic cultivation

Microfluidics, a technology for manipulating and processing liquid flow on a micrometer scale, has demonstrated significant potential in cultivating organoids in recent years. This technology is particularly effective for simulating the microenvironment of organs and controlling conditions for cell growth and differentiation. The following are several primary modes of organoid cultivation using microfluidic technology:

(i) Monolayer cell culture

By employing a microfluidic chip design, cells can form a monolayer membrane structure within microchannels. Through precise control of liquid flow, the required oxygen and nutrients for cell growth can be maintained, while metabolic waste is effectively eliminated. Microfluidic-mediated 2D monolayer gastric organoids can rapidly spread to form a uniform cell layer, offering an advantage for simulating the upward-growing columnar epithelium found in the gastric wall.[32]

(ii) Three-dimensional culture

By utilizing 3D structures within microfluidic chips, cells naturally form aggregates in the microenvironment, mimicking the 3D structure of organs. Microfluidic devices can provide uniform liquid flow and nutrient distribution, supporting long-term cell growth and differentiation. This method is highly suitable for cultivating complex 3D organoids, such as liver organoids.[33]

(iii) Dynamic fluidic culture

Dynamic fluid flow can be achieved through microfluidic systems, enabling continuous or pulsatile fluid movement that mimics physiological conditions, such as blood or lymphatic flow. This dynamic environment aids in maintaining the physiological state of cells and promotes the maturation of organoids. For instance, microfluidic devices can construct a sustained tumor microenvironment, facilitating interactions between tumor fragments and allowing a continuous flow of tumor-infiltrating lymphocytes. This setup simulates lymphocyte-mediated tumor immunity and infiltration, providing valuable insights into cancer research and immunotherapy.[34]

(iv) Multi-layered fluidic culture

The architecture of multi-layer fluidic channels within a microfluidic chip allows different types of cells or culture media to flow in distinct layers, thereby enabling cellular isolation or interaction. By regulating the fluid flow in these layers, it is possible to simulate the multi-layered structures found in organs. For instance, in the construction of patient-derived organoid (PDO) models for hepatocellular carcinoma, the tumor microenvironment necessary for organoid growth is created by the multi-layer microfluidic chip, which houses a co-culture system of mesenchymal stem cells (MSC) and peripheral blood mononuclear cells (PBMC), forming the MSC-PDO-PBMC model. Preliminary applications in drug experiments, including chemotherapy and molecular targeted therapies, have demonstrated the potential of this model in drug screening and cancer research.[35]

(v) Microenvironmental regulation

Microfluidics influences the development and functionality of organoids by regulating microenvironmental factors such as temperature, pH, and oxygen concentration, thereby providing culture conditions that more closely resemble physiological states.[36] This technology opens new possibilities in developing functional retinal tissue engineering by guiding the connectivity of various cell populations. The design of microchannels can direct axonal connections to grow exclusively in a single direction, mimicking the conditions found in native tissues. Furthermore, the integration of valves and pumping systems facilitates the functional maturation and long-term viability of the tissues, addressing one of the main challenges currently faced by 3D tissue engineering technologies.[37]

2.2.2.3. Air-liquid interface (ALI) culture

The ALI method is a technique that involves cultivating cells at the boundary between gas and liquid phases, which has garnered significant attention and widespread application in organoid research in recent years. In contrast to conventional submerged culture methods, this cultivation paradigm provides a more precise representation of *in vivo* conditions. It is significant for constructing epithelial cell organoids with specific functional and structural attributes.[38] The ALI culture model cultivates cells using a porous support system, where the bottom of the cells is immersed in a culture medium while the top remains exposed to air. This air-liquid interphase environment encourages cells to grow upward, forming a multilayered cellular architecture conducive to the differentiation and maturation of neurons. Brain organoids cultured at the air-liquid interphase exhibit enhanced electrophysiological activity, including inter-neuronal connectivity and signal transmission, which is important for studying brain function and disease models.[39] Furthermore, this model is well-suited for cultivating epithelial organoids, such as gastrointestinal or respiratory organoids, as these tissues naturally develop in an environment characterized by the interaction between gas and liquid *in vivo*.[40,41] Biocompatible porous materials, such as collagen, sodium alginate, or polylactic acid, are often used as scaffolds during cultivation. These materials support cell attachment and facilitate the exchange of nutrients and gases.[42] In summary, the ALI culture method, with its efficient oxygen supply and spatial utilization, has emerged as a crucial cultivation technique in modern bioscience and engineering research.

2.3. Signaling pathway activation

2.3.1. Wnt

The Wnt/β-catenin pathway regulates stem cell pluripotency and plays a crucial role in cell proliferation, differentiation, and embryonic development. Wnt, a secreted glycoprotein, is synthesized intracellularly and transported to the membrane, forming a protein complex with the membrane-bound *Frizzled* (FZD) receptors and *LRP5/6* co-receptors. The dimerization of this receptor activates kinases that phosphorylate the LRP tail and recruit the scaffold protein Axin.[43] β-catenin is a protein that mediates cell-cell adhesion through E-cadherin and participates in signal transduction. In its inactive state, it is sequentially phosphorylated by CK1 and GSK3, followed by ubiquitination and degradation by β-TrCP/Skp.[44] Typically, free β-catenin levels are maintained at low intracellular levels to prevent excessive malignant proliferation. However, in the membrane-bound complex, phosphorylated LRP inhibits *GSK3* activation, stabilizing intracellular β-catenin levels. This stabilization promotes its nuclear translocation, a critical step in initiating the Wnt/β-catenin signaling pathway.[45] During the determination of cortical neuron subtype proportions, downregulation of β-catenin increases *SATB2* expression in the subcortical region of the brain, aligning its proportion with *TBR1*$^+$ neurons and affecting the layered expression patterns between the cortical and subcortical regions of the brain.[46] In liver organoid culture, single *Lgr5*$^+$ stem cells can clonally expand into organoids that retain many characteristics of the original epithelial tissue. This expansion requires the Wnt agonist *Rspo1* in the culture medium, which serves as a ligand for *Lgr5*.[47]

2.3.2. Notch

The Notch signaling pathway plays a crucial role in maintaining stem cell gene expression and driving the differentiation of intestinal *Lgr5*$^+$ stem cells. These intestinal stem cells (ISCs) can be cultured homogeneously under various conditions that provide essential microenvironmental signals, such as Notch and Wnt signaling, supporting rapid proliferation and long-term self-renewal.[48] In the cultivation and formation of multilineage liver organoids, Kim *et al.*[49] utilized hepatic stellate cell-like cells to promote the formation of functional microvasculature within the organoids relying on Notch pathway-mediated endothelial interactions. This microvasculature facilitates the delivery of oxygen and nutrients to the inner layers of the organoids, thereby preventing necrotic core formation.[49] Similarly, pharmacological studies analyzed the role of the Notch signaling pathway in organoid differentiation. Notch inhibitors increased the expression of basal markers mRNA, upregulated *TP63*, and significantly reduced luminal cell markers (such as *Upk1b*, *Upk2*, *Upk3a*, and *Krt20* mRNA), indicating that Notch signaling plays a crucial role in the differentiation of urothelial epithelium.[50] In fallopian tube organoids, inhibiting Notch signaling, through the γ-secretase inhibitor dibenzazepine (DBZ), affects organoid growth and cell fate decisions. After DBZ treatment, 78 genes were significantly downregulated, including *Eph4A*, *RNF43*, *SMO*, and FZD receptors, *FZD2* and *FZD7*. The downregulated genes were enriched in "stem cell characteristic" genes essential for maintaining stem cell states, resulting in significant fold changes and alterations in global gene expression patterns within the organoids. These findings suggest that Notch-dependent regulation in fallopian tube organoids overlaps with the ISCs network, indicating shared regulatory mechanisms between the two systems.[51]

2.3.3. BMP

BMP4 are signaling proteins critical for cell growth, differentiation, and development. Initially recognized for their key functions in bone and cartilage formation, BMPs are now known to be essential in organ development and stem cell regulation. BMP signaling is primarily categorized into two; the classical BMP/Smad pathway and the non-classical pathways involving MAPK and PI3K.[52] Bustamante-Madrid *et al.*[53] analyzed the effects of BMP4 and Notch signaling blockade on organoids generated from adjacent normal tissue of patients with colorectal cancer. They verified BMP4 responsiveness by detecting phosphorylated SMAD1/5/8 proteins and the expression of BMP4 target genes, *ID2* and *DKK1*. The activity of DBZ was validated by detecting the decreased expression of the Notch target gene *HES1*. The results showed that BMP4 plays a primary role in promoting the differentiation of intestinal cells into intestinal epithelial cells, while Notch signaling strongly inhibits the mucus-secreting cell pathway.[53] In inner ear organoids, BMPs exhibit a dual role in stem cell differentiation. Under specific conditions (e.g., SB-431542 [SBB] treatment), BMPs promote the formation of non-neural epithelial cells, while under other conditions (e.g., SB-431542 [LSB] treatment), they inhibit neural epithelial cell formation. Specifically, treatment with LSB (a TGF-β inhibitor) alone upregulates non-neural markers, such as *TFAP2A* and *DLX3*, without inducing *CDX2* expression. However, co-treatment with BMP4 and SBB promotes the expression of *TFAP2A*, *DLX3*, and the extraembryonic marker *CDX2*. Conversely, co-treatment with the BMP inhibitors, LDN-193189 and LSB, leads to the upregulation of neuroectodermal markers, such as *PAX6* and N-cadherin, and the suppression of *TFAP2A* and *ECAD* expression, indicating that BMP activity plays a role in inhibiting non-neural differentiation.[54]

2.3.4. TGF-β

TGF-β is a homodimeric protein primarily composed of two identical subunits, each with a molecular weight of

approximately 25 kDa. In the body, TGF-β exists in an inactive pre-cursor form, consisting of a secreted pre-cursor protein complex with latent TGF-β-binding proteins. The protein is activated through enzymatic cleavage or other activation mechanisms when required by cells, producing biologically active TGF-β.[55] In a humanized skin organoid model, aggregating hPSCs into multicellular clusters, the addition of a matrix, BMPs, and LSB (a TGF-β inhibitor) in the culture dish inhibits TGF-β signaling and facilitates ectoderm induction from pluripotent stem cells, leading to the formation of initial epithelial cysts.[56] Similarly, in the formation of expanded neuroepithelium organoids (ENOs), a gradient of decreasing TGF-β concentrations is employed. Adding LSB during cortical neural induction suppresses TGF-β signaling, resulting in organoid morphology closely resembling cortical organoids. In contrast, organoids not treated with TGF-β inhibitors exhibit morphology similar to ENOs. Thus, generating ENOs from different hESC/iPSC lines is associated with varying levels of TGF-β inhibition.[57] The relevant pathways are presented in Table 1.

3. Application of different types of hydrogels in organoid culture

Compared to traditional 2D cell culture methods, organoid culture requires a specialized 3D culture system to facilitate cell growth and adhesion.[58] Conventionally, organoid culture has primarily depended on extracellular matrices derived from animal or tumor sources, which has resulted in poor reproducibility in organoid preparation and potential tumorigenic risks. However, with the advancements in tissue engineering, hydrogel scaffolds based on natural and synthetic polymers have become crucial components of organoid culture due to their unique advantages.[59] Natural matrix hydrogels are typically derived from biological materials and possess excellent biocompatibility, providing cells with a growth environment similar to their natural state. These hydrogels contain bioactive components, such as proteins and polysaccharides, that can enhance interactions among cells and between cells and the matrix, promoting cell growth, differentiation, and functional expression.[60,61] In contrast, synthetic matrix hydrogels are chemically synthesized, allowing for high tunability and precise control over their composition, mechanical properties, and degradation characteristics. In addition, synthetic matrices can be chemically modified or functionalized to precisely regulate cell behavior and organoid development.[62]

3.1. Components of natural hydrogels

3.1.1. Decellularized ECM (dECM)

The ECM is a complex network secreted by cells, primarily composed of proteins, glycosaminoglycans (GAGs), and bioactive factors, such as TGF-β1 and FGF. It plays a crucial role in the cellular microenvironment, contributing to cell survival and modulating cellular behavior.[63] ECM provides physical support to cells and conveys biological information while facilitating intercellular signaling. By decellularizing ECM to create dECM, potential immune responses can be avoided while retaining the functional properties of the ECM, which support organoid cultivation and functionality.[64] dECM provides a biomimetic microenvironment that enhances cell adhesion, differentiation, and proliferation. Decellularization also allows matrix customization, making it more suitable for various organoid cultures and clinical applications.[65] Kim *et al.*[66] utilized renal dECM hydrogels for the *in vitro* culture of kidney organoids, resulting in more extensive vascular networks than traditional Matrigel. Moreover, dECM provides enhanced support and guidance for cell growth and differentiation, facilitating the development of more mature and functionally complete kidney organoids.[66]

3.1.2. Alginate

Alginate, a natural polymer derived from seaweed, is known for its excellent non-immunogenicity and gelation properties, making it a popular choice in various medical biomaterials.[67] During organoid cultivation,

Table 1. Organoid-related signaling pathways

Signaling pathway	Receptor type	Ligand type	Key signal molecules	Main physiological functions	References
Wnt/β-catenin	Frizzled and LRP5/6	Wnt protein	β-catenin, etc.	Cell proliferation, differentiation, and embryonic development	43
Notch	Notch receptor (single transmembrane protein)	Jagged 1/2/Delta 1/3/4	Notch	Maintaining stem cell gene expression	48
BMP	BMP receptor type I/II	BMP ligand	Smad, MAPK, etc.	Cell growth, differentiation and development, organogenesis, and stem cell regulation	53
TGF-β	TGF-β receptor type I/II/III	TGF-β ligand	R-Smad, Co-Smad (Smad4)	Cell proliferation, differentiation, apoptosis, and stromatogenesis	55

Abbreviations: BMP: Bone morphogenetic protein, TGF: Transforming growth factor.

cells require suitable interconnected voids for effective communication. Alginate scaffolds can be prepared using gas foaming, microfluidic foaming, electrostatic spinning, leaching techniques, and freeze-drying, creating a suitable microenvironment for organoid culture. While alginate hydrogels have broad potential in cartilage regeneration, they have a few limitations, including limited mechanical properties, instability in degradation, and durability during application. Various methods have been developed to address these issues. For example, Fang *et al.*[68] utilized microfluidic droplet technology to achieve high-throughput generation of organoids within alginate microbeads. The tumor masses within these alginate microbeads exhibit both tubular and solid-like structures, demonstrating high similarity in cellular phenotype and lineage of the original tumors.[68] In addition, in a study where alginate hydrogels were used as an alternative to traditional Matrigel, it was found that alginate supported neurogenesis and gliogenesis in spinal cord organoids with similar efficiency. Furthermore, alginate reduced the expression of non-spinal cord markers, such as *FOXA2*, indicating its potential to regulate neural fate formation.

3.1.3. Chitosan

The chemical structure of chitosan closely resembles that of GAGs, which are key components of the ECM essential for cell-cell adhesion.[69] It exhibits excellent biocompatibility, biodegradability, mucoadhesive properties, and antibacterial activity while demonstrating rapid mechanical recovery under compressive loads.[70] This makes it an excellent support and growth environment for cells, with wide applications in tissue regeneration and drug delivery. However, chitosan still has certain limitations. Despite its toughness and flexibility, it lacks adequate mechanical strength and requires suitable crosslinking agents during a complex preparation process. Upadhyay *et al.*[71] cross-linked amine-enhanced hydrogels without chitosan using glutaraldehyde, which increased the rigidity and mechanical resistance of the chitosan hydrogel, providing a favorable environment for cell adhesion and proliferation of 3D-cultured MSCs and chondrocytes.[71]

3.1.4. Hyaluronic acid (HA)

HA is a GAG present in the human body, particularly in the ECM of bodily fluids and tissues. It is essential for maintaining tissue hydration and plays a key role in cell proliferation, differentiation, and inflammatory responses. It demonstrates significant biological activity, leading to the development of biomedical products based on its biocompatibility, biodegradability, non-toxicity, and non-immunogenicity properties.[72] However, HA hydrogels generally face challenges, such as inadequate mechanical strength and susceptibility to degradation by hyaluronidase.[73]

Due to its numerous reactive functional groups, HA can be readily functionalized, allowing for various structural modifications and novel crosslinking strategies to broaden its range of applications.[74] Wu *et al.*[75] utilized HA hydrogels as a 3D culture model to promote the self-assembly and neural differentiation of human iPSC (hiPSC)-derived neural progenitor cells. Soft methacrylated HA hydrogels significantly enhanced neural differentiation of neural progenitor cells, providing an effective platform for research into central nervous system disorders.[75]

3.1.5. Collagen

Collagen is the most abundant protein in the animal kingdom and a key component of the ECM. It can be easily and cost-effectively isolated from tissues, such as skin, tendons, and pericardium, and is widely used as a biomaterial. Collagen exhibits excellent biocompatibility, providing an ideal environment for cell adhesion and proliferation, making it an excellent material for tissue regeneration.[76] Randriamanantsoa *et al.*[77] successfully developed a complex tubular 3D architecture resembling pancreatic tissue by embedding single mouse pancreatic ductal adenocarcinomas within a collagen matrix, thereby capturing the growth process and morphological evolution of the tumors.[77] Although collagen is primarily responsible for the tensile properties of natural connective tissues, collagen hydrogels generally exhibit low mechanical performance without covalent crosslinking, which can be challenging for regenerating stiffer and stronger natural tissues, such as bone.[78]

3.1.6. Silk

Silk fibroin (SF) is a protein fiber secreted by arthropods and other organisms. Its unique properties allow it to be utilized in various biomaterial forms, including films, sponges, scaffolds, tubes, electrospun fibers, and hydrogels, making it widely applicable in the biomedical field. Gupta *et al.*[79] used SF to support the growth and differentiation of primary cells and iPSC-derived kidney tissues. The silk scaffolds supported the differentiation of key epithelial cell types and facilitated structural formation. Moreover, the epithelial cells in the SF scaffolds expressed appropriate molecular markers, indicating that the epithelial-mesenchymal transition process successfully produced epithelial cells resembling nephron units. This demonstrates the potential of SF as a scaffold material for iPSC-derived kidney tissues.[79]

3.2. Components of synthetic hydrogels

3.2.1. Polyethylene glycol (PEG)-based hydrogel

PEG is a versatile polymer with extensive pharmaceutical applications. Composed of repeating ethylene glycol units, PEG is widely used in various drug delivery systems due to its diverse chemical structures and molecular weights. It

enhances drug solubility through conjugation or formation of solid dispersions. In addition, PEG can be combined with other chemical groups to design drug delivery systems sensitive to pH, reducing environments, or enzymatic activity, enabling targeted drug release at specific sites. In organoid culture, the biocompatibility of hydrogels is crucial for the proper differentiation of stem cells. PEGylated drugs often exhibit reduced acute toxicity, and the "stealth" effect can minimize immune system recognition of the drugs, decreasing adverse reactions.[80] Klotz *et al.*[81] developed a PEG hydrogel platform that mimics the biological functions of the basement membrane while maintaining the simplicity, customizability, and reproducibility of synthetic materials. This platform successfully engineered large blood vessels and capillaries within liver organoids.[81]

3.2.2. Poly(N-isopropylacrylamide) (PNIPAAm) hydrogel

PNIPAAm is a temperature-responsive polymer known for its pronounced hydrophilic-hydrophobic transition at a specific temperature. This property has garnered significant attention in smart materials. Its chemical structure consists of long-chain polymers formed by the free radical polymerization of N-isopropyl acrylamide monomers. The distinctive isopropyl side chain gives it a low critical solution temperature (LCST) of approximately 32°C. Below this temperature, PNIPAAm hydrogels swell and exhibit hydrophilic properties, while above the LCST, they undergo a phase transition to a hydrophobic state, expelling water.[82] Physical cross-linking can occur near body temperature, enhancing the mechanical strength of the hydrogel and allowing for rapid *in situ* gelation, which is beneficial for cell transplantation.[83,84] Sun *et al.*[85] developed a porous hydrogel based on PNIPAAm as an integrated platform for spheroid bioinks preparation. The non-adhesive porous structure of the hydrogel facilitates the efficient fabrication of adipose-derived stem cell spheroids with excellent bio-preservation and chondrogenic differentiation.[85]

3.2.3. Polyisocyanate peptides (PIC)-based hydrogels

PIC hydrogels are polymeric materials formed by PICs. PICs are polymers with rigid main chains that self-assemble into hydrogels in solution. These molecular chains typically feature specific amino acid sequences or other functional groups that mimic the mechanical and chemical properties of the natural ECM.[86] Liu *et al.*[87] developed PIC hydrogels as simplified models of the ECM to investigate the impact of different parameters on cellular behavior. The study primarily altered the stiffness and stress sensitivity of the hydrogels, affecting the contacts between cells and polymers and the efficiency of stress transfer by regulating the polymer chain length and the density of peptide adhesive sites.[87]

The classification of hydrogels is presented in Table 2.

4. Clinical applications of organoid hydrogels

4.1. Circulatory system

4.1.1. Heart

The human heart is one of the most structurally complex organs, originating from the mesoderm into a cardiac crescent. It is composed of cardiomyocytes, accounting for

Table 2. Application of different types of hydrogels in organoid culture

Types of hydrogels	Components	Essence	Advantage	References
Natural hydrogel components	Decellularized extracellular matrix	Complex network structure secreted by cells	Low immunogenicity	58
	Alginate	Natural polymer derived from seaweed	Excellent non-immunogenicity, gelation properties	65
	Chitosan	The chemical structure closely resembles glycosaminoglycans	Excellent biocompatibility, biodegradability, mucoadhesive properties, antibacterial activity, and rapid mechanical recovery under compressive loads	68
	Hyaluronic acid	Glycosaminoglycan	Excellent biocompatibility, biodegradability, non-toxicity, and non-immunogenicity	70
	Collagen	A key component of the extracellular matrix	Excellent biocompatibility, economical	74
	Silk	Protein fiber	Unique properties	77
Artificially derived hydrogel	Polyethylene glycol-based hydrogel	Versatile polymer	high solubility, high targeting, low acute toxicity	78
	Poly (N-isopropyl acrylamide) hydrogel	A temperature-responsive polymer material	A pronounced hydrophilic-hydrophobic transition at a specific temperature	80
	Hydrogels based on polyisocyanate peptides	Polymeric materials formed by polyisocyanides	Emulate certain mechanical and chemical properties of the natural extracellular matrix	83,84

70 – 85% of its volume but only 25 – 35% of its total cells.[88] The remaining 65 – 75% consists of non-cardiomyocytes, including endothelial cells, vascular smooth muscle cells, fibroblasts, neurons, and immune cells.[89] These heart cells and the vascular system are surrounded by a 3D ECM network. The ECM is composed of collagen, laminin, and fibronectin secreted by heart cells, along with a plethora of cell adhesion molecules, growth factors, proteases, and glycoproteins.[90,91] Organoid hydrogels are ideal for cardiac scaffolds due to their customizability, unique mechanical properties, controlled release of biological factors, and the ability to replicate the heart's structure, function, and complex microenvironment.

Zhang *et al.*[92] developed an engineered endothelialized myocardial tissue platform by integrating 3D bioprinting and microfluidic technologies (Figure 2A). Endothelial cells were encapsulated within a microfibrous structure created by bioprinting, leading to a continuous endothelial layer (Figure 2B). The hydrogel scaffold facilitated the orderly localization of endothelial cells within the microfibrous structure. Subsequently, myocardial cells were seeded onto the macroscale anisotropic microfibrous structures, inducing the formation of well-aligned myocardial tissue capable of spontaneous and synchronized contraction (Figure 2C). Coupled with a microfluidic perfusion bioreactor, the endothelialized myocardial chip was used to screen for the cardiovascular toxicity of drugs. Finally, the potential of translating this model into endothelialized human myocardial tissue was explored, using hiPSC-derived cardiomyocytes for drug responsiveness testing.[92]

Lu *et al.*[93] introduced an innovative cardiac tissue engineering approach cultivating stem cell-derived

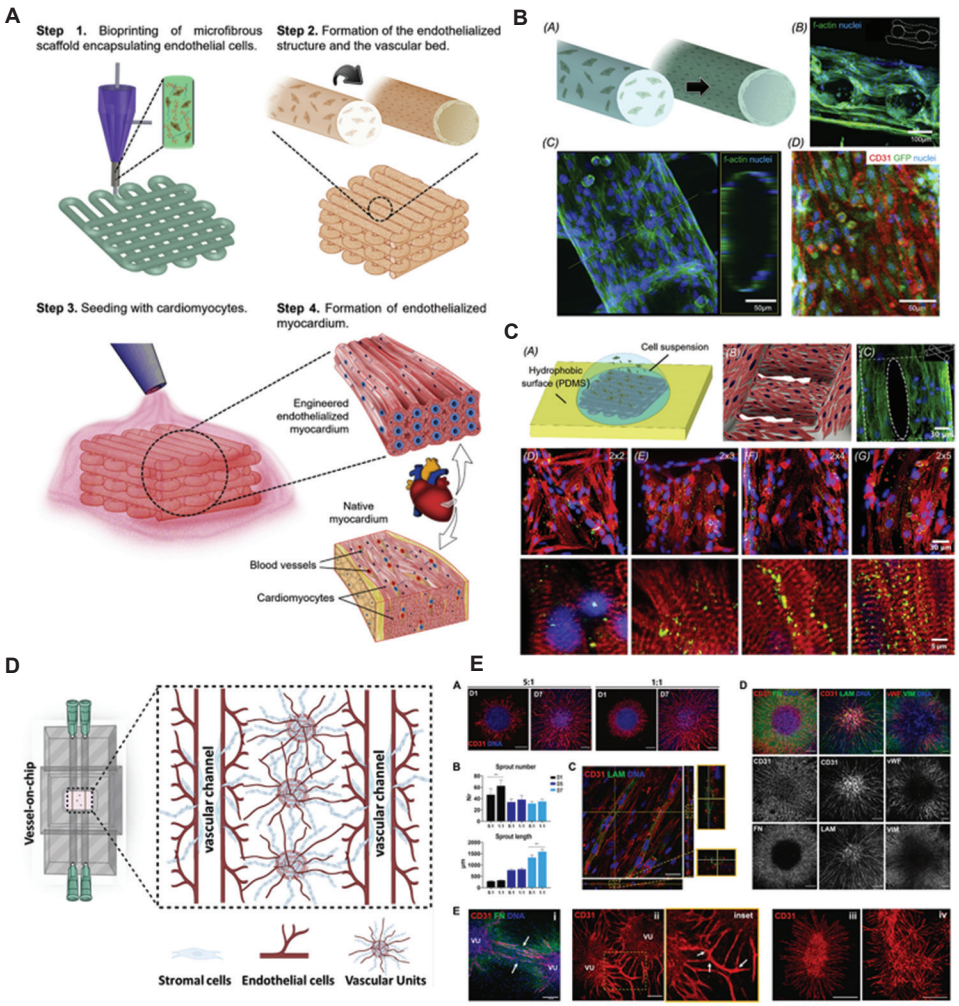

Figure 2. Engineering organoid culture models of the heart. (A) Schematic representation of the procedure for fabricating endothelialized myocardium using the 3D bioprinting strategy. (B) Endothelialization of the bioprinted microfibrous scaffolds. (C) Construction of the myocardium. Image used with permission from Zhang *et al.*,[92] Copyright © 2016, ELSEVIER. (D) Schematics showing the procedure of perfusable 3D microvascular networks. (E) VUs form extensive microvascular 3D networks when embedded in fibrin. Image used with permission from Orge *et al.*,[98] Copyright © 2024, ELSEVIER. Abbreviations: 3D: Three-dimensional; VUs: Vascular units.

myocardial tissue under progressive stretching conditions, promoting its growth and maturation. In the study, a collagen-fibrin hybrid hydrogel provided mechanical strength and passive tension. Given the constraints on the maximal stretch and size of myocardial cells and the impaired contraction performance of engineered heart tissues (EHTs), the study proposed a novel method for stretching and growing EHTs under defined diastolic loads to achieve more physiological growth. This method enhances cellular cohesion through high cell density and low ECM quality, inducing maturation and alignment of myocardial cells through progressive stretching under biomimetic conditions. The results significantly improved contraction force, tissue compliance, cellular alignment, electrophysiological properties, and excitation-contraction coupling of EHTs. This innovative tissue engineering approach generates highly mature human EHTs and provides insights into cardiac developmental biomechanics, thus addressing critical needs for disease modeling and therapeutic tissue replacement.[93]

The complexity of the heart's structure and function necessitates innovative approaches in cardiac tissue engineering incorporating advanced technologies, such as 3D bioprinting, microfluidics, and biomimetic culture systems. While these studies do not directly address the synthesis and application of cardiac organoids, they provide valuable tools for modeling and treating cardiac diseases and offer insights into the biomechanical mechanisms of heart development. Future research should focus on optimizing these technologies to enhance organ mimicry and facilitate clinical translation.

4.1.2. Vessels

The vascular system is one of the first to develop during embryogenesis, essential for nutrient transport and waste removal.[94,95] Capillaries, the most common blood vessels, consist of a single layer of endothelial cells supported by a basement membrane and pericytes.[96] They form a complex branching network distributed throughout the body, ensuring a stable supply of oxygen and nutrients while facilitating timely waste removal.[97] Maintaining the complex structure and 3D network of blood vessels is essential for cell viability in regenerative medicine.

Orge *et al.*[98] proposed an innovative microvascularization strategy by utilizing fibrin-based hydrogels for the 3D printing of blood vessels. They employed modular "vascular units" to construct a perfusable 3D microvascular network embedded within a matrix using a bottom-up approach.[98] Specifically, fibrin-based hydrogels formed hollow channels in the hydrogel using a needle-based template. Fibrin solution was injected into these channels to form a stable matrix (Figure 2D). Following the removal of the needle template, the hollow channels were established and coated with fibronectin and collagen solutions to promote endothelial cell adhesion and growth. A dual-channel vessel-on-chip device was employed to enhance the complexity and functionality of the system, enhancing its biomimicry performance. This setup allows hierarchical and interconnected microvascular network formation without imposing geometric constraints on vessel growth, effectively mimicking the physiological behavior of natural blood vessels (Figure 2E). This method is both straightforward and reproducible, with the needle-based template technique offering a simpler and more repeatable alternative to traditional sacrificial molding methods.

Enrico *et al.*[99] introduced a method for 3D printing blood vessels using collagen hydrogels, where microchannels and cavities are formed through femtosecond laser irradiation.[99] This approach allows for the creation of millimeter-long channels with diameters ranging from 20 to 60 μm, remaining stable for at least 8 days under physiological conditions. This technology enables the generation of 3D microchannels and cavities of arbitrary shapes and sizes while preserving cellular bioactivity within the hydrogel. Its advantage lies in its ability to provide biologically relevant yet controllable vascularization, enabling the development of 3D tissue models for studying complex tissue targets, such as tumors and neural tissues.

However, present 3D vascular models often lack physiological complexity, particularly in simulating dynamic behaviors and responses. Future research should focus on developing models that can dynamically simulate blood flow dynamics and enhance cell viability for applications in regenerative medicine and drug screening.

4.2. Respiratory system

4.2.1. Lungs

The lungs, essential for respiration, are located within the thoracic cavity, extending from the collarbone or the first rib to the sixth and seventh ribs. They contain a complex network of blood vessels, nerves, lymphatic vessels, alveoli, and connective tissue.[100] Lung development begins with ventral budding from the anterior foregut endoderm, leading to bronchial airways and alveolar progenitor cell formation.[101] During the primordial lung sac stage (around 4 weeks), the lungs are encased by ectodermal epithelial cells. By the 5th week, these sacs differentiate into smaller lung vesicles, with bronchi branching into finer bronchioles and alveoli. By the 8th week, smooth muscle in the airways begins to receive neural innervation. As blood vessels grow into the lungs, the alveoli mature as sites of gas exchange.[102] The lungs consist of bronchial epithelial cells, endothelial cells, macrophages, and smooth muscle cells.[103] This complexity necessitates advancements in lung organoid development using biocompatible composites that can mimic the lung microenvironment.

Traditional cell culture matrices, such as Matrigel, suffer from batch variability and complex compositions, limiting their application in high-precision studies. Loebel *et al.*[104] developed a novel HA hydrogel with a microporous structure, allowing for a controlled culture environment for AT2 cells. Cells cultured in this microwell hydrogel exhibited higher levels of mature AT2 marker expression and better-preserved functional characteristics than those grown in Matrigel. The microwell hydrogel offers a more accessible culture system for generating and maintaining primary and iPSC-derived lung progenitor cells. It can also be adapted to other epithelial progenitor cells and stem cell aggregates, using various hydrogel types and compositions. Furthermore, Loebel *et al.*[104] designed a microporous HA hydrogel by printing the micropore shape on the HA hydrogel pre-cursor solution through a crosslinking reaction triggered by ultraviolet light. They then implanted individual cells on micropores, allowing them to form alveolar spheres, namely 3D culture structures of alveolar epithelial cells (Figure 3A).[104] The study explored micropores of different sizes and observed their effects on the formation and growth of alveolar spheres. They found that the alveolar bulb area depended on micropore size; increasing the culture aperture could increase the efficiency and area of the alveolar bulb culture, while an excessively large aperture could create diffusion barriers for iAT2 and nutrients (Figure 3B). Dye *et al.*[105] utilized alginate hydrogels, cross-linked with calcium, to culture hPSCs, resulting in organoids resembling airway-like structures more closely than those cultured in traditional Matrigel.[105] In addition, organoids cultured with this hydrogel gradually undergo fibrosis upon treatment with TGF, making them suitable for modelling idiopathic pulmonary fibrosis (IPF). This novel IPF model can offer new insights into the progressive fibrosis of lung tissue, alterations in *ECM* gene expression, and the reduction in alveolar epithelial cell numbers during IPF disease progression.[105]

Research on hydrogels in the construction of lung organoids has made significant progress, particularly in simulating the pulmonary microenvironment and supporting

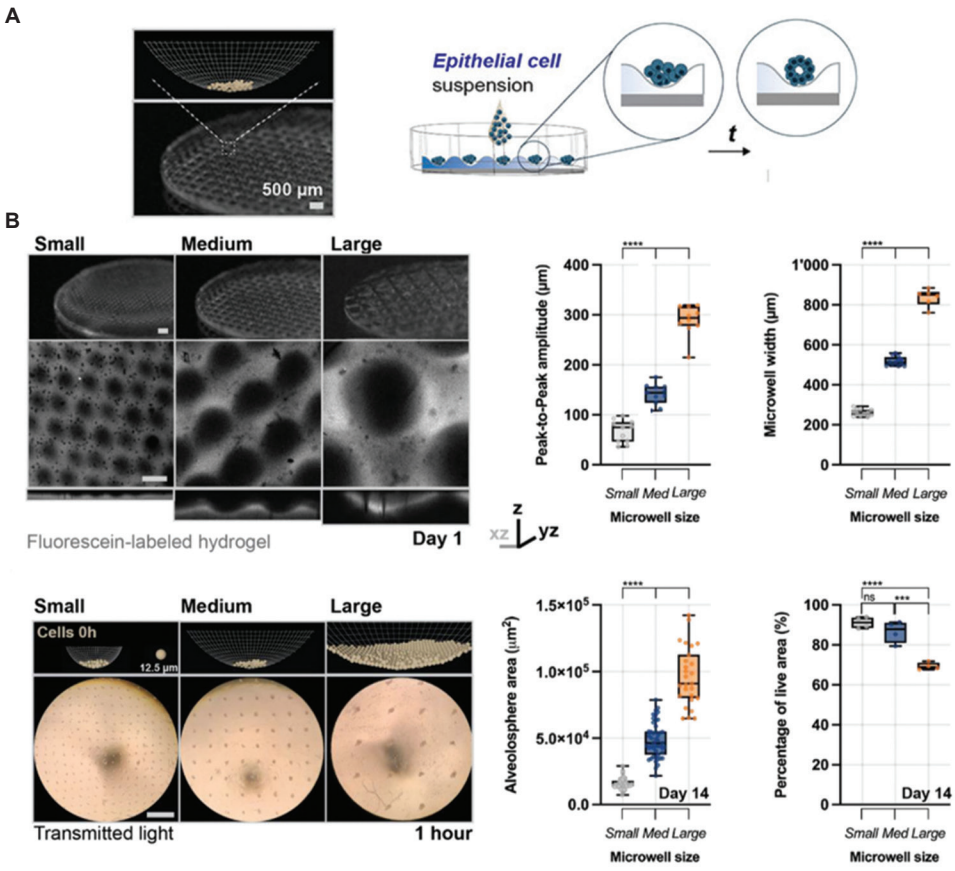

Figure 3. Hydrogel matrices provide support for the cultivation of lung organoids. (A) Formation of iPSC-derived alveolospheres within microwells of microstructured hydrogels. (B) Quantification of microwell hyaluronic acid hydrogels modified with fluorescein showcase variations in peak-to-peak amplitude and width after 1 day of swelling, with significant differences detected by ANOVA and Bonferroni's test ($P < 0.0001$). Simulated iAT2 cell localization and quantified alveolosphere areas at 14 days in culture highlight the impact of different microwell sizes on cell distribution and lung-like structure development.[104] Image used with permission from Wiley,[24] Copyright © 2022, Wiley.
Abbreviations: ANOVA: Analysis of variance; iPSC: Induced pluripotent stem cell.

cell-cell interactions. Hydrogels can effectively replicate the microenvironment of the lungs, providing necessary structural scaffolding and facilitating interactions between cells, thereby allowing a more accurate reconstruction of the 3D structure and function of lung tissue. However, while hydrogels can promote the formation of organoids, further studies are needed to determine whether they can maintain the functional integrity and viability of alveolar epithelial cells after transplantation into animal models. This involves investigating the biodegradability of hydrogels *in vivo* and their ability to provide long-term support to the cells.

4.3. Digestive system

4.3.1. Gastrointestinal tract

The primary functions of the gastrointestinal tract are digestion, absorption, excretion, and protection.[106] It develops from the endoderm into the foregut, midgut, and hindgut.[107] The foregut develops into the pharynx, esophagus, and stomach, the midgut develops into the small intestine and part of the colon, and the hindgut develops into the colon, rectum, and anus.[108,109] The stomach begins forming around week 4, with differentiation of its upper and lower regions. Its curvature and rotation establish its final morphology.[110] By the 8th week, the basic structures of the small and large intestines are established, followed by the differentiation of glands and mucosa. The immune system of the gastrointestinal tract, including gut-associated lymphoid tissue, continues to develop post-natal to protect the body from pathogens.[111] The intestinal epithelium undergoes multiple cycles of cell turnover in the crypts and villi of the small intestine. Sato *et al.*[23] pioneered the use of *Lgr5*$^{+}$ISCs, cultivated on a laminin-enriched matrix and supplemented with EGF, Wnt agonist *R-spondin 1*, and Notch agonist peptides. This approach led to the self-renewing epithelial structures formation, marking the inception of organoid technology for the intestine.[23]

Kim *et al.*[112] utilized gastrointestinal tissue ECM hydrogels as an alternative to cultured gastrointestinal organoids (Figure 4A).[112] By analyzing the composition and proteomics of ECM, the researchers found that gastrointestinal tissue-derived ECM hydrogels have highly similar properties compared to non-gastrointestinal Matrigel. They also compared the morphology, formation efficiency, organ-specific gene expression levels, and functionality of gastrointestinal organoids cultured in ECM hydrogels versus Matrigel. The results showed that when examining expression levels of gastric epithelial cell markers (main cell markers *Pgc* and parietal cell markers *Atp4a* and *Atp4b*) from organoid tissues cultured in decellularized stomach-derived ECM (SEM) hydrogel, the expression of *Pgc* and *Atp4a* was comparable to that of organoids in Matrigel, while the expression of Atp4b was higher in organoids cultured in SEM hydrogels. Furthermore, the researchers found that intestinal organoids grown in decellularized intestine-derived ECM (IEM) hydrogels showed comparable expression of core matrix protein-coding genes (*Col4a2*, *Nid1*, and *Lama3*), cytoskeleton-related genes (*Flna*, *Gsn*, and *Tuba1a*), and intestinal epithelial genes involved in thiamine uptake (*Tm4sf4*) to those of the original intestinal tissues. In addition, IEM organoids expressed genes associated with wound healing, inflammation, and immune response (*Procr*, *Mcpt2*, *Icam1*, *Cxcl10*, *Cxcl16*, and *Timp3*) at levels similar to the original intestinal tissue (Figure 4B), helping to maintain intestinal barrier homeostasis under physiological conditions. Overall, gastrointestinal tissue-derived ECM hydrogels can effectively replace Matrigel in gastrointestinal organoid culture. This alternative has similar properties to native gastrointestinal tissue, providing a more reliable and repeatable experimental platform for gastrointestinal research. This work, through proteomic analysis, has identified key matrix components necessary for the development of organoids, providing novel insights into the hydrogels required for organoid cultivation. In addition, it has addressed the issues of batch-to-batch variation, safety concerns, and high costs associated with traditional Matrigel, paving the way for potential commercialization and clinical applications in humans.

4.3.2. Liver

The liver is primarily composed of hepatocytes and biliary epithelial cells working in conjunction with stromal cells, endothelial cells, and mesenchymal cells to perform vital metabolic and endocrine functions.[113] Hepatocytes and biliary epithelial cells are differentiated from hepatic progenitor cells (embryonic liver pre-cursors) during organ development. Different cell types are strategically organized at specific locations along the liver lobules, essential for their individual functions and those of other cell types.[114,115] Hepatocytes are arranged in cord-like structures radiating from the central vein toward the portal vein. These polarized epithelial cells possess tight junctions and distinct polarity structures critical for their function. Bile is collected by tightly connected bile canaliculi, flowing in the opposite direction to blood and draining through the bile ducts.[116] Given this complexity, *in vivo,* the generation of organized lobular structures with multiple liver functions is challenging, particularly the long-standing issue of culturing and expanding primary hepatocytes (PHs) while maintaining liver functions.[117]

Wang *et al.*[118] introduced a novel method for the one-step fabrication of composite hydrogel capsules, based on interfacial complexation between oppositely charged sodium alginate and chitosan, for engineering stem cell-derived organoids in an oil-free microfluidic system (Figure 4C). This system enables continuous 3D culture,

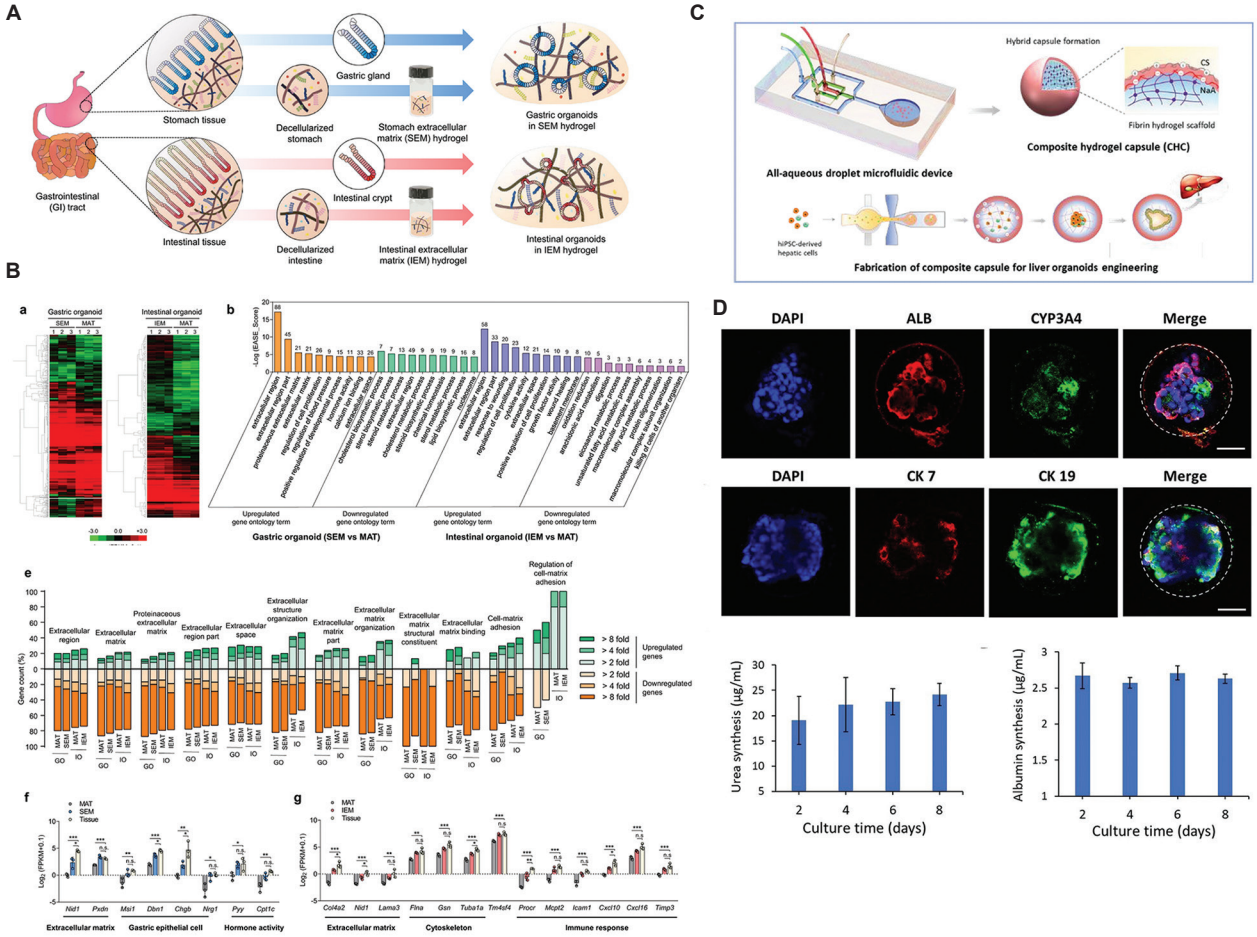

Figure 4. Hydrogels that adapt to the gastrointestinal physiology have become new alternatives for cultivation. (A) Preparation of GI tissue-derived ECM hydrogels for GI organoid culture. (B) Transcriptomic profiles of GI organoids cultured in GI tissue-derived ECM hydrogels. Image used with permission from Kim *et al.*,[112] Copyright © 2022, Nature Publishing Group. (C) One-step synthesis of CHCs that allows the engineering of hiPSC-derived liver organoids in an oil-free droplet microfluidic system. (D) Identification of differentiation and hepatic functions of liver organoids in CHCs. Immunohistochemical staining of hepatocyte markers (ALB, CYP3A4) and cholangiocyte markers (CK7, CK19) in liver organoids. Reprinted from image used with permission from Wang *et al.*,[118] Copyright © 2020, ROYAL SOCIETY OF CHEMISTRY.
Abbreviations: CHCs: Composite hydrogel capsules; ECM: Extracellular matrix; GI: Gastrointestinal; hiPSC: Human induced pluripotent stem cells.

differentiation, and generation of functional liver organoids from hiPSC. The composite hydrogel capsules exhibit high biocompatibility, stability, uniformity, and high-throughput capabilities, supporting 3D culture, assembly, and organoid formation in a mild aqueous environment. Liver organoids produced in these capsules show excellent growth, uniform size, and liver-specific functionality (Figure 4D). The hydrogel capsule system provides a controllable and stable 3D scaffold for high-throughput and reproducible organoid generation, addressing the limitations of traditional methods. This new system has significant implications for organoid research by integrating stem cell biology.[118]

The complex structure of the liver, particularly the organization of hepatic lobules, presents significant challenges for precise *in vitro* reconstruction. Generating multiple ordered lobular structures with liver functions is difficult. In addition, culturing and expanding PHs while preserving liver function remains a bottleneck in this field. PHs are prone to losing their functional characteristics and are difficult to culture and expand over extended periods. Advancing bioengineering technologies to reconstruct more complex liver structures is essential. This includes the accurate replication of hepatic lobule zonation and the development of sophisticated 3D multicellular systems to mimic the authentic liver environment.

4.4. Urinary system

4.4.1. Kidney

One of the most complex human organs, kidneys, features a sophisticated tree-like structure comprising 23 differentiated cell types. These cell types form distinct anatomical compartments, including blood vessels, stroma, collecting ducts, and nephron segments.[119,120] At present,

the primary challenges in engineering kidney organoids are safety, immaturity, and limited vascularization.[121] There is no available method to generate kidney organoids that fully recapitulate the complex structure and function of the kidney, limiting their efficacy in modeling kidney diseases and regenerative medicine.[66]

Nerger *et al.*[122] investigated how rigidity and viscoelasticity affect the 3D differentiation of human kidney organoids encapsulated in ion-cross-linked alginate hydrogels. Compared with traditional alginate hydrogels used for kidney organoid culture, the calcium ion-cross-linked alginate employed in their study has significantly higher viscoelasticity, resembling murine embryonic kidneys more closely. The use of alginate hydrogels as a culture matrix significantly impacts nephron morphology and function by encapsulating kidney organoids in viscoelastic gels with varying stress relaxation rates (Figure 5A), as encapsulated organoids exhibit more

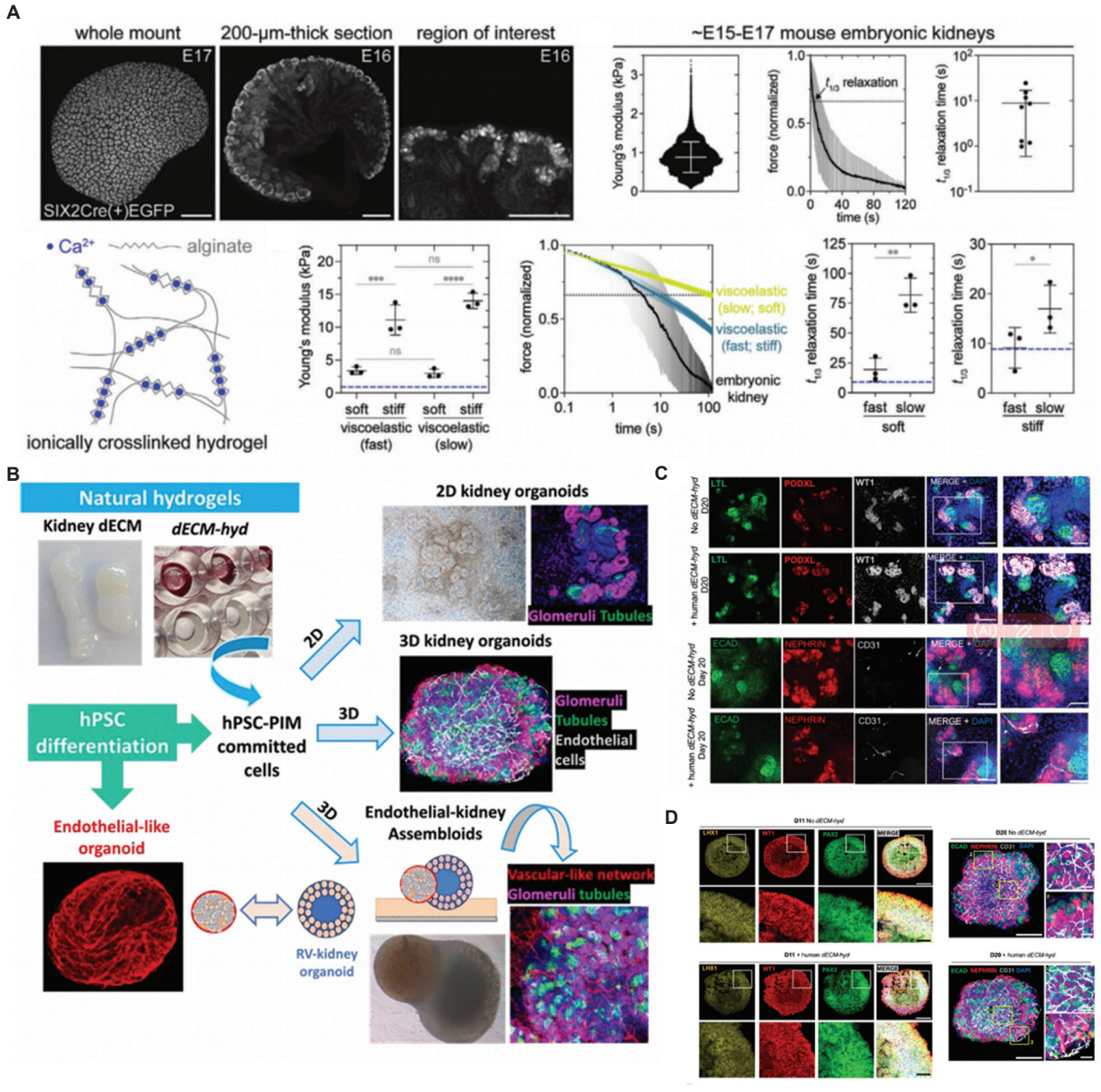

Figure 5. A new model for culturing kidney organoids with hydrogels. (A) Alginate hydrogels approximate the soft viscoelastic mechanical properties of the metanephric mesenchyme in the mouse embryonic kidney. Image used with permission from Nerger *et al.*,[122] Copyright © 2024, Wiley. (B) The scheme of a protocol for utilizing dECM-derived hydrogels to induce the differentiation of human pluripotent stem cells (hPSCs) into kidney organoids. (C) Human kidney dECM hydrogel enhanced the endothelial cell compartment in kidney organoids. Image used with permission from Garreta *et al.*,[123] Copyright © 2024, Wiley.

Abbreviations: dECM: Decellularized extracellular matrix; hPSCs: Human pluripotent stem cells.

pronounced curvature in their tubular segments than those in suspension culture.[122] The study revealed that mechanical and soluble signals generated by 3D packaging in alginate gel modulate nephron patterns and morphology of renal organoids, highlighting the importance of the 3D mechanical microenvironment in renal regenerative medicine and that the degree of cell-induced hydrogel deformation widely modulates epithelial morphogenesis during 3D organoid culture.

Strategies aimed at regulating cell-ECM interactions during organoid development have remained largely underexplored. Garreta *et al.*[123] used renal ECM-derived hydrogels transplanted into the chicken chorioallantoic membrane to demonstrate their angiogenic potential (Figure 5B). The isolation and aggregation of posterior intermediate mesoderm cells into spheres and the addition of dECM hydrogels to the culture medium every other day effectively induced a significant number of renal vesicles by day 11, with positive expression of intermediate mesoderm markers (*PAX2*, *WT1*, and *LHX1*). By day 20 of differentiation, the cells spontaneously patterned and segmented into typical nephron-like components, including renal tubules (ECAD$^+$), glomeruli (NEPHRIN$^+$), and endothelial cells (CD31$^+$) (Figure 5C). This indicates that dECM hydrogels enhance cell-ECM interactions, promoting the differentiation and angiogenesis of renal organoids (Figure 5D), thereby solving the problem of regulating the interaction between cells and ECM during organogenesis.[123]

The complexity of the structure and function of kidneys cannot be fully replicated by existing technologies, limiting their application in disease modeling and regenerative medicine. Nerger *et al.*[122] have demonstrated the significance of the 3D mechanical microenvironment in kidney organ development, while Garreta *et al.*[123] have showcased the potential of ECM in enhancing the vascularization of kidney organs. However, strategies to modulate cell-ECM interactions require further exploration. Future research should focus on developing more effective methods to regulate these critical factors to achieve kidney organ engineering that more closely mimics the organ.

4.5. Nervous system

4.5.1. Brain

During early embryonic development, the ectoderm forms the neural plate, which subsequently folds to create the neural tube, the pre-cursor of the central nervous system.[124] By the 4th week, the neural tube begins to close, forming the initial brain and the extending spinal cord structure. Around the 5th week, the anterior end of the neural tube expands into three primary brain vesicles: the forebrain, midbrain, and hindbrain. The forebrain differentiates into the cerebral hemispheres and diencephalon, the midbrain retains a relatively primitive structure, and the hindbrain differentiates into the cerebellum, pons, and medulla oblongata. Each brain region starts to develop preliminary functional areas.[125] During embryonic development and early post-natal periods, many cortical neurons are generated. In infancy and childhood, synaptic connections between neurons are established, leading to the proliferation of neural networks.[126] Subsequently, synaptic plasticity ensures the removal of unnecessary synapses through pruning, which warrants precision in brain function.[127] In addition, there are significant differences between human brain development and rodent models, including variations in developmental processes, brain volume, and genotype. In humans, the ventricular subependyma (SVZ) is divided by the inner fiber layer into the inner SVZ and the outer SVZ (OSVZ). However, the OSVZ is not present in commonly used rodent models. This discrepancy leads to insufficient model accuracy and experimental bias in present research, highlighting the need for brain organoid studies.[24]

Isik *et al.*[128] developed a biologically active hydrogel with tunable stiffness to cultivate and induce cerebral organoids (COs). The hydrogel was constructed from peptide amphiphiles (PAs) and HA. PAs offer well-defined chemical structures, tunable bioactivity, and nanofibrillar ECM-like architectures, making them an ideal support scaffold for organoid growth. Hydrogels with adjustable stiffness were successfully created by crosslinking PAs bearing the bioactive Ile-Lys-Val-Ala-Val peptide sequence with HA functionalized with tyramine groups. By employing multi-omics approaches, including transcriptomics, proteomics, and metabolomics, the study found that COs grown in these hydrogels exhibited morphological and biomolecular features similar to those grown in Matrigel. This hydrogel material shows promise as a safe synthetic ECM for CO induction and growth, providing a defined alternative to animal-derived matrices for CO-based basic and clinical studies.[128] Cho *et al.*[129] promoted the structural and functional maturation of human brain-like organs using microfluidic devices and the brain ECM (BEM).[129] They developed a microfluidic device that facilitates dynamic fluid flow and introduces BEM into organoids (Figure 6A). They found that using microfluidic devices and BEM significantly improved the structure and function of brain organoids. Brain organoids using microfluidic devices and BEM have better cell survival rates and proliferation than traditional culture methods. The microfluidic culture improved oxygen supply within the brain organoids promoting the increase of nerve cell population and thickening of neuroepithelium. Moreover, compared with BEM-cultured organoids, those grown in Matrigel exhibited smoother and smaller morphologies. This suggests that BEM promotes volume expansion, structural maturation,

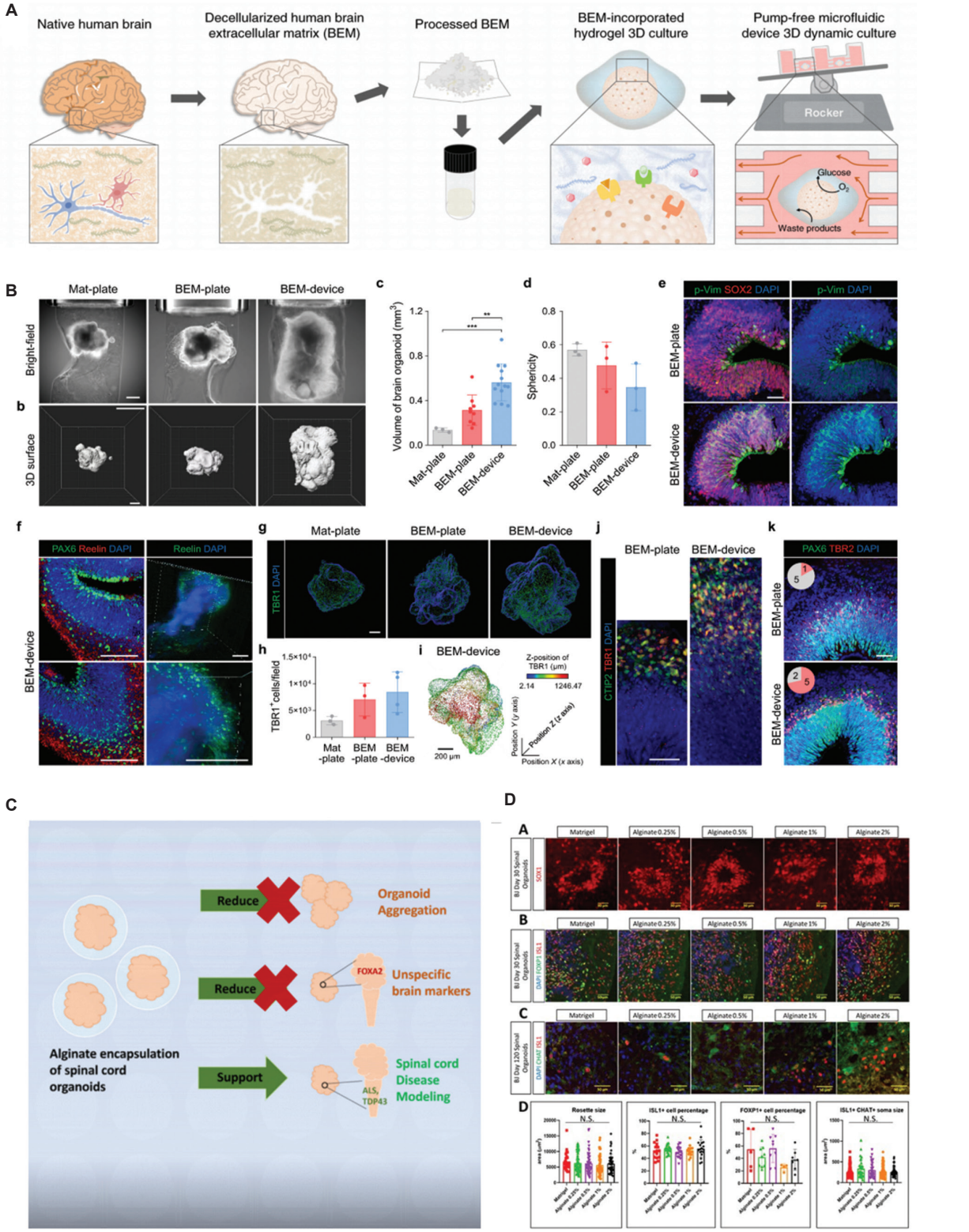

Figure 6. Hydrogels provide support for the cultivation of cerebral and spinal cord organoids. (A) Schematic illustration of the cerebral organoid culture system with a combination of 3D BEM hydrogel culture and the microfluidic device. (B) Bioengineering of the brain organoids by the microfluidic BEM system improves radial glial generation and cortical organization. Image used with permission from Cho *et al.*,[129] Copyright © 2021, Nature Publishing Group. (C) Schematic diagram of the effect of alginate encapsulation on maturation of spinal cord organoids. (D) Alginate supports neurogenesis in spinal cord organoids.[134] Image used with permission from Chooi et al.,[133] Copyright © 2023, Wiley.
Abbreviations: 3D: Three-dimensional; BEM: Brain extracellular matrix.

and the formation of elongated epithelium in organoids. Microfluidic devices amplify these effects by providing a favorable dynamic microenvironment for brain organoid culture (Figure 6B).

COs exhibit morphological and biochemical features similar to the human brain, allowing data acquisition closer to physiological conditions. Using biologically active and stiffness-tunable hydrogels, such as those incorporating peptides and HA, provides an ideal supportive structure that aids the growth and development of organoids. The integration of microfluidic devices with the ECM of brain cells enhances structural and functional maturation of organoids, improves cell viability and proliferation rates, and promotes neurogenesis. However, some differences exist between the development of brain organoids and the human brain, such as neurodevelopment, brain volume, and genomic type, which may impact the reliability of experimental results. Despite simulating certain brain functions, organoids have not fully reproduced the complexities of the neural network and higher cognitive functions. Therefore, it cannot replace genuine brain research. Nevertheless, these studies have provided a new platform for investigating neurological disorders and developing novel therapeutic strategies, necessitating continued technical research and improvements to enhance reliability in the future.

4.5.2. Spinal cord

The initial spinal cord structure is formed during embryonic development through the preliminary closure of the neural tube. The neural tube is divided into the inner grey matter and the outer white matter.[130] In the grey matter, clusters of neuronal cell nuclei develop into neurons, while glial cells, such as astrocytes and oligodendrocytes, begin to form and support neuronal function by providing nutrition, structural support, and forming myelin sheaths.[131] The white matter is primarily composed of myelinated nerve fibers. Spinal cord neurons, as permanent cells, have limited regenerative capacity and a long growth cycle. In addition, spinal cord nerve distribution differences between animal and human tissues make *in vitro* experiments extremely challenging. However, emerging techniques for preparing spinal organoids hold promise for advancing neuroscience.

Traditional matrices for organoid culture, such as basement membrane extracts or Matrigel, have unclear compositions, leading to reproducibility issues in spinal organoid development.[60,132] To address this, Chooi *et al.*[133] proposed using alginate hydrogels as an alternative (Figure 6C). Research has shown that by adjusting the concentration of alginate, the stiffness of hydrogels can be customized, facilitating the study of the effects of biophysical and biochemical signals on organoid maturation. Conventional Matrigel contains non-essential proteins, such as laminin, actin, proteoglycan, and growth factors, which lead to upregulated expression of other non-specific cell types, such as brain markers *FOXA2* and *FOXG1*. In contrast, alginate has the advantage of reducing the influence of these cofactors, improving the specificity and scalability of spinal cord organoids (Figure 6D). In addition, alginate hydrogels can minimize the impact of shear stress on organoids, preventing their aggregation and reducing variability in experimental results.[133] Wang *et al.*[134] employed dECM hydrogel from the human placenta (DPECMH) to promote the development of hiPSC-derived spinal cord organoids with distinct dorsal-ventral tissue structures. DPECMH prepared through a series of processing steps, has a complex ECM composition that supports the development and differentiation of spinal cord organoids better than traditional Matrigel. It facilitates the formation of key spinal cord regions within the organoids, including the floor plate, dorsal interneuron pre-cursor domain, ventral interneuron pre-cursor domain, and motor neuron pre-cursor domain. In addition, DPECMH promotes the generation of motor neurons, inhibitory neurons, and glial cells within the spinal cord organoids. The research provides a more comprehensive and accurate model for spinal cord organoid studies, with the potential to advance spinal cord development and pathology research.[134]

Given the limited regenerative capacity of spinal cord neurons, the study of organoids offers new avenues for neural regeneration. Utilizing alginate hydrogels and DPECMH provides enhanced support for organoid development. Compared to traditional Matrigel, these hydrogels reduce non-specific cell type expression, thereby improving the specificity and scalability of organoids. However, the extended growth cycles of spinal cord neurons may result in slower research progress. In addition, organoids may not fully replicate all biological characteristics of human spinal cords, limiting their effectiveness in certain applications. Therefore, while organoids provide a novel platform for spinal cord regeneration research, further research and technological advancements are necessary to enhance their biological relevance and application potential.

4.6. Skeletal system

Bone formation and development involve two primary processes: Bone development and remodeling. During early embryonic development, intramembranous ossification begins in the periosteum (a connective tissue membrane), where osteoprogenitor cells differentiate into osteoblasts, which mature into osteocytes and produce bone matrix.[135] The process of endochondral ossification involves chondrocyte proliferation and differentiation, bone mineralization, and chondrocyte apoptosis, ultimately forming primary bone.[136] Some bones, particularly

cranial and facial bones, form through intramembranous ossification, bypassing a cartilage model by allowing osteoblasts to produce bone matrix and trabeculae directly.[137] After skeletal development is complete, the bone undergoes remodeling in response to internal and external mechanical loads, establishing a robust bone structure. Within the bone, a dynamic equilibrium exists among osteoblasts, osteocytes, bone lining cells, and pre-osteoblasts.[138] Present experiments focusing on 2D cell cultures and single-cell interaction models have limited value in demonstrating bone repair. In contrast, the construction of bone organoids offers greater reference and application value for studying bone development and remodeling compared to traditional 2D cell culture models.

4.6.1. Bone

In large bone defects, insufficient callus tissue formation can lead to prolonged healing times or the failure of the defect to heal, leading to non-union.[139] Using MSC aggregates can enhance bone regeneration. However, differences in physiological callus tissue may limit further endogenous osteogenesis.[140] Xie *et al.*[141] employed digital light processing-based 3D bioprinting technology with gelatin methacrylate (GelMA) hydrogels loaded with bone marrow-derived stem cells (BMSCs). By sequentially inducing these microspheres to aggregate into callus-like organoids, they effectively addressed issues of nutrient deprivation and cell necrosis that are often encountered when preparing *in vitro* large-scale cell aggregates. This technique enables the rapid, large-scale production of cell-laden microspheres with precise control over organoid size and structure, supporting efficient bone regeneration.[141] Wang *et al.*[142] developed a novel method for fabricating self-mineralizing bone organoids to address large bone defects (Figure 7A). This approach utilizes a bioink inspired by natural bone matrix, constructed using 3D bioprinting technology to create complex bone matrix analogs. Composed of GelMA/alginate methacrylate (AlgMA)/hydroxyapatite, this bioink provides excellent mechanical support. With bioprinted scaffolds, large-scale bone organoids can be cultured and matured over extended periods. The self-mineralizing properties of this bioink enhance mechanical performance and allow for extensive *in vitro* and *in vivo* cultivation and multicellular differentiation (Figure 7B). These self-mineralizing constructs can replicate the structure and function of natural bone tissue, offering necessary mechanical support and mimicking the ECM, thereby overcoming the limitations of traditional bone regeneration methods.[142]

The utilization of MSC aggregates in conjunction with DLP 3D bioprinting technology offers a rational approach and strategy for effective internal nutrient supply to cells, significantly promoting bone regeneration. This technology supports rapid and large-scale production models. The biocompatible materials used, such as GelMA/AlgMA/hydroxyapatite, are derived from natural sources; exhibiting excellent biocompatibility and can reduce immune responses within the body, fostering cell growth and differentiation. By adjusting the ratios of GelMA and AlgMA, and the amount of hydroxyapatite, the material's mechanical properties and biodegradation rates can be controlled, meeting the diverse needs of various tissue engineering applications. Furthermore, incorporating additional bioactive molecules, such as growth factors, can further enhance the growth-promoting and regenerative capabilities of these composite materials.

4.6.2. Cartilage

Articular cartilage, which covers the ends of the bones at joint surfaces, is a smooth cartilage matrix that, along with calcified cartilage and subchondral bone, forms the basic structural unit of the joint. It consists of numerous chondrocytes and is enveloped by collagen fibers and GAGs. Cartilage lacks blood vessels, nerves, and lymphatic supply, limiting its self-repair capacity and rendering conventional treatment often ineffective.

Shen *et al.*[143] developed hydrogel microspheres with uniform size, porous surfaces, and excellent swelling and degradation properties using a microfluidic system combined with photopolymerization and self-assembly techniques (Figure 7C). These microspheres, composed of Arginine-Glycine-Aspartic acid (RGD), a short peptide sequence mimicking the natural cartilage microenvironment, SF, and DNA hydrogel, are referred to as RGD-SF-DNA hydrogel microspheres (RSD-MS). By simulating the cartilage microenvironment, RSD-MS promoted the proliferation, adhesion, and chondrogenic differentiation of BMSCs. The study demonstrated that RSD-MS primarily drives cartilage formation through integrin-mediated adhesion pathways and GAG biosynthesis. *In vivo* studies revealed that cartilage organoid pre-cursors formed by seeding BMSCs onto RSD-MS significantly enhanced cartilage regeneration (Figure 7D). Therefore, RSD-MS is an ideal material for constructing and long-term culturing cartilage organoids, providing an innovative strategy for cartilage regeneration and tissue engineering.[143] In addition, regenerating the complex hierarchical structure of cartilage and subchondral bone presents significant challenges. Yang *et al.*[144] developed customized gelatin-based microcryogels for cartilage and bone regeneration that self-assembled into osteochondral-like structures *in vivo*. These tailored microcryogels exhibited excellent cell compatibility and effectively induced MSCs to differentiate into cartilage and bone while facilitating self-assembly within the biphasic cartilage-bone structure. Gene expression analysis indicated that cartilage-type cryogels promoted chondrocyte differentiation and suppressed

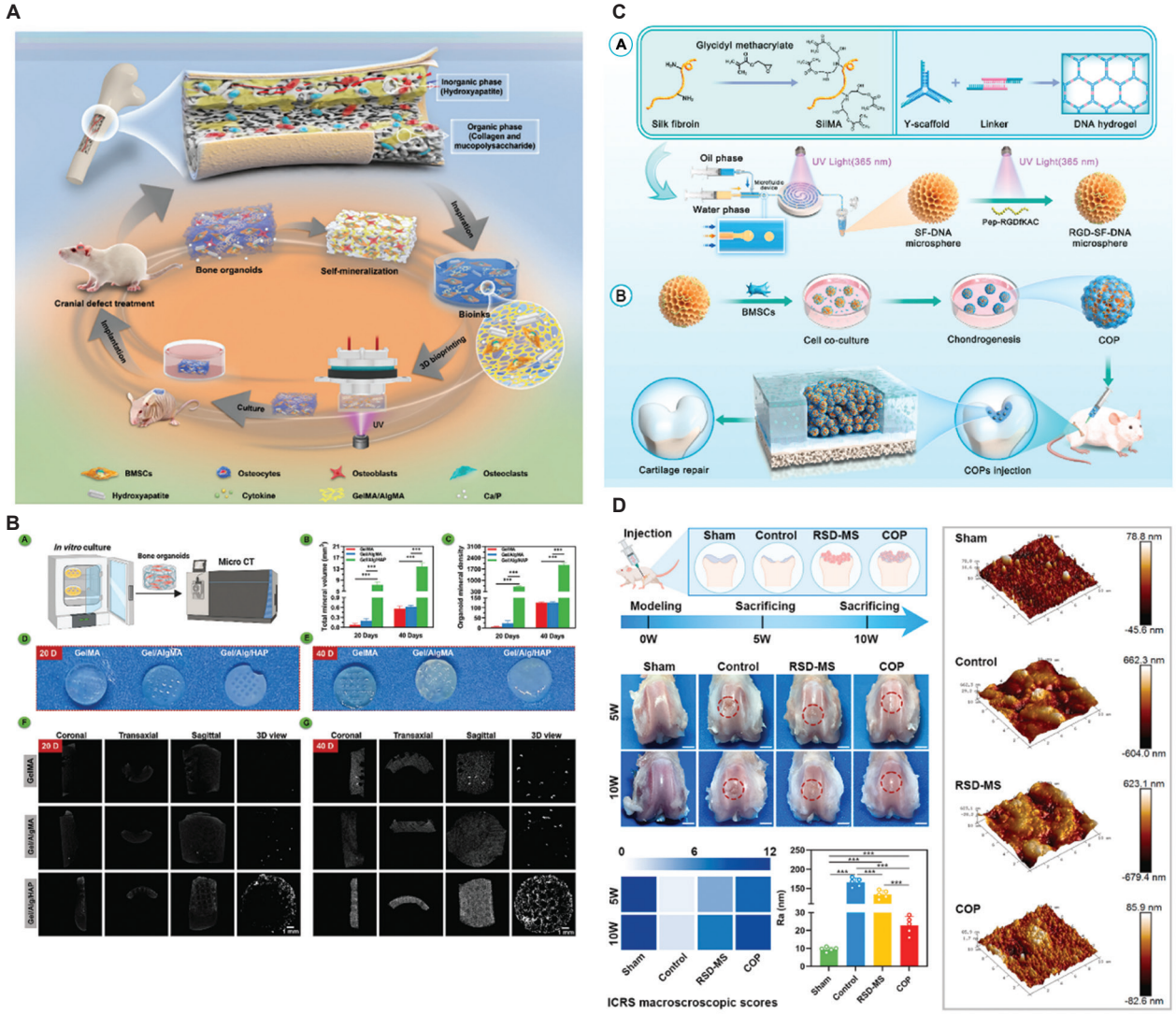

Figure 7. New methods for culturing bone and cartilage organoids. (A) Schematic diagram of research design and experimental process. (B) The self-mineralization of gelatin methacrylate (GelMA), GelMA/alginate methacrylate (GelMA/AlgMA) bioprinted scaffolds, and GelMA/AlgMA/hydroxyapatite (GelMA/AlgMA/HAP) bioprinted bone organoids *in vitro*. Image used with permission from Wang *et al.*,[142] Copyright © 2024, Wiley. (C) Schematic diagram of the RGD-SF-DNA microspheres (RSD-MS) synthesis and their application in preparing cartilage organoid precursors (COPs) and cartilage regeneration. (D) *In vivo* evaluation of cartilage repair by RSD-MS. Image used with permission from Shen *et al.*[143] Copyright © 2024, Wiley. Abbreviations: RGD: Arginine-glycine-aspartic acid; SF: Silk fibroin.

inflammatory responses, while bone-type microcryogels facilitated osteocyte differentiation and inhibited immune responses. Finally, transplantation of the pre-differentiated microcryogels into canine osteochondral defects successfully induced concurrent articular cartilage and subchondral bone regeneration. The study provides a novel approach for generating self-assembled osteochondral-like structures using customized microcryogels, advancing the field of tissue engineering.[144] The applications of hydrogels in organoid systems are presented in Table 3.

The composition of RSD-MS is well-designed, wherein the RGD peptide serves as a cell adhesion site that enhances the interaction between cells and the matrix, promoting the adhesion, proliferation, and differentiation of stem cells, thereby improving regenerative outcomes. SF, a natural polymer, possesses excellent biocompatibility and remarkable mechanical strength and elasticity, providing the necessary support to meet the mechanical performance requirements in tissue engineering applications. The degradation rate of RGD-SF-DNA hydrogels can be controlled by adjusting the amount of DNA and the crosslinking degree, allowing it to align with the tissue regeneration process and facilitating the timely degradation and replacement of the material. This hydrogel can be prepared using self-assembly and microfluidic technologies to create structures with diverse shapes and functions that

Table 3. Clinical role and application of organoid hydrogels

System	Organ	Role of organoid hydrogels	Examples of applications	References
Circulatory system	Heart	Good reproduction of the heart's natural environment morphology, structure, and function, as well as the complex microenvironment	Hydrogels as scaffolds for endothelialized myocardial tissue as platforms	88
	Vessels	Ensure complex tubular structures and precise 3D network distribution	3D printing of blood vessels and more using collagen hydrogels	94,95
Respiratory system	Lungs	Provides a more consistent and adjustable culture environment	microwell hydrogel	104
Digestive system	Gastrointestinal tract	Intestinal organoid formation and functionalized substrates	CS/GelMA composite hydrogel	106
	Liver	Controlled and stable 3D scaffolds for high-throughput and reproducible organoid generation	Specific composite hydrogel capsules	145
Urinary system	Kidney	Constructing substrates for renal organoids that encapsulate the complex structure and function of the kidney	Ionic cross-linked alginate hydrogels	112
Nervous system	Brain	For culturing and inducing the growth of cerebral organoids	Bioactive hydrogel with adjustable stiffness	124
	Spinal cord	As a substrate for spinal cord organoid cultures of well-defined composition	Alginate hydrogel	146
Skeletal system	Bone	As a long-term culture to mature large-scale bone organoid matrices	Bone matrix analogue	134
	Cartilage	As a matrix to mimic the cartilage microenvironment	Hydrogel microspheres	135

Abbreviations: 3D: Three-dimensional; CS: Calcium silicate; GelMA: Gelatin methacrylate.

mimic the microenvironment of natural cartilage or other tissues. Moreover, Yang's custom gelatine microcryogels can self-assemble *in vivo* into a bone-cartilage-like structure, effectively inducing stem cell differentiation. In addition, gelatine microcryogels can serve as drug delivery vehicles, encapsulating growth factors, cytokines, or other bioactive molecules within the microspheres, thereby promoting tissue repair and regeneration through controlled release.

5. Limitations

The advent of organoid technology has ushered in a revolutionary transformation in biomedical research, enabling the *in vitro* simulation of organ structures and functions, hence, being widely applied in drug screening, disease modelling, regenerative medicine, and other fields. Despite the immense potential demonstrated by organoid technology, it encounters numerous non-negligible limitations in specific applications across various tissues and organs.

Firstly, organoids exhibit significant shortcomings in model complexity. Although they mimic certain structures and functions of specific organs, they cannot fully replicate the intricacy of *in vivo* organs, particularly the absence of vascular networks and immune systems. The lack of complete vascular networks restricts oxygen and nutrient supply, affecting cell survival and function. In addition, the absence of an immune system limits organoids' utility in researching immune-related diseases or conducting drug screening. Secondly, organoids face limitations in maturity and functionality. While some organoids (e.g., brain and liver organoids) have made strides in morphology and function, they still fall short of the maturity levels *of in vivo* organs. For instance, brain organoids, while simulating certain brain structures and functions, still struggle with the complexity of neural networks and functional representation. Similarly, liver organoids, widely used in drug metabolism research, have notable gaps in metabolic function compared to real livers. Moreover, organoids' cellular heterogeneity is insufficient. Typically derived from a single type of stem cell, they lack the complex interactions of multiple cell types found *in vivo* organs. For example, intestinal organoids have a limited capacity to accommodate immune cells or neurons. Furthermore, organoids fail to fully replicate the biological microenvironment of the body, such as mechanical forces, electrical fields, and dynamic changes, which may result in differences in cellular behaviour and function.

Biocompatibility and transplantation issues also pose significant challenges. Transplanting organoids into host bodies may trigger immune rejection, necessitating further research to address this. In addition, organoids face difficulties in long-term survival and functional maintenance within the body. Standardization and reproducibility issues are other limiting factors in organoid technology development. Differences in media, growth

factors, and cultivation conditions among various labs lead to inconsistencies in organoid quality and functionality, affecting the reproducibility of organoid studies and limiting their application in clinical settings. Ethical issues are another major obstacle in organoid technology development. The generation of certain organoids requires the use of hESCs or other ethically contentious cell sources, potentially leading to ethical and legal controversies. Lastly, the high cost and technical complexities of organoid technology pose significant barriers to its widespread application.

In summary, despite the immense potential of organoid technology in various organ applications, it still faces numerous limitations in model complexity, maturity and functionality, cellular heterogeneity, environmental simulation, biocompatibility and transplantation, standardization and reproducibility, and ethical issues. These limitations hinder the further development of organoid technology and restrict its widespread application in clinical and research settings. Future research must continuously improve cultivation conditions, optimize organoid structures, introduce new cell types, and enhance organoid functionality to overcome these challenges and further advance organoid technology in various organ applications.

6. Conclusion and perspectives

Establishing *in vitro* disease models and cell culture systems is crucial for investigating tissue and organ diseases. While simple models yield favorable results in drug testing and clinical trials, their effectiveness is limited by the complexity of diseases, species variability, and individual genetic differences, are impeding clinical research progress. Organoid technology offers a solution by creating 3D cell models that mimic tissue architecture and replicate organ functions, making them a promising tool in contemporary biomedical research.

Organoids retain the genomic characteristics of their tissue progenitor cells and hold significant potential for regenerative medicine, particularly for transplantation.[147] However, genetic variations among patients, such as tumor heterogeneity, pose challenges to developing effective, personalized treatments. In addition, organoid cultures using engineered matrices often exhibit lower efficiency than traditional extracellular matrices, limiting their application in tumor organoid models.[148]

The complexity of human tissues results in single stem cell-derived organoids only partially replicating original organ characteristics. For example, in 3D cultures of brain organoids, neural progenitor cells differentiate and proliferate on the surface, forming suspended spherical structures. This enclosed environment can lead to inadequate internal energy supply and developmental delays.[149] Methods, such as sectioning organoid spheroids attempt to address these issues; however, organoids may still struggle to replicate complex neuronal circuits and specific cell types, limiting studies on neurodegenerative diseases, such as Alzheimer's disease.[150] Similarly, in 3D bone organoids, the lack of a replicable *in vivo* stress environment hinders the modeling of bone formation.[138] Vascularization remains a significant challenge, as continuous blood supply is essential for organoid development.

These innovations enhance organoid development by mimicking human tissues, offering new opportunities for disease modeling and drug screening. As the field evolves, it promises to translate breakthroughs into clinical applications that improve human health.

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Conflict of interest

The authors declare no conflicts of interest.

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REVIEW ARTICLE

Generation of vascularized brain organoids: Technology, applications, and prospects

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Abstract

Brain organoids have become important tools for studying neural development and disease modeling by closely mimicking in vivo brain architecture and function. However, despite their potential, conventional brain organoids lack a vascular system, limiting their physiological relevance and growth due to restricted oxygen and nutrient supply. Recent advances in generating vascularized brain organoids (V-Organoids) have sought to overcome such limitations, allowing for more accurate modeling of human brain development, neurovascular interactions, and blood–brain barrier (BBB) function. This article provides a comprehensive review of the methodologies employed to enhance the vascularization of brain organoids, including induction techniques, biomaterials, advanced bioengineering approaches, and in vivo implantation. The introduction of functional vasculature into brain organoids has not only enhanced their survival and maturation but also expanded their utility in disease modeling, drug screening, and regenerative medicine. We also discuss the applications of V-Organoids in the study of neurodevelopmental processes, BBB permeability in neurological disorders, brain cancer, and regeneration applications. Despite significant progress made in the development of V-Organoids, challenges such as vascular maturity, immune integration, longevity, and ethical considerations must be addressed to enable more accurate brain models, which could enhance our understanding of neurodevelopment and potential treatments.

Keywords: Vascularized brain organoids; Neurovascular interactions; Blood–brain barrier; Neurological disorders; Regeneration

1. Introduction

As the central organ of the nervous system, the human brain represents one of the body's most complex and mysterious organs, distinguished by its remarkable structural and functional intricacy. Its extraordinary diversity of neuronal cell types and synaptic connections underpins a wide range of functions, from cognition and emotional regulation to consciousness.[1] This intricate organization not only distinguishes humans from other species but also poses a significant challenge to unraveling the underlying mechanisms of brain function.[2] In addition, ethical limitations have restricted the capacity to conduct invasive research on the human brain, considerably hindering the advancements in understanding its complex function.[3] These restrictions have impeded progress in the development of treatments for neurological disorders, such as Alzheimer's disease (AD) and Parkinson's disease (PD).

The advancement of human pluripotent stem cells (hPSCs), including human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs), which exhibit pluripotency and the potential to differentiate into any cell type, has facilitated the creation of organoids.[4]

Under precisely optimized culture conditions, hPSCs can self-organize *in vitro*, recapitulating key processes of *in vivo* brain morphogenesis, thereby giving rise to three-dimensional (3D) brain-like structures known as brain organoids (BOs).[5] Establishing BOs *in vitro* has provided an invaluable platform for studying human central nervous system (CNS) development and the pathophysiology of neurological diseases.[6] By resembling the embryonic or fetal human brain, BOs exhibit intricate spatiotemporal complexity, mirroring dynamic structural and transcriptional changes over time.[7] Moreover, these organoids can effectively recapitulate critical aspects of human neurogenesis, including the differentiation of diverse neural cell types, the organization of neural progenitor zones, and the early formation of neural circuits.[8] These models may also offer important insights into the progressive development of neural network activities and the mechanisms underlying various neurological conditions.[9] In addition, patient-specific BOs derived from hiPSCs provide an invaluable tool for investigating neurodegenerative and neurodevelopmental disorders by closely mirroring the patient's genetic and cellular profile.[10] For example, these models have exhibited hallmark features of diseases such as AD and PD, including amyloid-beta (Aβ) plaques and dopaminergic neuronal degeneration, which have provided insights into disease progression at a mechanistic level.[11] In addition, BOs may provide important insights into neural circuit formation, cell-type specification, and brain region functionality, which could contribute to a deeper understanding of neurodevelopmental disorders such as autism and microcephaly, where disruptions in neurogenesis, synaptogenesis, and gliogenesis are evident.[12] Recent studies have also demonstrated the therapeutic potential of transplanting BOs *in vivo*. Medial ganglionic eminence-like organoids transplanted into brain injury sites have been shown to differentiate into GABAergic interneurons and promote neural repair after stroke.[13] Cortical organoids (COs) have also simulated cortical development, enhanced motor area reconstruction, and improved sensory and motor function recovery in stroke patients.[14] These findings suggest that BOs could model brain development and diseases and also hold promise for regenerative therapies for neurological injuries.

Despite the remarkable potential of BOs for neurobiological research and disease modeling, the absence of vascularization has remained a major constraint.[15] Vascularization begins around 30 days post-fertilization during human development, coinciding with neural tube closure and the deep penetration of blood vessels into the brain, ensuring the supply of oxygen and nutrients.[16] Especially in the organoid's deeper regions, the lack of a vascular system significantly restricts oxygen and nutrient delivery, leading to extensive cell apoptosis.[17] These insufficient supplies may halt organoid growth within several months.[18] Although slice cultures can provide oxygen and nutrients to inner regions, they may compromise the organoid's 3D structure, which is crucial for replicating the complexity of brain architecture.[19] Disruptions in vascularization can also lead to severe brain malformations. Without vascularization, organoids cannot fully replicate developmental processes, limiting their ability to model the structural and functional complexity of the human brain.[20] Furthermore, the interaction between the brain and vasculature is critical for forming the blood–brain barrier (BBB), which regulates the transport of substances between the bloodstream and the CNS, maintaining brain homeostasis.[21] A functional vascular system is essential for establishing the BBB. The successful vascularization of BOs cannot only enhance their physiological relevance but also enable the study of neurovascular interactions, BBB permeability for drug screening, and the development of more accurate disease models.[22]

Recent research has focused on developing strategies to incorporate functional blood vessel networks into these models to overcome the challenges of insufficient vascularization in BOs.[23-27] This review discusses the progress in generating vascularized BOs that more accurately replicate the structure and function of natural brain tissue. We examine various approaches to mimicking brain vasculature *in vitro*, including the use of dynamic bioreactor systems to promote oxygen and nutrient exchange, endothelial cells' (ECs') co-culture methods to stimulate vessel formation, organoid fusion techniques to integrate vascular and neural components, and *in vivo* transplantation to facilitate natural vascularization within a living host. Furthermore, we highlight the role of emerging technologies such as organ-on-chip systems and 3D printing, which can provide controlled microenvironments for studying neurovascular interactions and offer precision in constructing vascularized networks within organoids, respectively.[28] This review also explores the potential applications of vascularized BOs in both basic and clinical research, ranging from modeling neurodevelopmental processes and disease mechanisms to drug discovery and regenerative therapies.[15] Despite these advances, significant challenges remain, including achieving complete vascular network maturation, ensuring long-term survival and functionality, and replicating the complexity of the BBB *in vitro*. Finally, we propose future directions for the development of more physiologically accurate vascularized brain models, such as optimizing cell sources, enhancing vascular integration, and employing advanced imaging techniques. These efforts hold great promise for advancing our understanding of brain development, disease pathology, drug efficacy, and regenerative medicine (Figure 1).

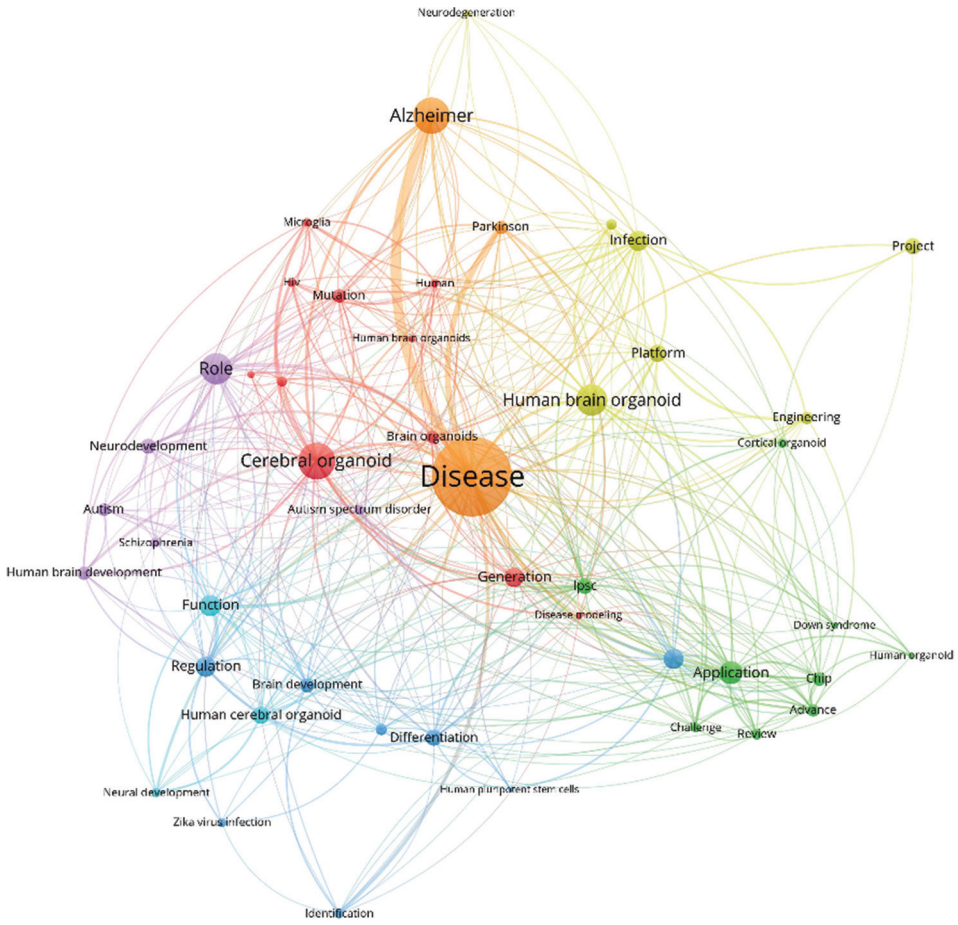

Figure 1. Mapping and clustering of terms in publications related to brain organoids. Three main clusters were identified: (i) The red cluster: Research on disease and brain organoids; (ii) the green cluster: Applications, challenges, and reviews of brain organoids; and (iii) the purple cluster: Research on neurodevelopment and brain organoids. Image created using Vosviewer 1.6.20.

2. Vascularization in the human brain

2.1. Human brain structure and function

Anatomically, the brain comprises three main parts: the cerebrum, cerebellum, and brainstem, each responsible for distinct aspects of behavior and bodily functions (Figure 2A).[29-31] The cerebrum consists of gray matter (the cerebral cortex), composed of neuron cell bodies responsible for processing information, and white matter, with myelinated axons for transmitting signals between brain regions (Figure 2B).[32]

As the outermost layer of the cerebrum, the cerebral cortex governs higher brain functions, including thought, perception, and voluntary movement. It is typically divided into four lobes, that is, frontal, parietal, occipital, and temporal, each of which is involved in distinct cognitive functions (Figure 2C). Structurally, the cerebral cortex is highly folded, known as gyri (ridges) and sulci (grooves), which allow for a greater number of neurons and synapses, thereby enhancing its complex processing capabilities.[33] The cerebral cortex is also organized into six distinct layers, each containing different types of neurons and supporting cells that contribute to its role in higher-order brain processes (Figure 2D).[34] The intricate anatomical structure of the brain serves as a key to understanding higher cognitive functions and provides critical insights into the development of BOs.[35] The brain's complex hierarchical organization and functional specialization are fundamental for advancing fields such as neuroscience, artificial intelligence, and brain–machine interfaces. These advances have opened new avenues for treating neurodegenerative diseases, cognitive impairments, and motor dysfunction by investigating signal transmission within cortical layers and exploring the relationship between cortical folding, neuron density, and neural network connectivity.[36,37]

2.2. The process of brain vascularization

Brain vascularization is a critical developmental process essential for providing the brain with the oxygen and nutrients necessary to support its high metabolic demands.

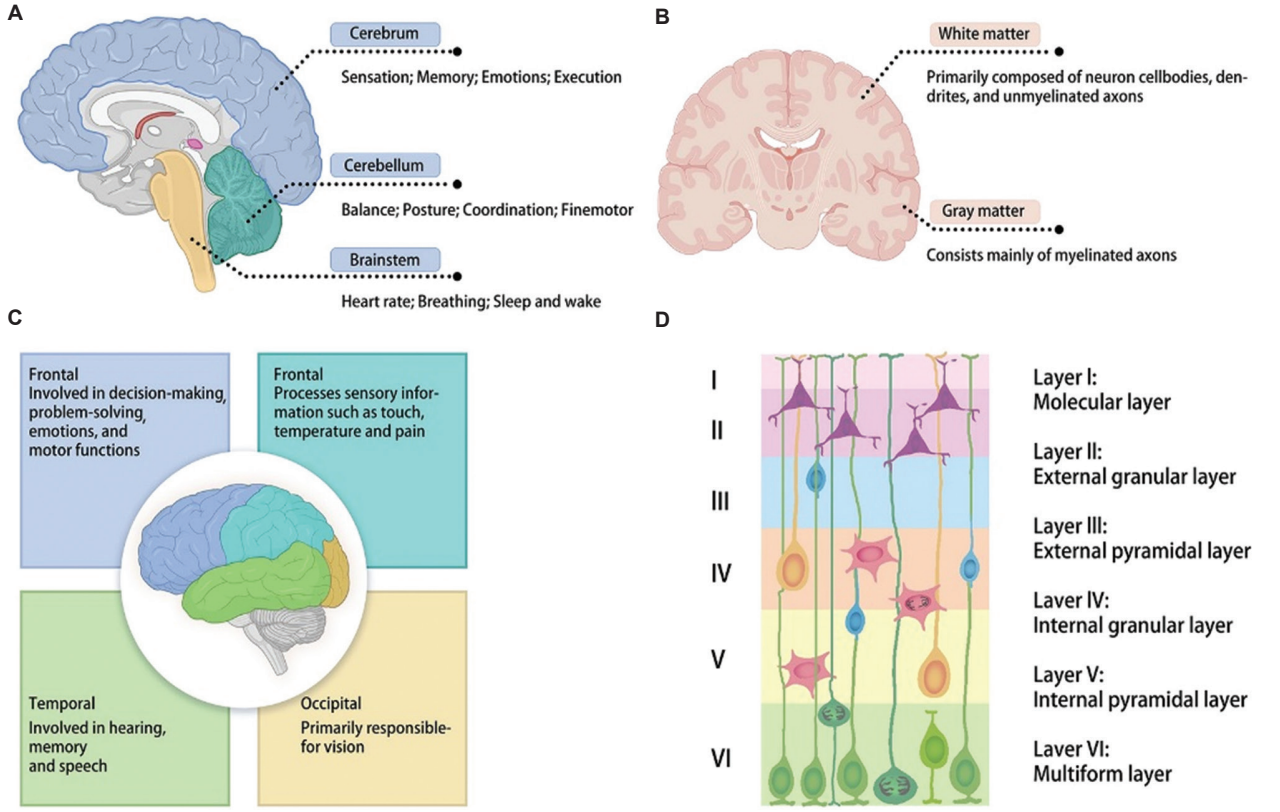

Figure 2. The structure and function of the human brain. (A) The composition of the brain. (B) The gray matter and white matter of the brain. (C) The main lobes and functions of the cerebral cortex. (D) The multilayer structure of the cerebral cortex. Image created using Photoshop.

This process begins early in embryogenesis and continues through postnatal development, ensuring the formation of a dense, intricate network of blood vessels that permeates the brain.[38,39] Blood vessel formation in the brain is initiated prenatally through angiogenesis, which originates from an external vascular network. One of the earliest contributors to this process is the perineural vascular plexus, an external network that serves as the starting point for blood vessel invasion into the brain, guided by signaling molecule gradients (Figure 3A).[40,41] This invasion results in the establishment of a dense and intricate vascular network capable of meeting the high metabolic demands of the developing brain. As the vessels penetrate deeper into the brain, the perineural vascular plexus forms the subventricular vascular plexus, which further branches out and establishes a hierarchical vascular network composed of arteries, veins, and capillaries (Figure 3B).[42] The establishment of the brain's vascular system is tightly regulated by a combination of cells, growth factors, and signaling pathways that guide the formation, patterning, and maturation of blood vessels within the brain.[43-45]

ECs play a crucial role in promoting brain vascularization by regulating both vasculogenesis and angiogenesis, the two primary processes involved in the formation of the brain's vascular network.[46] Vasculogenesis refers to the initial formation of blood vessels from endothelial progenitor cells, laying the foundation for the vascular network.[47] This process predominantly occurs during embryonic development, when endothelial progenitor cells differentiate into ECs to form the initial vascular framework. This foundational network provides the structural basis for subsequent angiogenesis and the sprouting of new vessels from existing ones. During angiogenesis, ECs migrate, proliferate, and differentiate to form new vascular branches, ensuring that the developing neural tissue receives adequate oxygen and nutrients. Tip cells lead the sprouting and migration of blood vessels, while stalk cells follow, proliferating to form the main structure and lumen of the new vessels.[48] The regulated growth of capillaries through angiogenesis is vital for nourishing neural precursors and developing neurons, supporting their rapid proliferation and differentiation. Together, these processes ensure that the brain's developing vasculature meets the metabolic demands of growing neural tissue, facilitating proper brain development and function.

Growth factors are critical regulators of brain vascularization, guiding both the formation and stabilization of blood vessels.[49] Vascular endothelial growth factor (VEGF) is one of the primary signals regulating angiogenesis in the brain. Produced by neurons, neuroglial

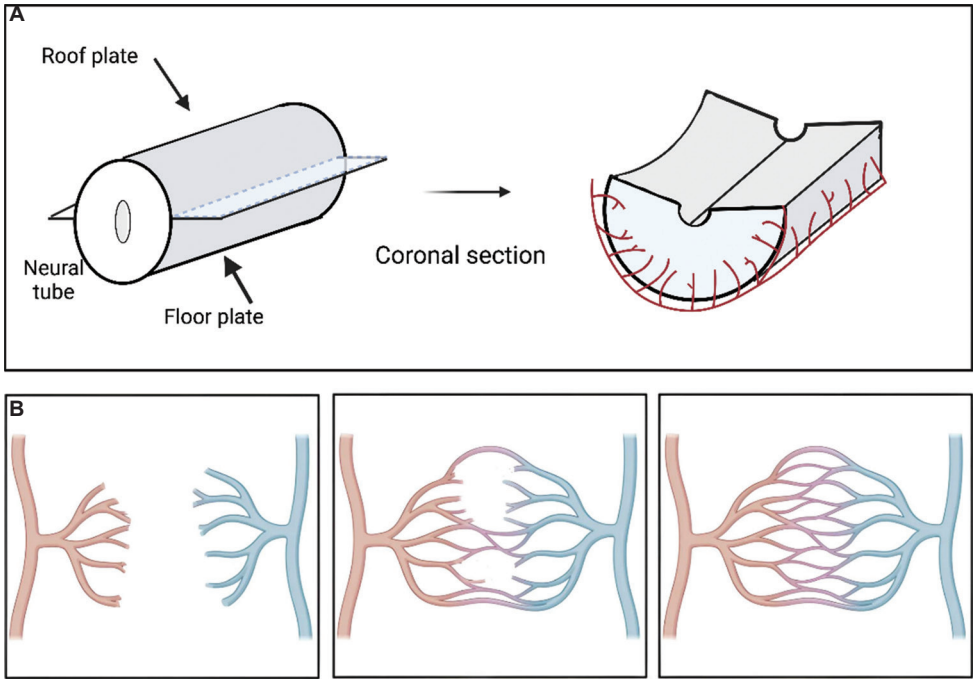

Figure 3. The process of brain neovascularization. (A) Formation of the neural tube during the embryonic stage. (B) Angiogenic sprouting into the neural tube throughout embryonic development. Image created using BioRender.com.

cells, and other neural components, VEGF binds to its receptors on ECs to promote their proliferation, migration, and the sprouting of new blood vessels.[50] Platelet-derived growth factor (PDGF) is another essential regulator of vascular development, particularly involved in recruiting pericytes, which wrap around ECs to stabilize blood vessels and maintain the integrity of BBB.[51] In addition, fibroblast growth factor (FGF) contributes by enhancing ECs' proliferation, working in tandem with VEGF to drive angiogenesis.[52] To further modulate vessel maturation and remodeling, angiopoietins (ANGs) – particularly ANG1 and ANG2 – are involved in the process. ANG1 strengthens the interactions between ECs and pericytes to stabilize vessels, while ANG2 helps to regulate vascular remodeling depending on VEGF levels.[53] The precise balance between these pro-angiogenic factors and stabilizing signals is crucial for proper vascular development.[54,55]

In addition to growth factors, various signaling pathways play essential roles in brain vascularization by regulating ECs' behavior, vessel formation, and stabilization.[56] Among the crucial pathways are Wnt, Notch, and transforming growth factor-beta (TGF-β), each contributing uniquely to brain vascular development. The Wnt/β-catenin pathway is particularly vital for ECs' proliferation, polarity, and differentiation, with a central role in forming the BBB.[57] Wnt activation promotes the expression of tight junction (TJ) proteins, which are essential for maintaining BBB integrity and regulating molecular transport between the bloodstream and neural tissue. The Notch signaling pathway functions alongside VEGF to regulate angiogenesis by controlling the differentiation of ECs.[58] It limits excessive tip cell formation, ensuring balanced branching and extension of blood vessels. TGF-β signaling plays a dual role by promoting ECs' proliferation during early vascularization and enhancing vessel stabilization through interactions with pericytes. It also contributes to BBB integrity by regulating extracellular matrix deposition and supporting vascular stability.[59] These pathways work synergistically with growth factors to ensure proper angiogenesis and vascular maturation. Disruptions in these pathways can result in developmental defects, vascular malformations, and pathologies, highlighting their crucial roles in maintaining a stable and functional vascular network during brain development. These molecular regulators collectively create a dynamic balance between vessel growth and stabilization, enabling the brain's vascular system to adapt to the expanding needs of the developing neural tissue.[60]

Brain vascularization is a complex, well-coordinated process that involves interactions among ECs, pericytes, neural progenitors, and various growth factors. It is essential for delivering oxygen and nutrients to the brain and forming critical structures, including the BBB. Proper vascularization supports neurogenesis and the development of intricate neural circuits. Defects in vascularization and angiogenesis dysfunction can lead to neurological disorders. Insufficient angiogenesis can cause hypoxia and neuronal death, while excessive angiogenesis

is often associated with brain tumors such as glioblastoma (GBM).[61] GBM, in particular, relies on extensive vascular networks to support its rapid growth. Understanding the molecular and cellular mechanisms underlying brain vascularization is crucial for gaining insights into brain development and identifying potential therapeutic targets for vascular-related conditions.[62]

2.3. BBB

The BBB is a complex, multi-component structure composed of various cellular and molecular elements that work in concert to regulate its selective permeability (Figure 4). The BBB regulates the brain's internal environment by selective permeability, allowing small, lipophilic molecules, such as oxygen and carbon dioxide, to pass freely while restricting larger or charged molecules unless they are actively transported.[63] This selectivity ensures the controlled entry of essential substances, such as glucose, amino acids, and certain ions.[21] In addition, the BBB serves as a protective barrier against neurotoxins and pathogens through its efflux transporters, effectively acting as both a physical and metabolic shield for the brain.[64]

ECs of the cerebral capillaries are the core structural component of the BBB. Unlike peripheral ECs, those in the brain are connected by TJs and comprised transmembrane proteins such as claudins, occludins, and junctional adhesion molecules that prevent paracellular transport, making the barrier highly selective. These cells also exhibit minimal transcytosis, further restricting the passage of unwanted substances. Pericytes, embedded within the basement membrane, provide additional structural support and regulate blood flow, permeability, and vascular stability. They are also crucial in controlling ECs' proliferation and differentiation, playing a key role in establishing and preserving the integrity of the BBB. Astrocytes, whose end-feet cover approximately 99% of the brain's capillary surface, secrete signaling molecules that help maintain BBB integrity and facilitate nutrient exchange between the blood and neurons. Furthermore, the basement membrane, an extracellular matrix surrounding ECs and pericytes, provides essential structural support, with components such as collagen and laminin contributing to the BBB's selective permeability and overall stability. Together, these elements form a highly regulated barrier that is crucial for brain homeostasis.

The BBB is not static; its integrity and function depend on dynamic interactions with surrounding cells. The expression and regulation of TJ proteins are crucial for maintaining BBB integrity. Pericytes help modulate the stability and permeability of the BBB by influencing the contractility of ECs and controlling the passage of macromolecules. Astrocytes release signals that strengthen ECs' barriers, while pericytes regulate the expression of molecules essential for maintaining BBB properties.[65] ECs can adjust their permeability in response to the brain's metabolic changes, highlighting the complex and adaptive nature of BBB maintenance.[66]

The BBB is fundamental to proper brain function. By restricting the entry of toxins, pathogens, and immune cells,

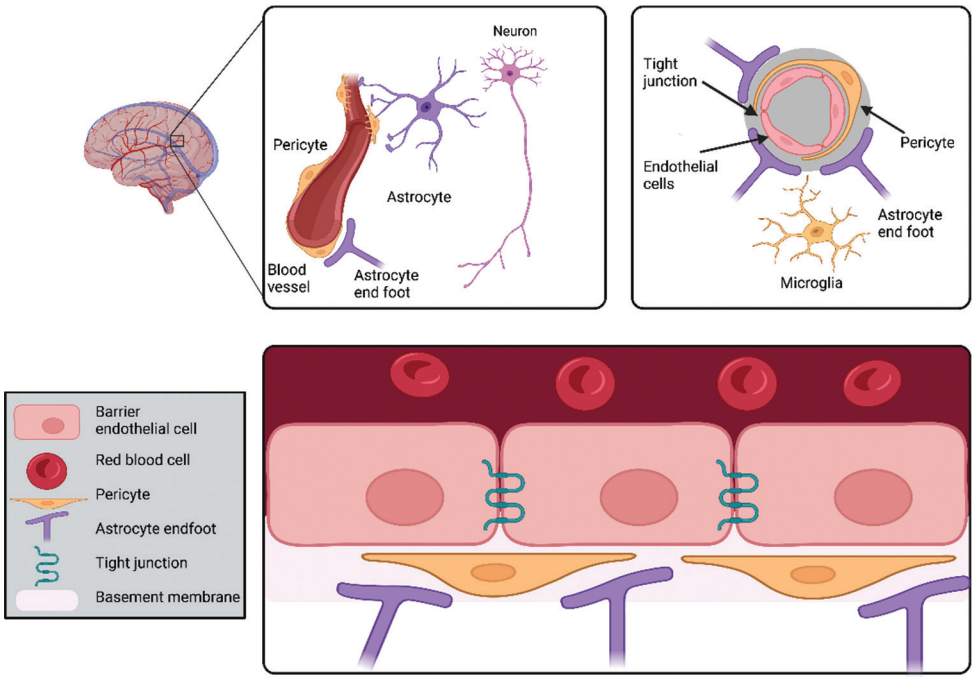

Figure 4. The structure and function of blood–brain barriers and neurovascular unit. Image created using BioRender.com.

it protects the CNS from infection and inflammation. The barrier shields the brain from harmful substances that could otherwise disrupt neuronal function or cause inflammation. Maintaining ion homeostasis is another critical role of the BBB. It carefully regulates ion concentrations, particularly potassium and sodium, in the brain's extracellular space to ensure proper neuronal excitability and function.[67] The BBB is also involved in neurovascular coupling, where astrocytes modulate cerebral blood flow (CBF) and direct blood to active brain regions according to metabolic demands.[68] Moreover, the BBB regulates the transport of essential nutrients, such as glucose and amino acids, into the brain while actively removing metabolic waste and toxins. This selective permeability is critical for supporting the high metabolic demands of neural tissue and preserving the brain's chemical environment, thereby safeguarding neural health and function.

BBB dysfunction is a key feature of many neurological disorders, such as multiple sclerosis, AD, stroke, and epilepsy.[69] Compromise of the BBB's integrity can result from physical damage, inflammation, and/or disrupted signaling pathways, leading to increased permeability and allowing harmful substances to infiltrate the brain.[70] In neurological diseases such as multiple sclerosis, BBB breakdown permits immune cells to invade the brain, causing inflammation and demyelination.[71] In AD, BBB dysfunction is associated with the accumulation of Aβ, which further contributes to neuronal damage.[72] Similarly, during a stroke, BBB disruption can lead to edema and ion imbalances, exacerbating neuronal injury.[73] When the BBB's permeability increases, larger molecules, such as proteins and immune cells, can enter the brain parenchyma, resulting in neuroinflammation, oxidative stress, and neuronal damage.[74] This dysfunction may also promote transcytosis, allowing non-specific transport of various molecules across the ECs, and increase the expression of adhesion molecules, facilitating leukocyte infiltration into the brain tissue, further exacerbating neurodegeneration. Therefore, understanding the mechanisms of BBB dysfunction is vital for developing therapeutic strategies aimed at restoring its integrity and preventing further neural damage. Advances in imaging and molecular techniques have provided valuable insights into BBB pathophysiology, guiding the development of targeted treatments and drug delivery methods for neurological diseases.[75]

2.4. The importance of neovascularization

Neurovascular interactions are critical for the development, maintenance, and function of the human brain. Such interactions occur between the brain's vascular system and neural cells, facilitating a delicate balance that is essential for various physiological processes, including neurogenesis, metabolic regulation, and the formation of complex neural circuits.[21,68] One of the key aspects of neurovascular interactions is the formation and maintenance of the BBB, a highly selective barrier that regulates the passage of substances between the bloodstream and neural tissue. The BBB's integrity relies on the neurovascular unit (NVU), which consists of ECs, astrocytes, pericytes, neurons, and extracellular matrix. This unit ensures that the brain receives adequate nutrients and oxygen while protecting it from toxins and pathogens. In addition, neurovascular interactions are essential in neurovascular coupling, a process that connects neuronal activity with blood flow. This mechanism ensures that active brain regions receive sufficient blood supply to meet their metabolic demands. Astrocytes and ECs within the NVU communicate through signaling pathways to modulate blood vessel diameter, allowing for precise control of CBF in response to neuronal activity.[70,76] Disruptions in neurovascular interactions can lead to various neurological disorders. For instance, imbalances in angiogenesis, particularly with the formation of new blood vessels, can contribute to conditions such as GBM, where excessive vascular growth supports tumor proliferation. Conversely, insufficient angiogenesis may result in hypoxia and neuronal death. Understanding the mechanisms governing neurovascular interactions is thus crucial for developing therapeutic strategies to treat vasculature-related neurological conditions.[77]

3. Methods for vascularization

Ideally, vascularization strategies for BOs should facilitate the formation of complex, branching vessels with active blood flow that dynamically respond to changes in oxygen demand and tissue growth. These strategies should also preserve the organoid's cellular structure and establish functional NVUs separated from neuronal tissue by the BBB. To achieve this, methods for vascularizing BOs can be classified into four categories: (i) vascular induction techniques that promote multi-lineage differentiation, (ii) the use of biomimetic materials, such as decellularized extracellular matrix (dECM) to support neurovascular growth, (iii) engineering approaches such as microfluidics and 3D printing to control the cellular microenvironment, and (iv) transplantation strategies that integrate organoids into host circulatory systems for nutrient and oxygen supply.

3.1. Vascular induction techniques

3.1.1. Co-culture techniques

Co-culturing with ECs or their progenitors has become a widely employed strategy to enhance vascularization in BOs (Figure 5A).[78] The integration of ECs into organoids has shown promise in creating human-specific vascular architectures in the liver,[79] intestine,[80] kidney,[81] and lung[82] organoids. Pham *et al.*[18] differentiated ECs from hiPSCs

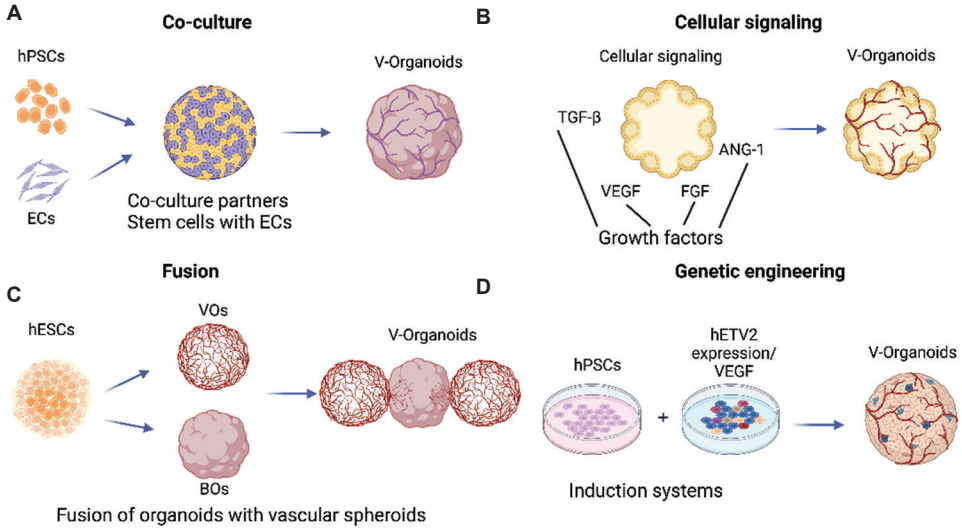

Figure 5. The generation of vascularized brain organoids using induction techniques. (A) Co-culture methods for generating pre-vascularized cerebral organoids. (B) Growth factor-induced brain vascularization. (C) Fusion for vascular and brain organoids to achieve vascularization of brain organoids. (D) Gene editing for vascularized brain organoids. Image created using BioRender.com.
Abbreviations: ANG-1: Angiopoietin 1; BOs: Brain organoids; ECs: Endothelial cells; FGF: Fibroblast growth factor; hESCs: Human embryonic stem cells; hETV2: Human ETS variant 2; hPSCs: Human pluripotent stem cells; TGF-β: Transforming growth factor-beta; VEGF: Vascular endothelial growth factor; VOs: Vascularized organoids; V-Organoids: Vascularized organoids.

and incorporated them into BOs; however, this approach often struggles to establish fully functional and mature vascular networks. Shi *et al.*[83] established a co-culture model by growing human BOs with human umbilical vein endothelial cells (HUVECs) to form tight, embryoid body-like aggregates, which subsequently underwent neural induction and differentiation. In the co-cultured organoids, the vascularization process occurred in synchrony with neurogenesis. Immunofluorescence analysis revealed that the major basement membrane glycoprotein laminin and the ECs' marker isolectin B4 co-labeled the vascular structures, indicating that HUVECs successfully formed a complex vascular network within the organoids. Compared to non-vascularized organoids, the vascularized organoids exhibited significantly increased size and a notably thicker neuroepithelium. Interestingly, HUVEC-vascularized organoids demonstrated a reduction in the hypoxia marker hypoxia-inducible factor 1-alpha (HIF-1α) and lower levels of apoptosis, indicating that vascularization plays a critical role in promoting cell survival. Moreover, HUVECs within the organoids expressed key proteins associated with vascular development, such as P-glycoprotein, suggesting that neural cells can modulate ECs' gene expression, contributing to the formation of the NVU. Single-cell RNA sequencing also revealed that vascularization accelerated neurogenesis, resulting in a higher number of spontaneously firing neurons in the vascularized organoids compared to their non-vascularized counterparts. Worsdorfer *et al.*[84] further advanced vascularization in BOs by fusing mesodermal precursor cell aggregates with the organoids. The resulting vessel-like structures displayed characteristic blood vessel ultrastructures, including basement membranes, EC–cell junctions, and microvesicles.

For the development of functionally vascularized BOs, multicellular models that incorporate pericytes, neurons, astrocytes, and neural stem cells are essential for achieving robust BBB characteristics.[85] Studies have shown that co-culturing astrocytes with brain microvascular cells significantly increases barrier tightness, while pericytes contribute by raising transendothelial electrical resistance and decreasing permeability – both of which are crucial for BBB integrity.[86,87] In addition, secreted factors from astrocytes and pericytes have been found to induce BBB-specific properties in endothelial progenitor cells, further supporting the establishment of a physiologically relevant BBB.[88] However, challenges remain in fully replicating the complex cellular interactions and signaling dynamics of *in vivo* NVUs. Advancements in bioengineering, such as microfluidic systems and tailored extracellular matrices, are emerging as promising methods to better simulate these interactions, facilitating the development of more accurate and vascularized BOs' models for studying brain function and disease.

Although co-culture methods effectively induce vascularization by integrating ECs or pericytes with BOs, their scalability remains challenging due to inconsistencies in cell interactions and structural organization. Maintaining consistent interactions between neural and vascular components becomes increasingly complex in larger cultures, potentially leading to variability in vascular network formation.

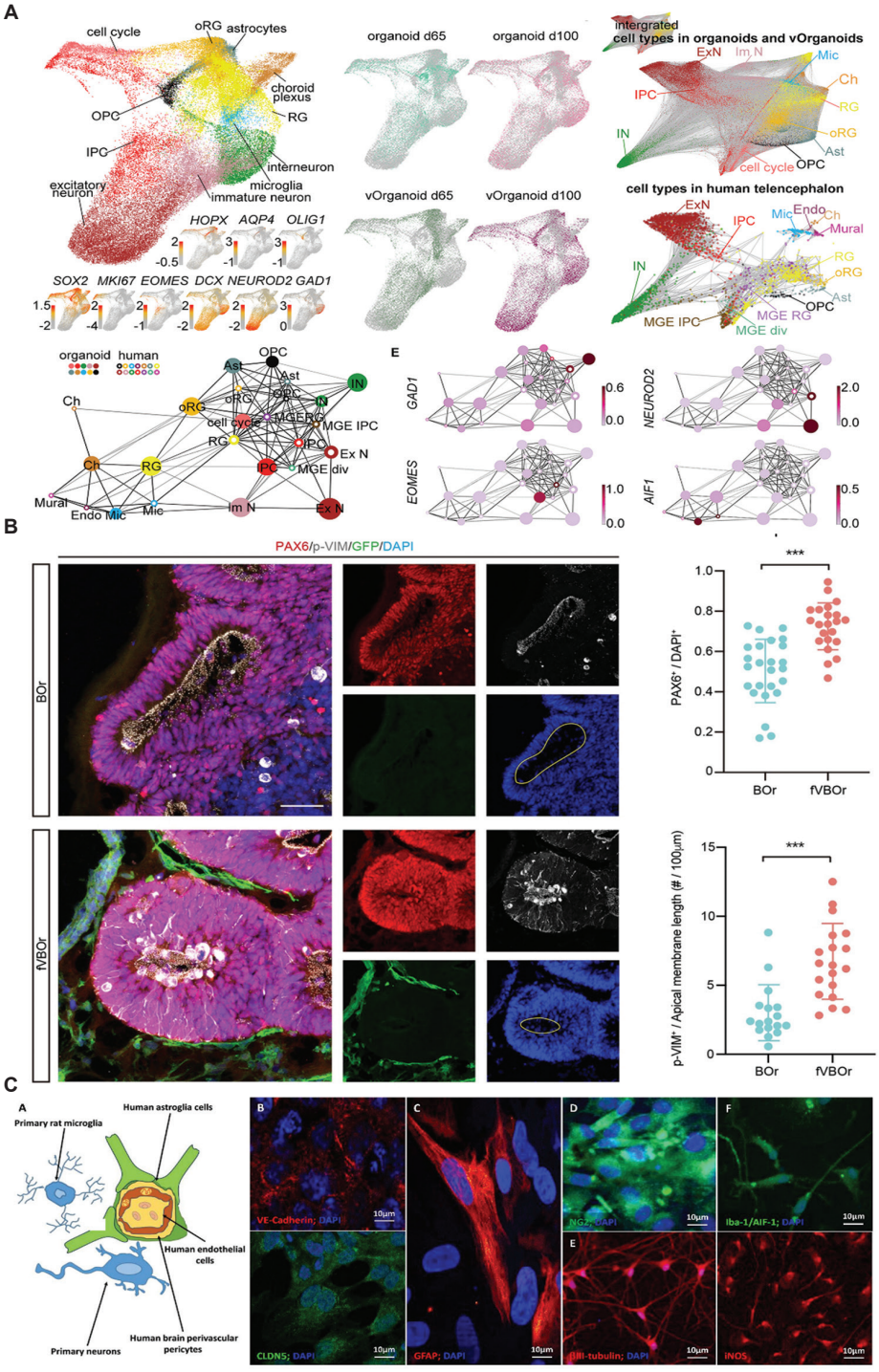

Figure 6. The application of vascularized brain organoids for the study of neovascularization. (A) Recapture of cell subtypes in neurogenesis and vascularization.[83] Copyright © 2020, Authors. (B) The relationship between neurogenesis and vascularization in brain organoid.[22] Copyright © 2022, Authors. (C) The reconstruction of neurovascular blood-brain barrier.[174,176] Copyrights © 2020 and 2021, Authors.
Abbreviations: AIF1: Allograft inflammatory factor 1; Ast: Astrocyte; *AQP4*: Aquaporin-4; BOr: Brain organoid; Ch: Choroid plexus; CLDN5: Claudin 5; DAPI: 4',6-diamidino-2-phenylindole; *DCX*: Doublecortin; Endo: Endothelial cell; *EOMES*: Eomesodermin; ExN: Excitatory neuron; fVBOr: Fused vasculature and brain organoids; *GAD1*: Glutamate decarboxylase 1; GFAP: Glial fibrillary acidic protein; GFP: Green fluorescent protein; *HOPX*: Homeodomain only protein X; Iba-1: Ionized calcium-binding adapter molecule-1; Im N: Immature neuron; IN: Interneuron; iNOS: Inducible nitric oxide synthase; IPC: Intermediate progenitor cell; MGE: Medial ganglionic eminence; MGE div: MGE dividing cell; Mic: Microglia; *MKI67*: Marker of proliferation Kiel 67; *NEUROD2*: Neuronal differentiation 2; NG2: Neuron-glial antigen-2; *OLIG1*: OPC: Oligodendrocyte progenitor cell; oRG: Outer radial glia; PAX6: Paired box protein 6; p-VIM: Phospho-vimentin; RG: Radial glia; *SOX2*: SRY box transcription factor 2; VE: Vascular endothelium; vOrganoid: Vascularized brain organoids.

Batch-to-batch inconsistencies can arise due to differences in cell sourcing, culture conditions, and the timing of co-culture integration. Standardizing protocols and employing automated systems may enhance reproducibility and scalability.

3.1.2. Growth factor-induced vascularization

As described in Section 2.2, the vascularization process in the brain is regulated by multiple growth factors that facilitate blood vessel formation and stability. The addition of specific growth factors to BOs' cultures has proven to be a crucial strategy for promoting vascularization (Figure 5B). Table 1 summarizes the primary growth factors strategies that have been explored to enhance vascularization in BOs.

Ham *et al.*[89] have made significant strides in introducing vascular differentiation cues within BOs by cultivating embryoid bodies in a VEGF-enriched medium. Their approach demonstrates that the addition of VEGF during the neuroectodermal differentiation stage could induce the formation of blood vessel-like structures with essential BBB characteristics. VEGF enhances vascular network complexity and stability in BOs while preserving neuronal integrity. It drives sprouting angiogenesis by activating the VEGF–VEGF receptor (VEGFR) signaling pathway, which initiates the Delta-like ligand 4–Notch cascade. This cascade regulates ECs' differentiation, ensuring the proper roles of tip and stalk cells, which are essential for vessel branching and stability in developing vascular networks.[42,90] Beyond VEGF, BOs' vascularization can be improved with key growth factors at different stages of development. FGF supports early vascular and neural growth, while the epidermal growth factor helps connect vascular and neural cells. As vessels mature, PDGF and TGF-β stabilize them by recruiting smooth muscle cells and forming supportive structures. ANG1 strengthens vessel walls, insulin-like growth factor promotes cell survival, and bone morphogenetic protein organizes vascular structures. These factors enhance vascular stability and integration, but further research is needed to confirm their functionality.[91,92]

While pro-angiogenic growth factors facilitate controlled vascularization in BOs and can be scaled by modulating their concentrations and exposure durations, their application presents challenges, including inconsistent

Table 1. Growth factors promoting vascularization in brain organoids

Growth factor	Function	Effect on vascularization	Signaling pathway (s)	References
VEGF	Stimulates endothelial cell proliferation and migration and promotes sprouting angiogenesis and vessel branching	Increases blood vessel formation within organoids	VEGF/VEGFR–DLL4–Jagged–Notch (for sprouting angiogenesis, key in endothelial tip cell guidance and vascular branching)	16,17,89,209
FGF	Promotes proliferation and differentiation of stem cells and endothelial cells	Supports angiogenesis and stabilization of vascular networks	FGF/FGFR, PI3K/AKT, and MAPK (essential for angioblast formation and vasculature differentiation)	22,42,210
EGF	Enhances proliferation of neural progenitors and endothelial cells	Promotes both neural and vascular growth, indirectly supporting vascularized organoid development	EGFR/MAPK and Notch (involved in cell survival and neurovascular growth in early stages)	158,211
PDGF	Aids in the maturation of vascular smooth muscle cells	Supports blood vessel maturation and stability	PDGF/PDGFR, YAP–TAZ, and MAPK (regulates pericyte recruitment and vessel maturation)	84,211-213
TGF-β	Involved in cell differentiation, immune response, and ECM production	Stabilizes vascular structures and promotes ECM deposition	TGF-β/SMAD (integrin αVβ8–TGF-β pathway important for vascular stability and ECM formation)	42,214,215
Angiopoietin	Stabilizes blood vessels by supporting pericyte–endothelial cell interaction	Enhances blood vessel stabilization and maturity	ANG–TIE2, MAPK, and PI3K (important for vessel stabilization and survival of endothelial cells)	83,216
IGF	Promotes cell survival, proliferation, and differentiation	Supports vascular development and neural survival within organoids	IGF/IGF1R (PI3K-AKT and MAPK pathways promoting cell growth and survival)	217,218
BMP	Regulates stem cell differentiation and vascular development	Promotes vascular differentiation and organization in brain organoids	BMP/SMAD and WNT (interaction with TGF-β for vascular differentiation)	219

Abbreviations: AKT: Protein kinase B; ANG: Angiopoietin; BMP: Bone morphogenetic protein; DLL4: Delta-like 4 protein; ECM: Extracellular matrix; EGF: Epidermal growth factor; EGFR: EGF receptor; FGF: Fibroblast growth factor; FGFR: FGF receptor; IGF: Insulin-like growth factor; IGF1R: IGF 1 receptor; MAPK: Mitogen-activated protein kinase; PDGF: Platelet-derived growth factors; PDGFR: PDGF receptor; PI3K: Phosphoinositide 3-kinase; TAZ: Transcriptional coactivator with PDZ-binding motif; TGF-β: Transforming growth factor beta; VEGF: Vascular endothelial growth factor; VEGFR: VEGF receptor; YAP: Yes-associated protein.

distribution in larger cultures, the risk of aberrant vessel formation, and the need for precise temporal and dosage regulation due to their transient bioactivity.

3.1.3. Organoid fusion for vascularization

Organoid fusion is a promising strategy to enhance vascularization in BOs by combining distinct cell populations to better mimic the NVU (Figure 5C). Song *et al.*[93] demonstrated this approach by fusing neural progenitor spheroids, EC spheroids, and induced pluripotent stem cell (iPSC)-derived mesenchymal stem cells to form hybrid NVU assembloids. These assembloids expressed key BBB markers, such as glucose transporter 1, TJ protein zona occluden 1 (ZO-1), and matrix metalloproteinases, while promoting VEGF-A secretion and cortical tissue development. Ahn *et al.*[94] utilized hiPSCs to generate blood vessel organoids and investigated their interaction with COs. The results demonstrate that blood vessel organoids could infiltrate COs and form functional vessel-like structures. These structures were composed not only of ECs (cluster of differentiation 31-positive [$CD31^+$]) but were also surrounded by smooth muscle cells (smooth muscle actin-positive) and pericytes (PDGFR-positive), exhibiting molecular marker characteristics of the BBB. Similarly, Sun *et al.*[22] fused brain and vascular organoids to form neurovascular assembloids, enabling the study of neurovascular interactions (Figure 5B). The fused organoids developed functional vascular networks that coexisted with neurons and promoted the generation of neural progenitor cells. In addition, the fused organoids exhibited structures resembling the BBB, and microglial cells, on immune stimulation, displayed synapse phagocytosis, indicating their role in immune regulation. These studies highlight the feasibility of vascularizing BOs through vascular organoids, offering a new model for investigating neurovascular diseases. However, challenges such as the absence of a perfusion system – leading to necrotic cores in the organoids – still persist.

Organoid fusion has shown the potential to generate more physiologically relevant tissue architectures. However, its scalability remains constrained by the efficiency of the fusion process and the challenges associated with sustaining viability and functionality in larger fused organoids. Reproducibility is further impacted by variations in organoid size, cellular composition, and the precision of spatial alignment during fusion. Advances in bioengineering technologies, including microfabrication and bioprinting, offer promising strategies to improve the scalability, standardization, and structural consistency of this approach.

3.1.4. Gene editing for vascularized BOs

Gene editing offers a promising strategy for inducing vascularization in BOs, particularly through transcription factor-mediated differentiation of hESCs into vascular cells. A key example is the overexpression of ETS variant 2 (ETV2), which promotes the formation of complex vascular-like networks within COs (Figure 5D). ETV2-expressing cells exhibit essential BBB characteristics, such as nutrient transport and TJ formation, facilitating organoid maturation. Cakir *et al.*[17] demonstrated this approach by genetically modifying hESCs to express ETV2 under a doxycycline-inducible promoter. By overexpressing the ETV2 transcription factor in hESCs, ECs were induced, and a 20% ETV2 expression cell ratio was optimized to successfully generate V-Organoids. In V-Organoids, ECs formed stable vascular networks, which persisted for 30, 70, and 120 days of culture, with significantly greater vessel area and network complexity compared to control organoids. In addition, V-Organoids exhibited BBB-like characteristics, confirmed by TJ markers such as α-ZO-1, occludin, and kinase insert domain receptor. After 120 days of culture, V-Organoids showed reduced apoptosis and significantly lower HIF-1α levels, indicating that the vascular network effectively supported oxygen and nutrient transport. Electrophysiological patch-clamp experiments revealed that neurons in V-Organoids could generate multiple action potentials at 80 – 90 days, unlike those in control organoids. Single-cell RNA sequencing further demonstrated that V-Organoids exhibited gene expression patterns resembling those of human fetal brain development at 16 – 19 weeks, while control organoids were closer to the 10 – 12-week stage. This method provides a powerful tool for refining BOs' models by generating accurate BBB components and promoting functional vascularization, all while allowing precise control over cell differentiation.

Gene editing techniques offer high precision and the potential for consistent induction of vascular features across organoid batches. Scalability is feasible, as gene-edited stem cells can be expanded and differentiated into organoids with vascular potential. However, challenges include ensuring the efficiency and specificity of gene edits, as well as addressing ethical considerations. Standardizing gene editing protocols and conducting thorough validation are essential for reproducibility and scalability.

3.2. Biomaterial for vascularized BO

Biomaterials can facilitate cell growth within organoids and regulate the microenvironment, thereby influencing the development of neural networks and vascular structures within the organoids. As a result, biomaterials offer a promising approach for generating vascularized BOs. Recent advancements in biomaterials, including the use of natural and synthetic hydrogels, have contributed to improved maturation and vascularization of BOs. This section focuses on key developments in the biomaterials field that may enhance the formation of BOs, particularly their capacity to support both neural and vascular

characteristics (Table 2).

3.2.1. Matrigel

Matrigel, a widely used biomaterial in cell culture systems, plays a pivotal role in BOs' culture. Rich in growth factors and ECM proteins, Matrigel provides essential biochemical cues for cell differentiation, polarization, and the formation of organoid-like structures.[95] Lancaster *et al.*[4] first demonstrated that Matrigel enables hiPSCs to self-organize into 3D BOs, mimicking the cellular complexity of the developing human brain. In addition to providing structural support, Matrigel mimics the properties of the native brain ECM, facilitating the maturation of neural networks. Kim *et al.*[96] showed that BOs cultured in 3D Matrigel promoted the aggregation of Aβ peptides. Similarly, Park *et al.*[97] incorporated astrocytes and microglia into a Matrigel culture system, successfully modeling AD-related neuroinflammation. Matrigel is a popular choice for creating vascularized organoids due to its ability to imitate the extracellular matrix, thus creating a supportive environment for cell growth and differentiation.[98,99] It promotes the formation of vascular networks by supporting ECs' growth and organization, allowing these cells to self-organize into blood vessel-like structures. However, Matrigel alone has been found to be inadequate for supporting vascular lumens. Therefore, researchers combined Matrigel with ECs, microfluidics platforms, and *in vivo* transplantation to construct vascularized organoids more effectively.[100]

Table 2. Biomaterials supporting brain organoid development and vascularization

Biomaterials	Stem Cells	Methods	Key founding	Advantages	Limitations	References
Matrigel	iPSCs and hiPSCs-derived ECs	Co-culture and *in vivo*	Vascularization of brain organoids with UC Davis patient's own ECs	Rich in laminin, collagen IV, and growth factors, closely resembling the native basement membrane; Supports neural and endothelial attachment, migration, and lineage commitment; Promotes endothelial network formation and host vasculature integration *in vivo*	Extracted from EHS mouse sarcoma; Matrigel exhibits compositional inconsistencies; Matrigel variable bioactive components limit reproducibility and standardization; As a murine-derived ECM, it may not fully replicate human physiological conditions	18
	hESCs/hiPSCs and HUVECs	Co-culture and *in vivo*	V-Organoids are integrated with the mouse vasculature, creating functional human-mouse blood vessels			83
	hiPSCs and MPC	Co-culture and *in vivo*	Directed incorporation of MPCs into neural organoids leads to the formation of organized blood vessels with a hierarchical structure, mimicking natural vasculature			84
dECM	hiPSCs	3D culture with microfluidic system	BEM enhances neurogenesis and cortical structure formation and reduces cell death; Ventricle-like structures and neural development are optimized in a dynamic culture	Provides a bioactive and physiologically relevant microenvironment; More biomimetic than Matrigel; Promotes a long-term culture	Preparation complexity and batch-to-batch variability limit reproducibility	144
	hiPSCs	Embedded in B-ECM-based hydrogel	Supports neurogenesis and structural formation, similar to Matrigel but with a more native-like cellular environment			117
Collagen	hiPSCs	Embedded in collagen type I with growth factors	The application of neuronal plasticity and modeling of disease	Well-characterized structure; Widely used for scaffold-based neuroengineering; Promotes endothelial cell migration and vascular integration	Lacks the biochemical complexity required for full ECM mimicry	132
	hiPSCs	Embedded in collagen type I with VEGF and FGF-2	Microenvironmental role in brain tumor progression			133

Abbreviations: BEM: Brain extracellular matrix; B-ECM: Brain-derived extracellular matrix; dECM: decellularized extracellular matrix; ECM: Extracellular matrix; ECs: Endothelial cells; EHS: Engelbreth–Holm–Swarm; FGF: Fibroblast growth factor; hESCs: Human embryonic stem cells; hiPSCs: Human induced pluripotent stem cells; HUVECs: Human embryonic stem cells; iPSCs: Induced pluripotent stem cells; MPC: Mesodermal precursor cell; UC: University of California; VEGF: Induced pluripotent stem cells; V-Organoids: Vascularized organoids; 3D: Three-dimensional.

3.2.2. dECM

The ECM supports brain development by providing structural support and regulating biochemical and mechanical signals essential for angiogenesis and neurodevelopment.[101] The dECM scaffold preserves key ECM components, maintaining the biochemical and structural properties of the original tissue.[102,103] This preservation enables dECM to closely replicate the native microenvironment, making it an effective scaffold for promoting BOs' maturation and vascularization.[104] Brain-specific dECM has demonstrated particular benefits for BOs' cultures. Its tissue-specific biochemical cues significantly accelerate neuronal network formation and enhance cell differentiation.[105] Fetal brain-derived dECM, enriched with higher glycosaminoglycan content and tissue-specific signaling molecules, more effectively promotes dense axonal network formation and enhances neuronal activity compared to adult brain-derived dECM. This is likely due to its closer resemblance to the *in vivo* environment of the developing brain.[106] The adaptability of dECM has further been highlighted in various disease modeling.[107] In GBM research, patient-derived GBM cells cultured in a brain dECM hydrogel successfully recapitulated the tumor microenvironment, aiding in the investigation of tumor behavior, progression, and therapeutic resistance.[108] Furthermore, dECM from different brain regions introduces unique biochemical properties that influence tumor invasion dynamics. For example, Koh *et al.*[109] observed that GBM cells adapt their migratory strategies based on local ECM characteristics, illustrating how the biochemical composition of the ECM impacts tumor progression. The complexity and specificity of dECM, derived from various brain regions, offer a physiologically relevant microenvironment for BOs' cultures.

Extracellular matrix-cell interactions also direct vasculogenesis. Acting as a scaffold that retains key biochemical cues, dECM plays a pivotal role in stimulating ECs and facilitating blood vessel formation in both *in vitro* and *in vivo* environments. Small intestinal submucosa dECM hydrogel has demonstrated its ability to accelerate tissue repair and enhance angiogenesis, making it a promising material for vascularization applications.[110,111] While the addition of peptides from native tissue ECM has enhanced vascular network formation and stability in other organoid types, this approach has not yet been tested in Cos.[112] Natural dECM-based hydrogels have been utilized to support kidney organoid development and promote angiogenesis *in vitro*, with assays such as the chick chorioallantoic membrane assay confirming their angiogenic potential.[113] Using stomach-derived dECM to construct patient-derived tumor vascularized organoids provides a favorable microenvironment for angiogenesis. The tissue-specific and collagen-rich composition of dECM effectively supports ECs' sprouting, promoting vascularization within the organoids. This vascularization not only enhances the physiological function of the organoids but also improves their predictive capacity in personalized cancer treatment, allowing the model to more accurately reflect patients' responses to anticancer therapies.[114] Despite the validation of dECM in promoting vascularization in other organoid types, brain ECM has yet to be tested in COs for similar effects. Given the sensitivity of ECs to ECM signals, brain ECM may encourage the specification of ECs toward brain microvascular ECs or enhance their compatibility for vascularizing COs. However, further research is necessary to comprehensively understand the extent and mechanisms by which exogenous matrix influences cell behavior within COs.[115,116]

3.2.3. Other natural hydrogels

Beyond native ECM, individual components derived from it can be isolated from animal sources or synthesized as recombinant proteins. Natural hydrogels, crafted from these specific ECM components, have emerged as invaluable tools in BOs' culture systems, providing a controlled microenvironment that closely replicates native ECM properties.[117] Compared to complex and compositionally variable matrices such as Matrigel or dECM, natural hydrogels allow for precise modulation of their mechanical and biochemical characteristics, facilitating enhanced consistency and reproducibility in experimental setups.

Collagen type I is one of the most commonly used natural hydrogels in neural culture systems. While collagen is not abundant in the native brain ECM, it is biocompatible, provides cell-binding sites, and supports neuronal differentiation, axonal growth, and network formation.[118] Collagen hydrogel systems have been applied to study microenvironmental influences in both pediatric and adult GBM.[119] This approach represents an early advancement in creating bioengineered BOs under customizable conditions for disease modeling.[120] Collagen hydrogel systems have also been used to explore the microenvironmental role in pediatric and adult GBM.[121] In AD models, collagen hydrogels have been used to recreate key pathological features such as Aβ aggregation and BBB dysfunction, highlighting their potential for disease modeling.[122]

Hyaluronic acid (HA), a major component of the brain ECM, is another widely studied hydrogel in BOs' research. Since HA lacks inherent cell-binding sites, it is often functionalized or combined with other ECM proteins, such as collagen, fibronectin, or laminin, to promote cell adhesion and network formation. HA hydrogels have been shown to support neuronal development and the formation of functional neural networks.[123] In addition, HA-based hydrogels have been used to model neurological disorders, such as investigating cell migration and synaptic defects in

Rett syndrome.[124] HA in spongy-like hydrogels promotes neovascularization by releasing HA fragments through controlled enzymatic degradation. These fragments interact with specific ECs' receptors, such as a cluster of differentiation 44 and a receptor for hyaluronan-mediated motility, supporting ECs' proliferation and the formation of new neurovascular networks, particularly beneficial in ischemic or damaged neural tissues.[125]

Laminin, an essential ECM protein, is often used to enhance the bioactivity of hydrogel systems. Although laminin alone cannot form a hydrogel, it is frequently combined with other hydrogels such as HA or collagen to support neural stem cell maintenance and guide cell differentiation, particularly toward the neuronal lineage.[126] This ability to influence cellular behavior without altering the hydrogel's mechanical properties makes laminin a valuable additive in BOs' cultures.[127,128] When combined with collagen in hydrogels, laminin significantly enhances endothelial function, particularly through the upregulation of TJ protein ZO-1, which is crucial for vascular barrier integrity. This configuration fosters an environment conducive to neovascularization by stabilizing ECs' connections and improving overall vascular barrier function. One study has shown that laminin enhances vascular network formation within 3D collagen scaffolds by modulating VEGF uptake.[128] Laminin increases the expression of VEGFR2 on ECs, leading to more efficient VEGF absorption and promoting the formation of interconnected ECs' networks. This effect facilitates the development of more robust neurovascular structures, suggesting that laminin plays a critical role in vascularization by improving the bioactivity of ECs within scaffold environments.

Overall, natural hydrogels offer a more controlled and tunable platform for BOs' development. They provide the ability to precisely manipulate the cellular microenvironment, facilitating studies of neural differentiation, disease modeling, and the bioengineering of BOs. Their versatility and adaptability make natural hydrogels an invaluable tool in advancing our understanding of neural development and disorders.

3.2.4. Synthesized materials

Synthetic hydrogels provide a highly controlled environment for BOs' cultures, addressing issues such as variability and limited flexibility often encountered with natural materials. These hydrogels can be engineered to mimic the brain's ECM while allowing precise control over important factors such as stiffness and biochemical properties, which are crucial for cell growth and tissue formation.[95]

Among synthetic hydrogels, polyethylene glycol (PEG) and its derivatives are among the most widely used due to their biocompatibility, hydrophilicity, and tunable mechanical properties.[129] PEG can be modified to include cell adhesion sites and can be broken down by cells, allowing them to remodel their surroundings. This makes PEG an effective tool for studying brain development and diseases like AD, where Aβ accumulation affects neural stem cells.[130] Recently, Schwartz *et al.*[131] combined neural progenitors, ECs, mesenchymal stem cells, and microglia precursors on chemically defined PEG hydrogels to create 3D neural constructs with integrated microglia and vascular networks.

Beyond PEG, other synthetic polymers, including poly(lactic-co-glycolic acid) (PLGA), polylactic acid, poly(vinyl alcohol), poly(ε-caprolactone), polyacrylamide, and polydimethylsiloxane, have been explored in BOs' engineering. These materials offer distinct mechanical and degradation properties and can be functionalized with extracellular matrix proteins (e.g., laminin), bioactive peptides (e.g., arginylglycylaspartic acid), and soluble factors (e.g., VEGF, FGF2, and bone morphogenetic proteins) to enhance vascularization and neurogenesis.[132] For instance, PLGA fiber scaffolds have been successfully incorporated into COs' models to support progenitor cell expansion and cortical layer development when combined with microfluidic platforms.[133]

In addition, self-assembling peptides (SAPs) offer another synthetic hydrogel option, specifically the HYDROSAP scaffold, which is used to create a standardized 3D culture system for human neural stem cells (hNSCs). Unlike conventional animal-based matrices, HYDROSAP provides a brain-like environment that consistently supports the differentiation of hNSCs into mature neurons, astrocytes, and oligodendrocytes. Across various hNSC lines, the SAP-based scaffold showed reliable results, enabling the formation of complex, mature neural networks that are not achievable in two-dimensional cultures. This consistency and ability to mimic natural brain tissue suggest that SAP-based scaffolds could become essential tools in neural tissue engineering, disease modeling, and the development of therapies for CNS disorders.[134] By adding ECs and growth factors, these hydrogels can enhance the development of vascular networks *in vitro*, improving neurovascular modeling.[95] In summary, synthetic hydrogels offer a flexible and consistent platform for BOs' research, advancing the study of neurodevelopment, disease modeling, and vascularization in the brain.

3.3. Engineering strategies for vascularized BOs

3.3.1. Microfluidic chips for vascularized BOs

Microfluidic technology has emerged as a useful method for promoting BOs' vascularization by enabling the precise reconstruction of complex vascular networks.[135,136] Unlike

conventional static cultures, microfluidic platforms provide controlled fluid dynamics, ensuring consistent nutrient flow and enhancing organoid development.

Conventional static cultures often suffer from uneven oxygen and nutrient distribution, particularly in thicker tissues. In contrast, microfluidic platforms use miniaturized channels that replicate the structure and function of blood vessels, allowing continuous perfusion, precise nutrient delivery, and effective waste removal – essential for the maturation and long-term viability of BOs (Figure 7A).[137,138] Gong *et al.*[139] developed human retinal organoids using a controllable perfusion microfluidic chip, which enhanced retinal organoid growth by optimizing oxygen and nutrient distribution through improved perfusion. Abdulla *et al.*[140] developed a 3D microfluidic platform with dynamic fluidic perturbation and oxygen supply, demonstrating that the controlled fluidic environment mitigated hypoxia and ensured uniform nutrient distribution, thereby enhancing COs' viability and uniformity. Seiler *et al.*[141] also reported that automated microfluidic platforms minimize glycolytic and endoplasmic reticulum stress COs, supporting neurogenesis and promoting organoid maturation.

Microfluidic platforms also facilitate the co-culture of BOs with other cell types, such as ECs and stromal cells, to promote vascularization by providing precise spatial and temporal control over cell interactions and growth factor delivery. Through multi-channel designs, these platforms enable distinct yet interconnected environments, allowing BOs to receive localized stimulation from neighboring cells and growth factors. This co-culture system mimics natural tissue organization, promoting the development of functional vasculature within organoids. Osaki *et al.*[138] successfully co-cultured human embryonic stem-derived spheroids and ECs in microfluidic devices, leading to the migration of ECs into the organoids and the formation of an organized vascular network. The microfluidic platforms support the formation of 3D vascular networks that interweave with neuronal structures, facilitating direct cell-cell interactions. These interactions occur through mechanisms such as paracrine signaling (e.g., growth factors such as brain-derived neurotrophic factor) and juxtracrine signaling pathways (i.e., the Delta–Notch pathway). Salmon *et al.*[142] described how microfluidic platforms enhance the vascularization of organoids by facilitating spatially and temporally synchronized interactions between cerebral and vascular cells. Using a custom-designed 3D-printed microfluidic chip, the study enabled the co-culture of organoids with pericytes and ECs, promoting the formation of organized vascular networks. These networks self-assembled around COs, creating integrated neurovascular structures. Similarly, Osaki *et al.*[143] found that continuous perfusion in microfluidic devices enhances both neuronal differentiation and vascular network formation, reducing metabolic stress and improving cell health. Microfluidic devices also offer advanced platforms for studying neurovascular interactions, particularly the BBB. These systems provide continuous perfusion and allow the integration of ECs, pericytes, and astrocytes to replicate key features of the BBB. Maoz *et al.*[144] developed an NVU-on-a-chip to study the metabolic coupling between neurons and vessels, providing valuable insights into BBB dysfunction in neurological diseases. Grebenyuk *et al.*[145] used a two-photon-mediated 3D microfluidic device to create neural spheroids, showing that perfusion reduces necrosis and enhances BBB function compared to static cultures.

Moreover, microfluidic platforms allow precise control over fluid dynamics, molecular gradients, and shear forces, which are vital for studying developmental processes such as neural tube formation and understanding the mechanisms of neurodegenerative disease.[146] These platforms enable the replication of neural processes and facilitate the study of neurodegenerative disease mechanisms. For example, the *in vitro* compartmentalized microfluidic device described by Miny *et al.*[147] offers valuable insights into neurodegenerative diseases by recreating minimalistic neural circuits and allowing detailed studies of the molecular aspects of neurodegeneration. This setup underscores microfluidics' role in improving our understanding of neurodegenerative pathophysiology by mimicking neural circuits and enabling dynamic molecular studies. Further advancements in brain-on-a-chip technology, as highlighted by Amirifar *et al.*,[148] support studies on neural tissue responses to environmental stressors, fluid dynamics, and molecular gradients, which are central to disease progression and therapy development. In neurovascular applications, BOs and brain-on-chip technologies model the complex microenvironments essential for disease studies and therapeutic screening, particularly in conditions such as AD and PD.

Unlike traditional culture plates, microfluidic systems require specialized fabrication techniques, such as soft lithography or 3D printing, as well as additional flow control equipment, which increase initial costs.[149] Nevertheless, advancements in mass production, injection molding, and 3D printing are reducing manufacturing costs, enabling the production of disposable and reusable microfluidic devices.[150] The integration of these chips into commercially available organoid culture platforms further simplifies their adoption, bridging the gap between traditional static cultures and advanced dynamic systems. However, accessibility remains a significant challenge, as microfluidic platforms require expertise in fluid dynamics and bioengineering, limiting their adoption in conventional biological research settings. In addition, integrating them into existing workflows

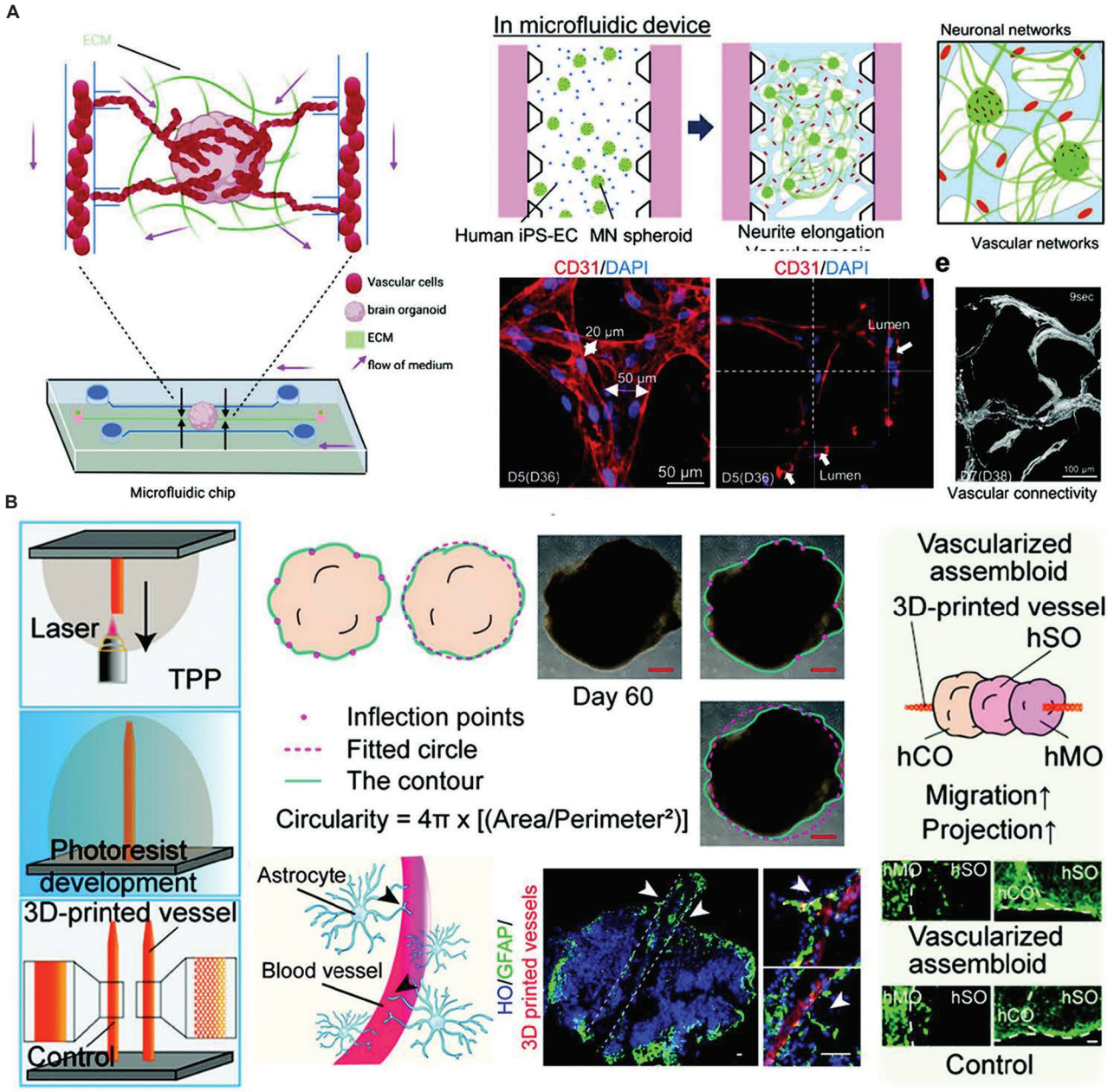

Figure 7. The generation of vascularized brain organoids using engineering strategies. (A) Formation of the vascular system in a microfluidic device.[138] Copyright © 2018, Authors. (B) Three-dimensional (3D) printing vessel-induced human brain organoids.[158] Copyright © 2024 The Authors. Abbreviation: CD31: Cluster of differentiation 31; DAPI: 4',6-diamidino-2-phenylindole; ECM: Extracellular matrix; GFAP: Glial fibrillary acidic protein; hCO: Human cortical organoid; hMO: Human medial ganglionic eminence organoid; HO: Hoechst 33258 dye; hSO: Human striatal organoid; MN: Motor neuron; TPP: Two-photon polymerization.

necessitates specialized training, protocol adaptation, and access to microfluidic equipment, presenting barriers for non-specialized laboratories.[151] To enhance accessibility, standardization and commercialization of ready-to-use microfluidic chips compatible with conventional multiwell plates can streamline adoption by reducing reliance on custom fabrication.[152] These measures, coupled with cost-effective mass production techniques, collectively improve scalability, affordability, and usability, thereby driving the broader adoption of microfluidic platforms in vascularized BOs' research.[153]

In summary, microfluidic technology provides a versatile and powerful platform for improving the vascularization and physiological relevance of BOs. By enabling continuous perfusion, supporting co-culture systems, and recreating the BBB, microfluidic devices are indispensable tools

for advancing research in brain development, disease modeling, and drug discovery.

3.3.2. Application of three-dimensional printing in the vascularization of BOs

Three-dimensional printing technology has integrated advanced bioengineering techniques with the self-organizing properties of cells to construct bioengineered organoids with enhanced reproducibility and improved structural fidelity. This approach leverages the ability to precisely control the spatial arrangement of multiple cell types, biomaterials, and bioactive molecules, enabling the fabrication of highly complex tissue models, including organoids, engineered tissues, and even functional organs. By employing 3D bioprinting, it is possible to accurately define the external and internal geometries, spatial organization, and cellular orientation of tissues, thereby effectively mimicking the structural and functional characteristics of their *in vivo* counterparts. This ensures the interconnectivity of different regions within the organoids and supports adequate perfusion, which is crucial for tissue development, maturation, and repair.[154] Recent applications of 3D printing in BOs' research have included the use of printed scaffolds to support organoid formation and maturation. 3D-printed scaffolds have been used to generate planar BOs that exhibit gyrification. In particular, fibrous scaffolds fabricated using electrospinning or cryogen techniques have been shown to enhance neuronal cultures by providing structural support as well as improving oxygenation and nutrient delivery to the tissue.[155,156] In addition, by employing PLGA microfilaments as scaffolds, researchers have successfully induced the formation of elongated embryoid bodies, leading to well-defined neuroectoderm and cortical development, with organized cortical plates and radial structures.[157]

Three-dimensional bioprinting has also significantly enhanced the development of vascular networks within BOs by creating intricate, biocompatible vascular structures that closely resemble native vasculature. Xu *et al.*[158] used two-photon polymerization 3D printing to create high-resolution meshed vessels with micropores that facilitated the diffusion of nutrients and oxygen, thereby reducing hypoxia and apoptosis in the core of BOs (Figure 7B). Such vascularized organoids exhibited enhanced growth, maturation, and functional integration of different brain regions when assembled into multi-regional structures, supporting complex neurovascular interactions. Meanwhile, vascular structures within organoids also promoted dimensional breaking growth and enabled the co-culture of various neural regions, such as cortical, striatal, and medial ganglionic eminence regions, fostering cellular migration, projection, and signaling pathways.

In addition to direct organoid fabrication, 3D printing can also be applied to create microfluidic chips that enhance scaffold biocompatibility and optimize fluid dynamics within organoid cultures. Using hydrogel-based bioinks, complex multicellular structures, including precisely positioned ECs and pericytes, can be printed. This approach supports the formation of perfusable vascular networks that integrate with organoids and promote tissue maturation. Salmon *et al.*[142] demonstrated the potential of 3D-printed microfluidic chips, which allowed for the formation of organized and perfusable vascular networks within organoids. These networks supported essential nutrient flow and oxygenation, thereby promoting organoid maturation and functionality. The 3D-printed chips are customizable, allowing organoid-specific designs that facilitate vascular growth and integration, overcoming limitations associated with conventional polydimethylsiloxane-based methods. By enhancing nutrient supply, waste removal, and mechanical fluid flow, these chips support the self-organization and development of BOs.[159] The integration of 3D printing with microfluidics has enabled more efficient modeling of physiological processes, offering a better platform for disease modeling, drug screening, and the study of neurodegenerative disorders such as AD and PD.

3.4. *In vivo* vascularization of BOs

In vivo vascularization of BOs has emerged as a critical strategy for overcoming the limitations posed by the lack of perfusion and nutrient supply in organoid cultures.[160] Early efforts to vascularize BOs involved transplantation into host animals, particularly immunodeficient rodents, where the host's vascular system supported the engraftment and integration of the transplanted tissue. Daviaud *et al.*[161] transplanted hESC-derived COs into the cortex of immunodeficient mice, creating an *in vivo* environment that promoted neuronal maturation and vascularization (Figure 8A). Mansour *et al.*[162] developed a vascularized model by grafting hESC-derived BOs into the retrosplenial cortex of immune-deficient, non-obese diabetic-severe combined immunodeficiency mice. Within a week, the mouse blood vessels migrated toward the grafts, and vascular networks were confirmed by dextran dye injection. Notably, 85.4% of the grafts were vascularized, while non-vascularized organoids failed to survive. This observation suggests that blood flow was essential for delivering oxygen and nutrients. Vascularized organoids were larger, exhibited reduced apoptosis, and had more mature Fox-3, Rbfox3, or hexaribonucleotide binding protein-3-positive neurons compared to those cultured *in vitro*. Subsequently, Revah *et al.*[163] transplanted COs into the retrosplenial cortex of adult mice. By integrating transparent microelectrodes with two-photon microscopy, they monitored the organoids over time. Their results demonstrate the successful integration of the organoids into the mouse brain, with vascularization and functional responses to visual stimuli confirmed by electrophysiological recordings and imaging.

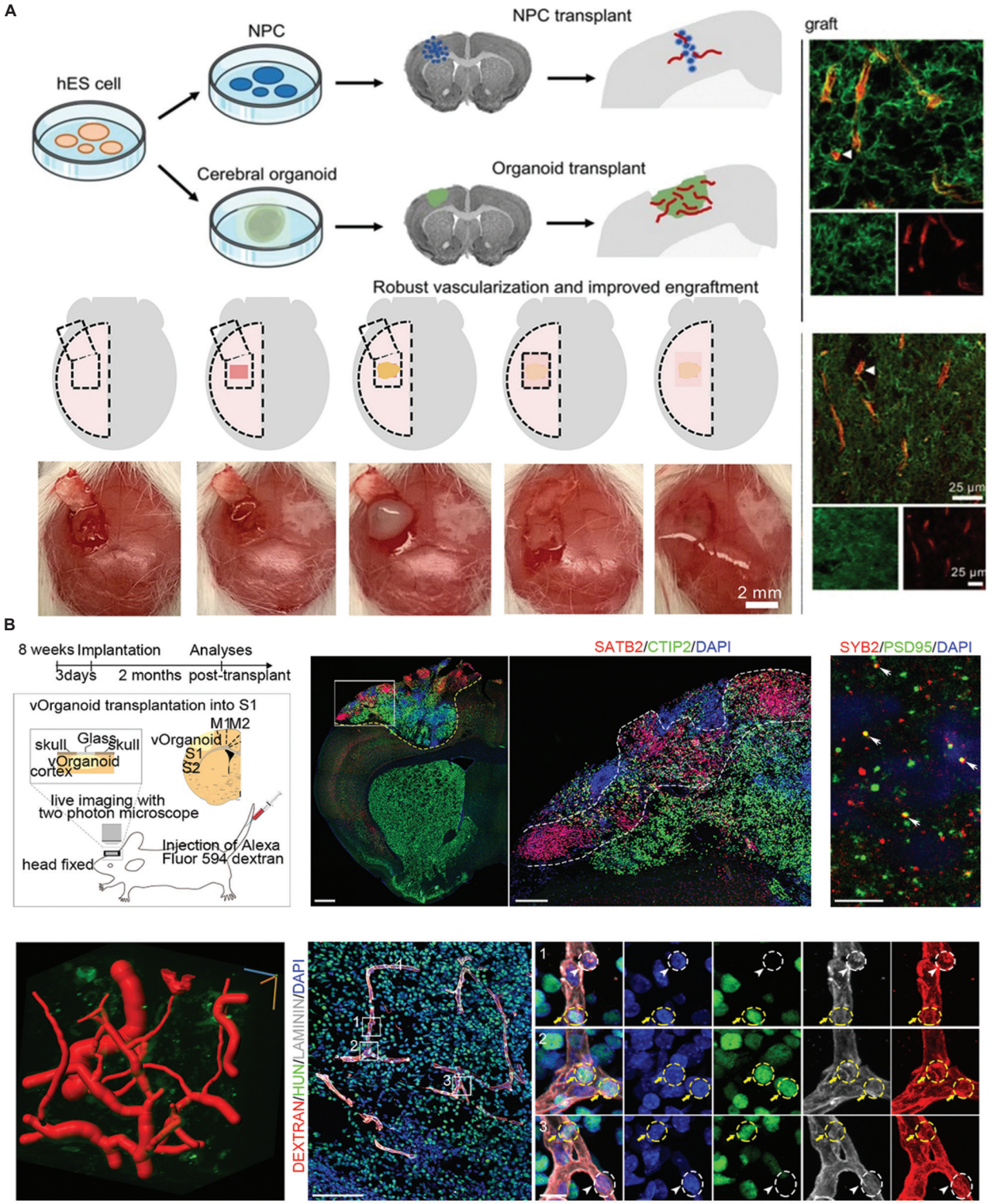

Figure 8. Generation of vascularized brain organoids through *in vivo* implantation. (A) Induction of vascularized brain organoids by implantation into the mouse brain.[161] Copyright 2018, Authors. (B) Enhanced V-Organoids through implantation.[83] Copyright © 2020, Authors.
Abbreviations: CTIP2: Chicken ovalbumin upstream promoter transcription factor-interacting protein 2; DAPI: 4',6-diamidino-2-phenylindole; hES: Human embryonic stem cells; HUN: Human nuclear; NPC: Neural progenitor cells; PSD95: Postsynaptic density protein-95; SATB2: SATB homeobox 2; SYB2: Synaptobrevin 2; vOrganoid: Vascularized brain organoids.

Meanwhile, other researchers have pre-vascularized BOs using various co-culture techniques with ECs or endothelial-like cells to induce vascularization before transplantation. For example, Shi *et al.*[83] co-cultured BOs with HUVECs for several weeks before transplantation into immunodeficient mice (Figure 8B). This pre-culturing resulted in enhanced vascular integration and improved perfusion within the organoids. 3D reconstruction revealed blood vessel structures in the V-Organoid grafts, with human ECs from the V-Organoids and mouse-derived ECs from the host forming a cohesive vascular network. Functional blood flow was observed in the grafts using live two-photon microscopy after injecting Alexa Fluor 594-labeled dextran, indicating integration between the graft and host vascular systems. Compared to non-vascularized organoids, V-Organoids exhibited earlier angiogenesis after implantation. Similarly, Pham *et al.*[18] used iPSC-derived ECs to pre-vascularize BOs, which has led to higher survival rates and more efficient vascularization post-transplantation. These approaches highlight the potential of co-culture systems to promote vascularization and improve the physiological relevance of organoids.

Advances in genetic engineering have also enabled the prevascularization of BOs. Cakir *et al.*[17] developed a novel approach by overexpressing the transcription factor ETV2 in iPSCs, which enabled the generation of functional ECs within the BOs. These pre-vascularized organoids, when transplanted into mice, integrated with the host's vascular system, supporting long-term organoid survival and neural development. The V-Organoids showed clear MRI contrast and evidence of vascularization compared to controls, which exhibited limited host vessel invasion. Dynamic contrast enhancement imaging indicates that the V-Organoids were vascularized, though the structures displayed lower permeability than the adjacent muscle tissue. Immunostaining with human-specific CD31 and fluorescein isothiocyanate, following dextran perfusion, identified functional blood vessels within the V-Organoids, which were absent in the control organoids. In addition, the V-Organoids contained significantly more $hCD31^+$ and fluorescein isothiocyanate-dextran-filled vessels, highlighting the importance of *in vitro*-generated vascular networks in establishing blood flow and functional integration with the host vasculature. This method circumvents the need for complex *in vitro* vascularization protocols, offering more precise control over vascular network formation and improving the overall function of the organoids.

Despite these advancements, *in vivo* vascularization of BOs remains associated with several limitations. One significant challenge is that the vascular networks formed are often of murine origin, which limits the translatability of such models to human physiology. Species-specific differences between murine and human vasculature can lead to inconsistencies in nutrient delivery, signaling pathways, and organoid maturation, potentially compromising the physiological relevance of the model.[164] Furthermore, while the integration of host vasculature can support basic organoid functions, the differences between human and rodent vascular cells may affect organoid development, limiting the applicability of such models for studying human-specific neurological conditions. In addition, immune compatibility challenges persist even in immunodeficient mouse models, where residual immune responses may affect organoid survival and function. Ethical concerns and biological variability associated with animal models further complicate data interpretation and limit the translational applicability of findings to human systems.[165]

These challenges underscore the need for further refinement of vascularization strategies to generate fully humanized vascular systems within BOs. To address these issues, we discuss alternative strategies, including humanized animal models, in which murine hosts are engineered to express human vascular endothelial markers, thereby improving compatibility between the host and transplanted organoids. Moreover, advances in *in vitro* vascularization techniques, such as microfluidic perfusion systems and co-culture approaches with human ECs, provide promising alternatives to reduce reliance on murine models.[166] In addition, 3D bioprinting and synthetic scaffolds used to pre-vascularize organoids before transplantation show the potential to enhance vascular network formation and reduce species-related discrepancies.[167] These advancements aim to provide a more comprehensive understanding of the limitations of murine vasculature in human-specific organoid research and offer methods to improve the translational relevance of such models.[168]

In summary, the development of vascularized BOs represents a significant advancement in recapitulating the complex structure and function of the human brain *in vitro*. Through innovative engineering strategies such as microfluidic technologies, 3D bioprinting, and *in vivo* transplantation, researchers have made considerable progress in overcoming the limitations posed by the lack of vascularization in BOs' cultures. Microfluidic platforms have facilitated continuous nutrient and oxygen supply, improving organoid viability and maturation, while 3D bioprinting has enabled the construction of intricate tissue models with functional vascular networks. *In vivo* transplantation into rodent models has further demonstrated the critical role of host vasculature in supporting long-term organoid survival and functionality. Nevertheless, challenges remain, particularly regarding the

need for fully humanized vascular systems within organoids to ensure their relevance to human physiology and disease modeling. While murine vasculature may provide a temporary solution, future work must focus on developing techniques that promote the formation of human-specific vascular networks, allowing for more accurate modeling of neurodevelopmental processes, neurovascular interactions, and neurological diseases. The integration of emerging technologies, such as gene editing and biomaterials, holds promise for addressing these challenges, potentially leading to more sophisticated organoid models that closely mimic *in vivo* brain environments. These advancements will not only enhance our understanding of brain development and disease but also pave the way for innovative therapeutic approaches, including drug screening and regenerative medicine applications.

4. The application of vascularized BO

Vascularization is essential for replicating the complex relationship between neurons, blood vessels, and the immune system within the brain. Compared to conventional BOs that lack this critical component, vascularized BOs enhance both their physiological relevance and research applications. This advancement has opened new avenues for studying neurodevelopment and brain development, providing a platform for BBB and disease modeling, as well as offering novel approaches for regenerative applications. The integration of vascularization into BOs represents significant progress in understanding the complex dynamics of the brain, developing more effective treatments for neurological diseases, and enabling *in vivo* implantation for brain regeneration.

4.1. Neurodevelopment and brain development

BOs have become indispensable *in vitro* models for studying neural and brain development.[169] Their 3D architecture closely replicates the early phases of brain development, including neuron and glial cell production, neural progenitor cell differentiation, and the establishment of organized brain regions.[35] Qian *et al.*[5] used miniature spinning bioreactor techniques to generate brain-region-specific organoids, such as forebrain, midbrain, and hypothalamic organoids. These organoids recapitulated key features of region-specific brain development, including the organization of progenitor zones and neurogenesis, offering a rough analysis of neurodevelopment. They also provide an accessible and versatile platform for studying neurological diseases, including modeling the effects of the Zika virus, human immunodeficiency virus, and severe acute respiratory syndrome coronavirus 2 exposure on neural progenitors, which resemble microcephaly.[170,171]

The vascular system is crucial for regulating neural stem cell proliferation, differentiation, and migration. Incorporating vascular networks into BOs may expand their potential in neurodevelopmental research by simulating the role of the *in vivo* vascular system in brain development.[101] Shi *et al.*[83] developed vascularized human COs by integrating ECs, which formed a functional vascular network within the organoids. Vascularization played a critical role in promoting neural differentiation and accelerating the maturation of neural circuits, closely mimicking *in vivo* cortical development. Similar to the natural process of brain development, where blood vessels regulate the proliferation of NSCs and their differentiation into neurons and glial cells, the presence of a vascular system in V-Organoids enhanced neurogenesis and provided an environment that supported more efficient neuronal maturation (Figure 6A).

Another research direction has involved using vascularized BOs to explore the intricate relationship between brain vasculature development and neural network formation (Figure 6B). During development, the vascular and nervous systems may collectively influence neuronal migration, axonal guidance, and the construction of neural circuits. Salmon *et al.*[142] constructed vascularized COs by utilizing microfluidic technology to co-culture hPSC-derived pericytes and ECs. Vascular networks were established through microfluidic perfusion, enabling the investigation of neurovascular organoid formation on a 3D-printed microfluidic chip. By day 15, co-differentiation of βiii-TUBULIN$^+$ neurons, CD31$^+$ ECs, and PDGFRβ$^+$ pericytes was observed, exhibiting a well-organized architecture. After 30 days of culture, neuronal axons aligned with the vascular network, and a complex, layered structure emerged between the neuronal and ECs' networks. Furthermore, pericytes and ECs integrated with the organoids, indicating robust cellular interaction. Sun *et al.*[22] explored the regulatory role of brain vasculature in neural stem cell differentiation using vascularized BOs. Their findings demonstrate that signaling molecules secreted by ECs could promote the differentiation of neural stem cells into mature neurons and astrocytes, highlighting the importance of blood vessels in brain development. In addition, they discovered that only the vasculatures connected to or near the BOs expressed TJ markers, including Claudin 5 and ZO-1. These observations reveal that vasculatures could be regulated by neural networks. Further research by Sun *et al.*[24] confirmed that ECs could regulate brain vascularization through interactions with neural progenitor cells and influence neurodevelopment. This underscores the role of neurovascular interactions in neurodevelopment and offers novel insights into the pathological mechanisms underlying neurodevelopmental disorders such as microcephaly and autism.[100,172]

Vascularized BOs have offered an invaluable model for investigating the BBB in the context of neurodevelopment.[173] Recent advancements in the development of "neurovascular

assembloids," achieved by fusing vascular networks with BOs, have created a powerful model for studying neurovascular interactions and BBB formation (Figure 6C). Studies have shown that ECs and pericytes in neurovascular assembloids can help recapitulate the BBB by forming TJs and controlling molecular permeability, thereby mimicking the selective barrier properties of the BBB.[172,174] Furthermore, microglia, the resident immune cells of the CNS, have been found in such models. These cells exhibited phagocytic activity, a process involved in synaptic pruning, and are critical for maintaining synaptic plasticity and neuronal health.[175] Kumarasamy and Sosnik[176] documented the role of microglia in neurovascular assembloids, where they participated in synaptic clearance and responded to external stimuli, potentially opening new avenues for studying neuroinflammatory responses. In addition, assembloids have been used to model neurodegenerative diseases like AD by impairing microglial function and inducing BBB breakdown.[177] These models have highlighted the complexity of neurovascular interactions and their critical role in both development and disease pathogenesis, offering novel insights into cellular and molecular mechanisms.[178]

BOs have provided versatile platforms for studying neural development, and the advent of vascularized BOs has further expanded their applications. Compared to conventional BOs, vascularized organoids more accurately replicate *in vivo* neurovascular interactions and serve as vital models for exploring neural stem cell proliferation, differentiation, and migration. In addition, the inclusion of vascular networks within the BOs has enabled in-depth research into the formation and function of the BBB during development. As technological advances continue to enhance the vascularization of organoids, future studies may explore the impact of neurovascular interactions on the onset and treatment of neurological diseases.

4.2. Vascularized BOs in BBB and disease modeling

As previously mentioned, BBB serves as a crucial regulatory interface that protects the brain by controlling the transfer of substances between the bloodstream and neural tissue. While its selective permeability may play a vital protective role, it also presents a considerable challenge for the delivery of therapeutics aimed at treating CNS disorders. In recent years, V-Organoids have emerged as an advanced model for studying the BBB's intricate structure and function. These cultures have been used to model neurological diseases such as neurodegenerative disorders (Figure 9A), ischemic stroke (Figure 9B), and brain cancers (Figure 9C), providing a platform for assessing the efficacy and safety of potential therapeutics in disease-like conditions.[179]

Neurodegenerative disorders, such as AD, are closely associated with BBB dysfunction. As the permeability of the BBB increases and CBF decreases, cognitive decline often follows.[180] Traditional BOs' models lack functional vasculature, limiting their ability to accurately recapitulate BBB-associated pathophysiology. In contrast, vascularized V-Organoids provide a more physiologically relevant platform by integrating a functional NVU, which is essential for modeling BBB integrity, neurovascular interactions, and disease progression in AD. The presence of perfusable vasculature enhances oxygen and nutrient delivery, supporting extended culture durations, advanced neuronal differentiation, and improved cellular maturation compared to static organoid cultures. These advantages make V-Organoids superior for investigating AD-related BBB dysfunction, enabling researchers to study vascular contributions to neurodegeneration, neuroinflammation, and therapeutic targeting in a controlled and scalable system. In a study by Shin *et al.*,[122] a 3D *in vitro* AD model was developed by co-culturing AD neurons with brain ECs, resulting in increased BBB permeability and reduced expression of key TJ proteins, including claudin-1 and claudin-5. The deposition of Aβ on the vascular endothelium has been identified as a major contributor to vascular damage and BBB breakdown. Furthermore, the study observed elevated levels of matrix metalloproteinase-2 and reactive oxygen species, which exacerbated the weakening of the BBB. Similarly, Ko *et al.*[181] developed a microfluidic platform that integrated a self-assembled microvascular network of the BBB composed of pre-differentiated neurospheres originating from neural progenitor cells specific to AD. This co-culture system facilitated direct interactions between BBB components, for example, brain ECs, astrocytes, pericytes, and the AD-neurospheres, allowing for the study of AD-specific neurovascular alterations. The model effectively recapitulated neurovascular phenotype characteristics of AD, including Aβ-induced BBB dysfunction, and provided a valuable tool for investigating AD pathology, highlighting neurovascular contributions. In addition, this platform holds significant potential for screening therapeutic compounds aimed at reducing BBB disruption and Aβ accumulation, offering a more physiologically relevant model for therapeutic discovery in AD research.

V-Organoids have also opened a new avenue for studying stroke-induced BBB alterations. Conventional BOs face limitations in stroke modeling due to the absence of a vascular system and microglia, both of which are essential for replicating the pathophysiological changes observed in stroke.[9,182] Integrating a vascular network into organoids introduces more realistic conditions, allowing researchers to assess the critical roles of blood circulation, factors secreted by vascular cells, components of the blood, and microglia in stroke progression. Vascularized BOs cultured for extended periods (6 months or longer) have shown the potential to model stroke-related phenotypes

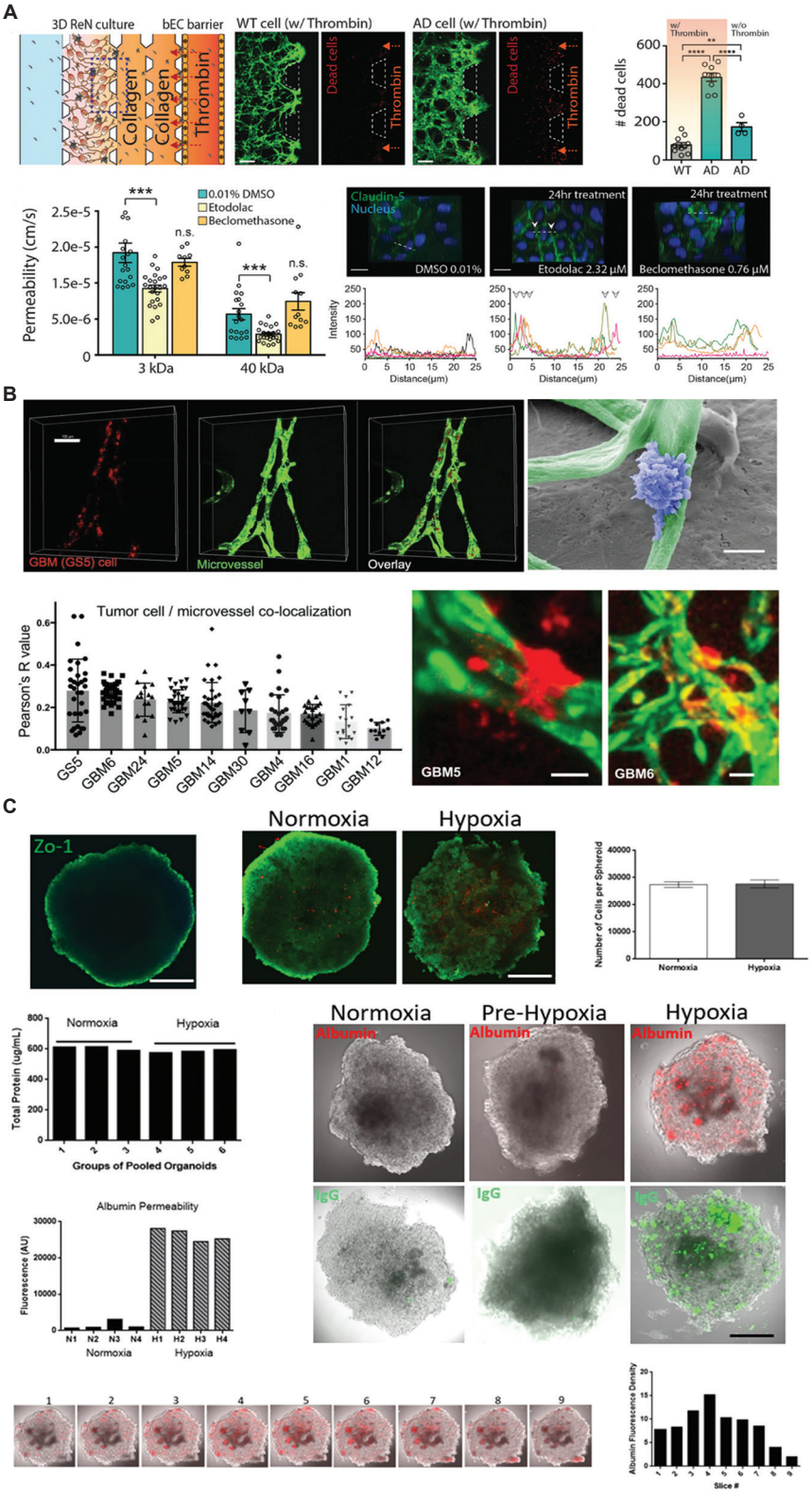

Figure 9. Vascularized brain organoids for disease modeling. (A) Alzheimer's disease model.[122,181] Copyright © 2019, Authors. Copyright © 2023, Authors. (B) Ischemic stroke model.[183] Copyright © 2020, Authors. (C) Glioblastoma multiforme model. Reproduced from Xiao *et al.*[208] Copyright © 2019, Authors.

Abbreviations: AD: Alzheimer's disease; bEC: Brain endothelial cells; DMSO: Dimethyl sulfoxide; GBM: Glioblastomas; IgG: Immunoglobulin G; ReN: Human neural progenitor cells; WT: Wild-type; Zo-1: Zona-occluden 1; 3D: Three-dimensional.

more accurately. For example, Nzou *et al.*[183] developed 3D NVU organoids using a hanging-drop culture method. Such organoids, which comprise neurons, oligodendrocytes, astrocytes, microglia, human brain microvascular ECs, and pericytes, were exposed to hypoxic conditions to simulate stroke-induced BBB dysfunction. This model successfully recapitulated features such as increased permeability, inflammatory responses, and oxidative stress, providing a physiologically relevant platform for stroke research. Although studies on 3D stroke-related organoid models are limited, advancements in organoid technology suggest a promising future for stroke modeling.

The presence of functional vasculature in V-Organoids provides a more physiologically relevant platform for studying brain cancers, particularly in modeling the blood–brain tumor barrier, which arises as the BBB undergoes a pathological transformation during tumor progression. Unlike traditional organoid models, which lack perfusion and vascular remodeling, V-Organoids closely mimic the tumor microenvironment by integrating dynamic vascular networks. This allows researchers to investigate tumor-endothelial interactions, angiogenic signaling, and barrier permeability changes in GBM. This vascular complexity is critical for evaluating anti-angiogenic therapies, which target tumor-induced neovascularization, as well as for testing BBB-penetrating drugs that must cross the blood–brain tumor barrier to reach malignant cells. By providing a controlled yet physiologically relevant *in vitro* system, V-Organoids enable high-fidelity drug screening and therapeutic development, bridging the gap between preclinical research and clinical applications in brain cancer treatment. Moreover, V-Organoids have enabled researchers to more accurately replicate the complex interactions between GBM cells and the neurovascular environment. Grebenyuk and Ranga[184] used V-Organoids to demonstrate how GBM cells may compromise BBB structure, resulting in alterations of TJ proteins and increased permeability, thus mimicking the *in vivo* tumor microenvironment. Moreover, the integration of microglia into V-Organoids has allowed for the investigation of cross-talk between GBM cells and immune cells, offering insights into how tumors modulate the immune response to support their growth. This multifaceted approach advances our understanding of GBM biology, including tumor angiogenesis, immune evasion, and BBB disruption, all of which are critical for developing more effective therapies for brain cancers.[185]

In addition to disease modeling, V-Organoids have a wide range of applications in CNS drug development. As previously mentioned, the treatment of CNS diseases may be hampered by the BBB.[186] To address this, Bergmann *et al.*[186] discussed how BBB organoids can be utilized to determine whether compounds can cross the BBB. The permeability of the compound can be quantified by measuring the mean fluorescence intensity through confocal fluorescence z-stack images. This method allows for rapid testing of different compounds, such as angiopep-2, phosphatidylinositol 3-kinase inhibitor BKM120, and dabrafenib. In addition, V-Organoids can be used to screen anti-inflammatory compounds and identify agents that restore BBB integrity and counteract the invasive properties of GBM cells.[127] V-Organoids can simulate the crucial aspects of BBB, providing a low-cost approach to evaluate the brain-permeable molecules for a wide range of diseases. Future research may benefit from the ongoing refinement of V-Organoids, particularly through the incorporation of vascular and immune components to more accurately simulate *in vivo* conditions.

4.3. Regeneration applications of vascularized BOs

Current cell transplantation therapies for brain injuries face limitations in regenerating multiple damaged cell types. Most cell transplantation research has employed single NSCs or neuronal cell types to repair brain injuries, which may be insufficient for regenerating the wide array of cell types lost during injury.[182,187] BOs, however, contain a diverse range of neural cell types, presenting a rich source of cells for transplantation, brain injury repair, and regeneration. Wang *et al.*[188] assessed the effect of COs' transplantation in a rat model of ischemic stroke (Figure 10A). Their findings demonstrate that transplanted COs could differentiate into neurons, glial cells, and other cortical cell types, effectively mimicking cortical regeneration. This process supports neurogenesis, synaptic reconstruction, and axonal regrowth, thus enabling the formation of functional neural circuits within the damaged brain region. Meanwhile, Cao *et al.*[13,189] showed that COs' transplantation is more effective in repairing structural damage compared to individual neural stem cell transplants, providing a novel strategy for stroke rehabilitation. In their ischemic stroke mouse model, transplanted medial ganglionic eminence organoids survived in the infarcted cortex, differentiated into specific cortical neuron subtypes, and projected axons to establish connections with host neural circuits, leading to significant improvements in sensorimotor functions. Furthermore, Cao *et al.*[13] transplanted COs enriched in GABAergic interneurons into stroke-affected mouse cortices. These organoids integrated effectively within the damaged cortex, differentiated robustly, and restored sensorimotor function in the stroke-affected mice. These observations suggest that specific types of organoids, particularly those containing inhibitory neurons, can facilitate recovery post-stroke by replenishing lost neuronal populations and reconstructing functional neural circuits (Figure 10B).

Given most brain injuries result from ischemia, vascularization in BOs could significantly enhance their

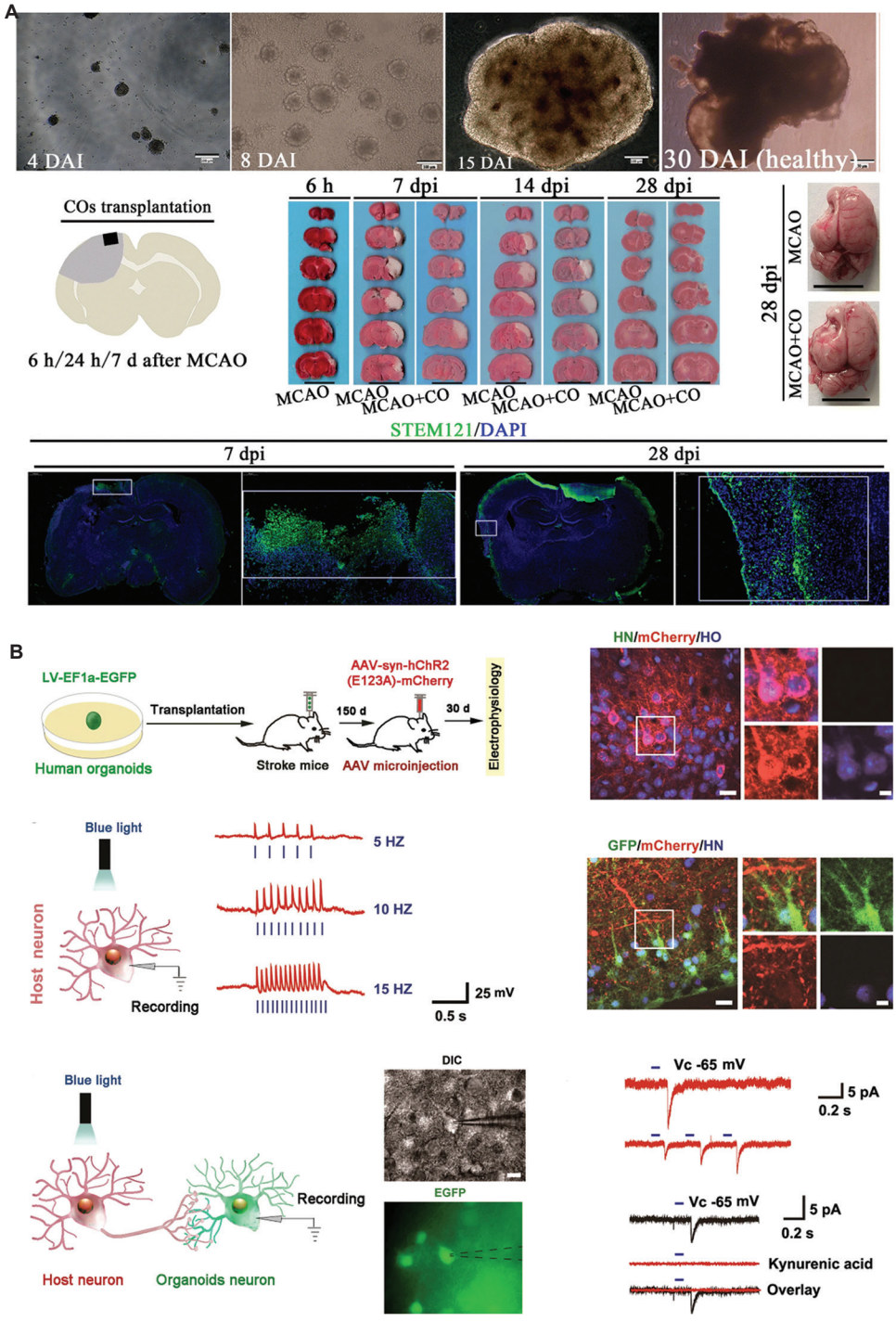

Figure 10. Brain organoids transplantation for stroke brain injury. (A) hMGEO transplantation in stroke. Reproduced from Wang *et al.*[188] Copyright © 2020, Authors. (B) Cerebral organoids transplantation after stroke.[13] Copyright 2023, Springer Nature.
Abbreviations: AAV: Adeno-associated viruses; COs: Cerebral organoids; DAI: Days after induction; DAPI: 4',6-diamidino-2-phenylindole; DIC: dpi: Dots per inch; EF1a: Elongation factor 1-alpha 1; EGFP: Enhanced green fluorescent protein; hChR2: Human channel rhodopsin 2; HN: Human nuclei; HO: Hoechst 33258 dye; LV: Lentivirus; MCAO: Middle cerebral artery occlusio.

regeneration by restoring blood supply, supporting tissue repair, and improving survival and functional recovery post-transplantation. In a pivotal study, Daviaud *et al.*[161] transplanted hESC-derived vascularized BOs into the cortex of immunodeficient mice. The results showed that the transplanted V-Organoids integrated with the

host vascular system, establishing functional NVUs. The V-Organoids survived for several months post-transplantation, formed complex cortical structures, and expressed mature neuronal markers. Notably, the host vasculature infiltrated the transplanted organoids, creating a stable blood supply system that supported long-term survival and reduced necrosis. In addition, the study reported the formation of functional synaptic networks and the activation of neuronal circuits, suggesting that V-Organoids can integrate into the host brain tissue and potentially replace lost or damaged neuronal populations following brain injury. To further expand the regenerative applications, Shi *et al.*[83] used the V-Organoids for repairing brain injuries. On transplanting V-Organoids into mouse models, they observed that the organoids successfully integrated with the host's vascular system, forming new blood vessels and promoting tissue regeneration within the infarcted cortex. Functional assessments, including motor coordination tests, revealed significant improvements in motor function recovery in mice receiving V-Organoid transplants compared to control groups. Histological analysis confirmed the presence of newly formed cortical layers in the infarcted regions, indicating that V-Organoids could not only support structural regeneration but also restore the functionality of the damaged cortex.

The inclusion of vascularization in BOs has significantly broadened their regenerative potential. By incorporating functional blood vessels, V-Organoids can better simulate the brain's *in vivo* environment, which is crucial for supporting transplanted cells, reducing necrosis, and promoting integration with host tissues. This represents a promising direction for regenerative medicine, offering a multifaceted approach to repairing neural damage caused by stroke, traumatic brain injury, and other neurological conditions. Future research on V-Organoid transplantation may focus on enhancing their vascularization and exploring the use of additional factors, such as neural progenitor cell-derived extracellular vesicles, to further improve their regenerative capacity.[83,190]

While V-Organoids hold significant promise for regenerative medicine, their clinical application faces critical challenges. Immune rejection remains a primary obstacle in tissue transplantation, as the host immune system may recognize the organoids as foreign entities, leading to graft rejection. The complexity of the immune system exacerbates this challenge, as various immune cells, including T-cells, macrophages, and dendritic cells, can identify and target the transplanted organoids. This immune response significantly limits the long-term survival and functional integration of the grafts, ultimately hindering the successful clinical application of V-Organoids in regenerative therapies.

Several strategies to mitigate immune rejection include the use of iPSCs to create autologous organoids, thereby reducing immunogenicity. In addition, gene editing techniques, such as human leukocyte antigen engineering, aim to diminish immune recognition and promote graft acceptance. To further enhance compatibility with host tissue, the use of autologous iPSCs, derived from the patient's own cells, ensures that the organoids are recognized as "self" by the immune system, thus reducing the risk of rejection.[191] Genetic modifications of the organoids, such as the introduction of immunosuppressive factors such as programmed death-ligand 1 or TGF-β, can suppress immune responses and facilitate better integration with the host tissue.[192] Pre-conditioning the organoids and host tissue in a controlled immune environment before implantation can also help the organoids adapt to the host's immune system.[193] Immunoprotective biomaterials, including coatings with PEG or self-healing materials, can reduce immune recognition and provide additional protection. Finally, clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) gene editing can be used to alter the organoid's genetic makeup, lowering its immunogenicity and improving its survival within the brain. Together, these strategies form a comprehensive approach to overcoming immune rejection and enhancing the success of BOs' transplantation.[194]

Another critical challenge in the long-term integration of vascularized BOs (V-Organoids) into host tissue is ensuring stable vascular anastomosis and functional incorporation into the host circulatory and neural systems.[184] While vascular networks may form within organoids before transplantation, their structural maturity, perfusion efficiency, and endothelial compatibility with host vasculature remain key determinants of successful engraftment. Inadequate integration can result in insufficient blood flow, hypoxia, and necrotic core formation, particularly in larger organoid constructs where diffusion alone is insufficient to sustain deep tissue regions.

To enhance vascular integration, prevascularization with perfusable endothelial networks facilitates early vessel formation within organoids, enabling more efficient host–organoid vascular connections.[195] Co-transplantation with supportive stromal cells, such as pericytes and astrocytes, has been shown to stabilize vascular structures and contribute to BBB formation. In addition, bioreactor-based dynamic culture systems improve endothelial maturation and vascular complexity before transplantation, increasing the likelihood of successful integration with the host tissue.[196] The use of angiogenic biomaterials and bioactive hydrogels provides a supportive microenvironment that promotes endothelial migration, vessel remodeling, and sustained perfusion post-implantation. Further advancements in these approaches are essential for optimizing the structural and functional incorporation of V-Organoids into host tissue, ultimately enhancing their potential in regenerative medicine.

Despite these challenges, ongoing advancements in tissue engineering, genetic engineering, and stem cell biology continue to bring us closer to overcoming these obstacles, paving the way for the clinical application of V-Organoids in regenerative medicine.

5. Challenges and future perspectives of vascularized BOs

The development of V-Organoids represents a significant step toward modeling the human brain's structure, development, and disease mechanisms.[162,197] Despite their potential, several challenges must be addressed to fully realize the application of V-Organoids in neuroscience research and clinical therapies.[198]

A primary challenge in creating V-Organoids is to achieve a fully functional vascular system that accurately reflects the complexity of *in vivo* brain vasculature. Current approaches, including co-culture with ECs, growth factor induction, and microfluidic systems, have led to the formation of rudimentary vascular networks.[17,199] However, these methods often result in immature and non-humanized vasculature, lacking the full characteristics of the BBB and the NVU. Enhancing the maturity and integration of the vascular system remains a major technical hurdle. In addition, the absence of proper immune components, such as microglia and other immune cells, in most V-Organoid models limits their ability to fully replicate *in vivo* neurovascular interactions, particularly in disease contexts such as neuroinflammation, stroke, and GBM.[200] Future research should aim to incorporate such elements to provide a more comprehensive model of the brain's microenvironment.

While V-Organoids hold promise for modeling late-onset diseases and long-term brain development, sustaining their growth and maturation over extended periods presents significant difficulties.[200] The cultivation of V-Organoids for several months is crucial for developing complex neural structures and establishing mature vascular networks.[201] However, achieving long-term maintenance without the formation of necrotic cores due to oxygen and nutrient limitations remains a critical obstacle.[202] Advanced bioengineering techniques, such as perfusion systems and dynamic microfluidic platforms, need further optimization to support the longevity and complexity of V-Organoids.[203]

Transplanting V-Organoids for regenerative applications has demonstrated potential for integrating with host tissue and promoting functional recovery. However, ensuring consistent integration with host vasculature, proper neuronal network formation, and functional synaptic connections remains challenging. Another key issue in transplantation is overcoming immune rejection, which has led to the development of immunocompatible organoids and/or the use of immunosuppression strategies.

Ethical implications of using human-derived stem cells to create BOs, particularly those with vascular networks that closely resemble aspects of human brain functionality, cannot be ignored. As V-Organoids become more complex, ethical concerns surrounding consciousness, sentience, and their potential use in transplantation may arise.[204] Furthermore, translating V-Organoids research into clinical therapies will likely involve navigating regulatory challenges to ensure safety, efficacy, and ethical compliance.

Despite such challenges, the future of V-Organoids appears promising. Advanced gene-editing techniques, like CRISPR/Cas9, could be employed to engineer more precise and humanized vascular components within organoids.[205,206] Combining V-Organoids with other organ-on-a-chip systems, such as liver or gut models, may provide insights into systemic interactions affecting the brain.[207] Moreover, the integration of immune cells into V-Organoids cultures will enhance their utility in modeling neuroimmune interactions and developing therapies for neuroinflammatory and autoimmune diseases.

Ongoing research efforts should focus on optimizing the vascularization process, improving long-term cultivation techniques, and enhancing the functional integration of transplanted organoids. Collaborative, interdisciplinary approaches that merge biology, engineering, and medicine will be key to overcoming current limitations.[5] With these advancements, V-Organoids hold the potential to revolutionize our understanding of the human brain, offering a versatile platform for disease modeling, drug screening, and the development of novel regenerative therapies.

6. Conclusion

Vascularized BOs have emerged as a transformative tool in neuroscience, bridging the gap between traditional models and the complex physiology of the human brain. The incorporation of functional vasculature enhances their ability to model neurodevelopment, neurovascular interactions, and disease mechanisms, providing valuable insights into conditions such as stroke, GBM, and neurodegenerative disorders. Despite substantial progress, key challenges persist, including the development of fully mature vascular networks, long-term stability, and effective integration following transplantation. As these models continue to advance, ethical considerations must evolve in parallel to address their increasing complexity. Future innovations in bioengineering – such as microfluidics and CRISPR-based gene editing – will be critical for optimizing vascularization strategies and improving translational relevance. Overcoming these barriers could enable V-Organoids to revolutionize personalized disease

modeling, drug discovery, and regenerative medicine, firmly establishing them as indispensable tools for bridging the divide between basic research and clinical application.

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Conflict of interest

Hui Qi Xie is an Editorial Board Member of this journal but was not involved in any way in the editorial or peer-review process conducted for this paper, either directly or indirectly. Separately, the other authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

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Ethics approval and consent to participate

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Consent for publication

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Availability of data

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ACCSCIENCE PUBLISHING

OR

REVIEW ARTICLE

Organoid research breakthroughs in 2024: A review

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Abstract

Organoid research has experienced significant advancements in 2024, revolutionizing the fields of disease modeling, drug discovery, and regenerative medicine. Key innovations include the refinement of culture protocols for generating more physiologically relevant organoids derived from a wide range of human tissues, facilitated by improved differentiation protocols for induced pluripotent stem cells and adult stem cells. These advancements have led to organoids that better mimic *in vivo* tissue architecture and function, making them more suitable for studying complex diseases. The integration of microfluidics and biomaterial scaffolds into organoid cultures has further enhanced the replication of organ-specific microenvironments. In addition, the application of cutting-edge genomic tools, such as CRISPR/Cas9 gene editing, single-cell RNA sequencing, and high-throughput screening, has enabled the generation of organoid models with precise genetic mutations, facilitating the exploration of disease mechanisms and the screening of therapeutic agents. Artificial intelligence and machine learning have played a pivotal role in analyzing organoid data, enabling high-throughput screening and the development of personalized treatment strategies. While challenges remain in scalability, reproducibility, and vascularization, the innovations made in 2024 have set the stage for future clinical applications of organoid technologies, offering new possibilities for personalized medicine, drug development, and regenerative therapies.

Keywords: Organoids; Induced pluripotent stem cells; Disease modeling; Drug discovery; Biomaterials

1. Introduction

Organoid technology has emerged as a transformative tool in biomedical research, offering unprecedented opportunities to replicate the structure and function of human tissues *in vitro*.[1-3] By providing physiologically relevant, three-dimensional (3D) models and organoids provide platforms that bridge the anatomical and physiological gaps between traditional two-dimensional (2D) cell cultures and animal models, enabling a deeper understanding of complex diseases and the development of advanced therapeutic strategies.[4,5] In 2024, remarkable breakthroughs have been achieved in organoid research across various fields, pushing the boundaries of their applicability in disease modeling, drug discovery, regenerative medicine, and personalized health care.[6-8]

The literature selected for this review was guided by specific criteria: First, we focused on original research published in 2024, prioritizing studies that introduced significant breakthroughs and advancements within the year. The research was selected based on its innovative

application of organoid models in disease mechanism analysis, drug screening, and regenerative medicine, whereas excluding studies that primarily addressed basic technical optimizations without substantial applied contributions. We emphasized articles from high-impact journals that had been widely cited, particularly those featuring methodological innovations such as artificial intelligence (AI) integration and organoid functionalization. Finally, only studies with robust experimental validation, including single-cell RNA sequencing and comprehensive *in vivo* and *in vitro* assessments, were included to ensure the reliability and credibility of the findings.

One of the major advancements of 2024 was the development of brain organoids with anatomical region specificity, which replicate distinct brain regions, such as the cortex and hippocampus, with high fidelity. These organoids provide an advanced platform for studying neurodevelopmental disorders, such as autism and schizophrenia, offering novel insights into the genetic and environmental factors affecting specific brain regions. Similarly, the creation of amniotic fluid (AF)-based organoids revolutionized prenatal diagnostics by enabling the modeling of fetal development and the non-invasive detection of congenital diseases. In oncology, 2024 saw the emergence of organoid models capable of identifying carcinogenic factors and simulating tumor progression with unprecedented precision. For instance, engineered "mini-colons" demonstrated spatiotemporal tumorigenesis *in vitro*, facilitating the study of cancer heterogeneity and the role of gut microbiota in tumor development. In addition, immune-competent organoids integrated tissue-resident immune cells, allowing researchers to explore immune-mediated processes in gut inflammation, autoimmune diseases, and cancer immunotherapy. The integration of AI with organoid technology has marked another significant milestone. AI-assisted organoids enabled high-throughput drug screening, improved the identification of therapeutic targets, and paved the way for precision medicine by tailoring treatments to individual genetic profiles. Furthermore, advances in bioengineering, such as the development of large-scale, self-mineralizing bone organoids and DNA microbead technologies for spatiotemporal control, addressed critical limitations in scalability, vascularization, and tissue complexity.

This review highlights the latest advancements in organoid technology, focusing on breakthrough strategies for organoid construction, enhanced disease modeling, and transformative applications (Figure 1). By addressing critical challenges and showcasing innovative approaches, this work underscores the pivotal role of organoids in reshaping the future of biomedical research and therapeutic development.

2. Breakthrough strategies for organoid construction

Recent advancements in organoid construction have gone beyond traditional stem cell-derived models, exploring innovative approaches to develop organoids from various tissue sources.[9] This paradigm shift enables the creation of organoids that better capture the unique characteristics and functions of specific tissues, providing more accurate models for studying development, disease mechanisms, and therapeutic responses.[10] In addition, breakthroughs in material science have paved the way for precisely regulated organoid development.[11] By designing biomaterials that mimic the native extracellular matrix (ECM) and modulating mechanical and biochemical cues, researchers can guide organoid self-organization and maturation.[12] These strategies represent a significant leap forward, offering new possibilities for personalized medicine, tissue engineering, and regenerative therapies.

2.1. Brain organoids with anatomical region specificity

Recent advancements in brain organoid technology have led to the development of organoids with anatomical region specificity, a significant leap beyond traditional models that are derived from pluripotent or adult stem cells.[13] Unlike conventional organoids, which generally provide a generalized representation of the brain, these new models are generated directly from fetal brain tissue, enabling the creation of organoids that exhibit the distinct cellular composition and functional characteristics of specific brain regions, such as the cortex, hippocampus, or basal ganglia.[14] This innovation allows for a more accurate replication of the complex architecture and cellular organization seen in the human brain, making it a valuable tool for studying regional brain development and pathology.[15] Region-specific brain organoids hold particular promise for investigating the underlying mechanisms of neurodevelopmental disorders, including autism spectrum disorders, schizophrenia, and intellectual disabilities, as they provide insights into how genetic and environmental factors affect different brain regions.[16] Thus, brain organoids with anatomical region specificity represent an important advancement in the field of neuroscience, offering new opportunities for understanding brain function and disease.

During development, the human brain exhibits a unique capacity for expansion while concurrently establishing cellular differentiation, diversity, and complex tissue architecture.[17,18] However, the successful translation of these intrinsic brain characteristics into long-term, expanding, 3D *in vitro* culture systems has not been achieved. Benedetta Artegiani and Hans Clevers led their research team in the Netherlands to generate long-term expanding central nervous system (CNS) organoids from human fetal tissue.[19] They demonstrated

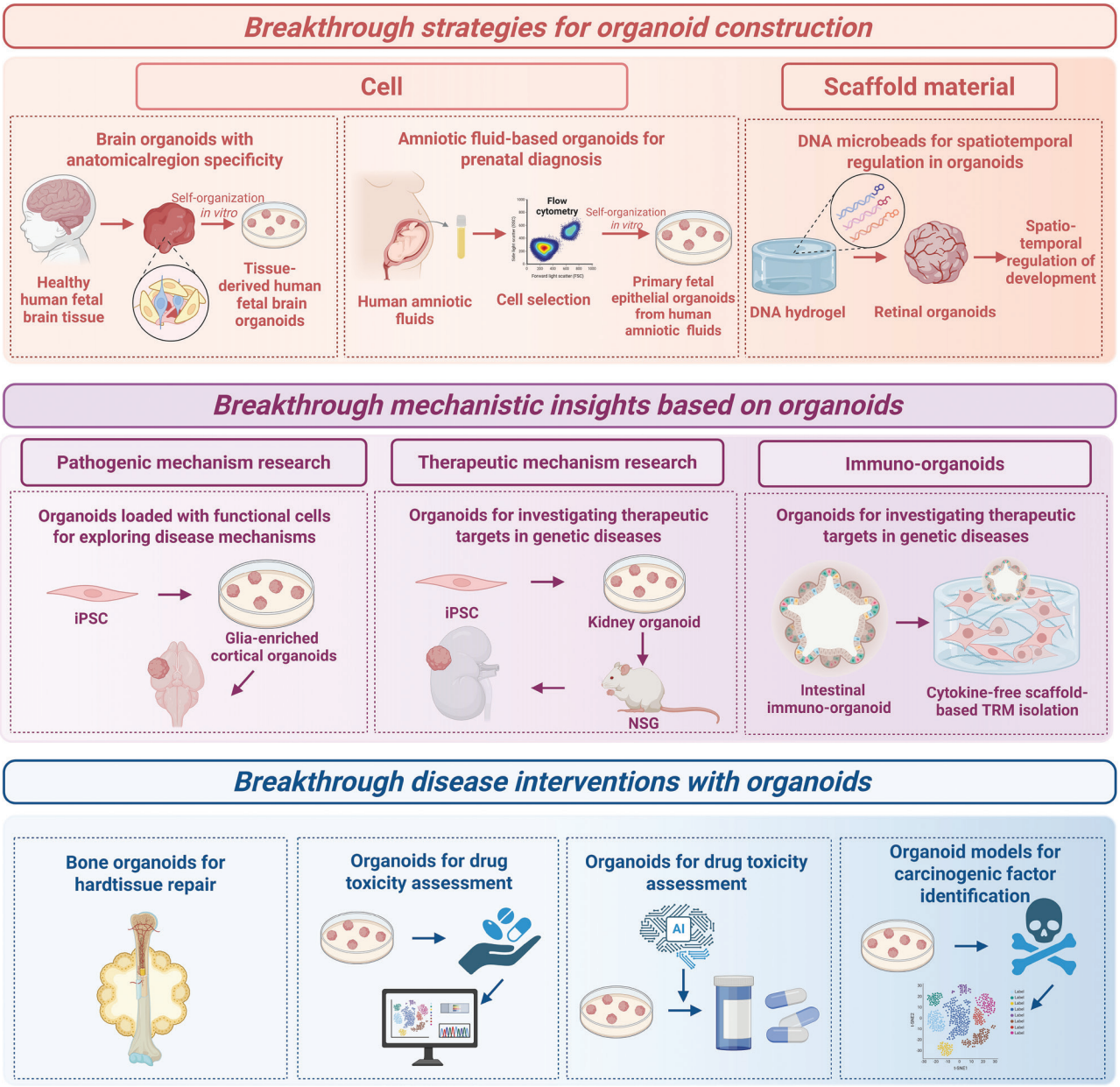

Figure 1. Recent breakthroughs in organoid technology highlight innovative methods for organoid construction, advancements in disease modeling, and transformative applications in medical research. Created with BioRender.com.

Abbreviations: AI: Artificial intelligence; iPSC: Induced pluripotent stem cell; NSG: NOD.Cg-Prkd^{cscid} IL2rgtm^{1Wjl}/SzJ; TRM: Tissue-resident memory T cell.

that healthy human fetal brain tissue can self-organize into organoids (fetal brain organoids, FeBOs) *in vitro*. Notably, unlike traditional organoids generated from pluripotent stem cells (PSCs) or tissue stem cells, this is the first successful generation of organoids directly from fetal brain tissue. Meanwhile, FeBO lines derived from various regions of the CNS, such as the dorsal and ventral forebrain, maintain their regional identity and enable the investigation of positional identity (Figure 2). By altering the culture conditions, FeBOs can further exhibit characteristics closer to those of the mature brain, such as synapse formation and neuronal activity. The study detailed the gene expression changes during the maturation of FeBOs through single-cell sequencing and transcriptomic analysis. In addition, using CRISPR-Cas9, they successfully generated syngeneic mutant FeBO lines for studying brain tumors.

2.2. AF-based organoids for prenatal diagnosis

AF-based organoids for prenatal diagnosis offer a cutting-edge, non-invasive alternative to traditional prenatal screening methods. Instead of relying on invasive techniques such as amniocentesis or chorionic villus sampling, which carry some risks, this approach uses AF itself as a source

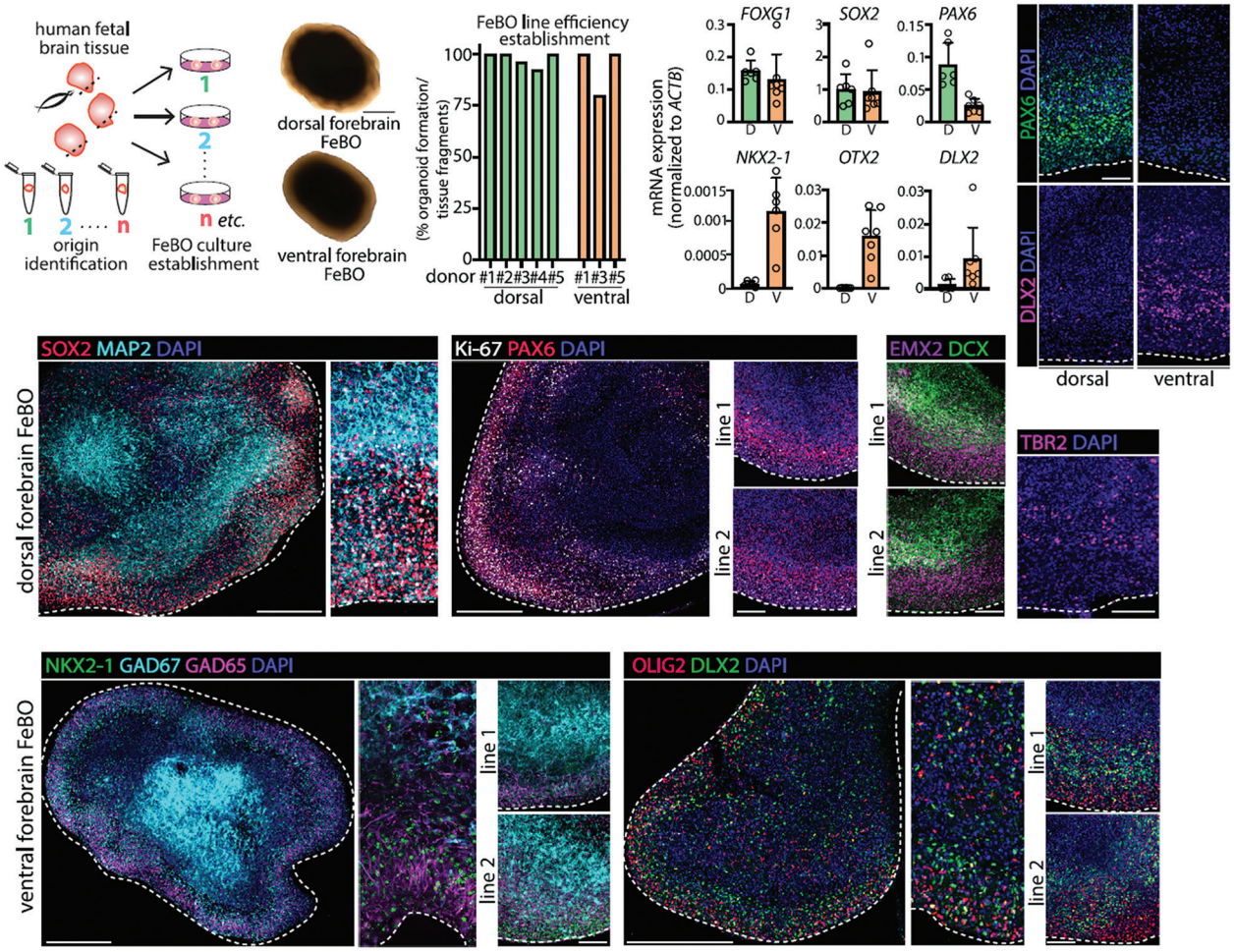

Figure 2. Derivation and characterization of regional FeBOs from different brain regions. The brightfield and immunofluorescence images of regional forebrain-derived FeBOs illustrate the expression of PAX6 (dorsal forebrain marker) and DLX2 (ventral forebrain marker). Quantification of the efficiency of FeBO line establishment from dorsal and ventral forebrain regions is presented, and differences in mRNA expression of specific regional markers across varying culture ages are highlighted. In addition, the figure also demonstrates the reproducibility of marker expression in dorsal and ventral forebrain FeBOs across different lines.[19] Copyright © 2023 The author(s).

of cells to create 3D organoid models that closely resemble fetal tissues. These organoids can be cultured to represent various fetal organs, such as the brain, lungs, and kidneys, providing a more accurate and physiologically relevant model of fetal development. By studying these organoids, researchers can gain insights into how genetic mutations and environmental factors impact fetal tissues at the cellular and molecular levels. This method allows for early and precise detection of genetic disorders such as Down syndrome, cystic fibrosis, or congenital heart defects, all without the need for invasive procedures. In addition, AF-based organoids offer the potential for drug screening, enabling the testing of therapeutic interventions in models that closely mimic fetal development. Overall, this innovative technique represents a significant advancement in prenatal diagnostics, providing a safer and more comprehensive means of assessing fetal health.

Traditional methods of fetal stem cell isolation and organoid culture often depend on fetal tissue samples obtained after the termination of pregnancy. This limitation restricts the investigation of fetal development and congenital diseases prenatally. Therefore, there is a need for new patient-specific *in vitro* models to overcome these limitations.[20] AF, which contains cells from multiple developing fetal organs, and tracheal fluid, which is closely related to fetal lung development, offer alternative sources for such models. Through single-cell analysis technology, Gerli *et al.*[21] identified fetal epithelial stem/progenitor cells in both amniotic and tracheal fluids and cultured tissue-specific organoids from these cells. Through RNA sequencing and immunostaining, the research team confirmed the tissue specificity of the organoids (Figure 3). For example, small intestine organoids (SiAFOs) exhibited

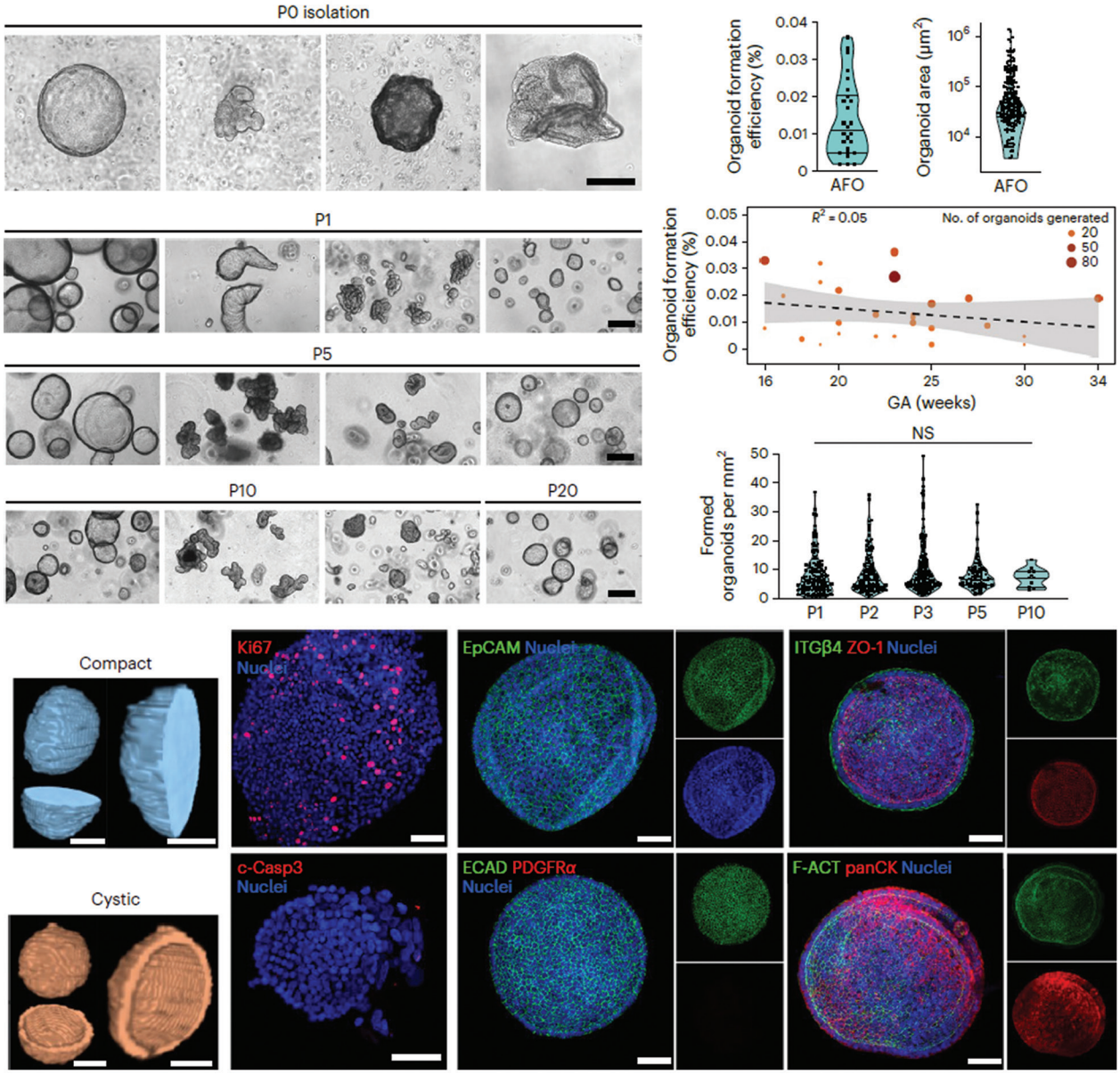

Figure 3. Characterization of primary fetal epithelial amniotic fluid organoids (AFOs). Phase-contrast images show organoid formation from amniotic fluid cells, with varying morphologies observed by day 14. Organoid formation efficiency and size at P0 are quantified, with minimal correlation to gestational age. X-ray PC-CT reveals compact and cystic phenotypes. Immunofluorescence at P3 confirms proliferation (Ki67), epithelial marker expression (EpCAM, ECAD, pan-cytokeratin), and polarization (ZO-1, ITGβ4). PCA and scRNA-seq identify distinct clusters corresponding to organoids from various fetal tissues.[21] Copyright © 2024 The author(s).

Abbreviations: GA: Gestational age; PC-CT: Photon-counting computed tomography; PCA: Principal component analysis; scRNA-seq: Single-cell RNA sequencing.

characteristics of intestinal epithelial cells and digestive enzyme activity upon maturation. Kidney organoids (kidney AF organoids, KAFOs) showed features of renal tubule cells and displayed functional voltage-gated potassium channels and tight junctions. Lung organoids (lung AF organoids, LAFOs) demonstrated traits of lung epithelial cells, such as normal cilia structure and surfactant secretion after differentiation. They further cultured lung organoids from the AF and tracheal fluid of fetuses with congenital diaphragmatic hernia (CDH). These organoids exhibited disease-related features of CDH, such as increased expression of *SOX9* and changes in the expression of surfactant-related genes. The study provides a novel approach to obtaining fetal stem cells from AF and tracheal fluid and to culture tissue-specific organoids without the need to terminate the pregnancy. This breakthrough offers new possibilities for prenatal diagnosis and treatment of congenital diseases.

2.3. DNA microbeads for spatiotemporal regulation in organoids

The regulation of organoid development through matrix materials involves the precise design and adjustment of ECM composition, structure, and physical properties, which directly influence the formation, differentiation, and functional performance of organoids.[22] The ECM not only provides essential support and a 3D growth environment for cells but also regulates cellular behaviors, migration, proliferation, and gene expression through interactions with cell-surface receptors.[23] By controlling the hardness, porosity, surface chemistry, and bioactivity of matrix materials, researchers can fine-tune the developmental processes of organoids, enabling them to more closely mimic the morphology, function, and tissue characteristics of native biological tissues.[24]

The lack of spatial organization of morphogen gradients is one of the critical factors limiting organoids from fully replicating the corresponding organs, thereby hindering their development as physiologically relevant model systems.[25] Morphogen gradients are typically achieved through methods such as microfluidic devices and hydrogel patterning technologies incorporating biochemical signals.[26,27] However, these approaches primarily provide unidirectional gradients from the exterior to the interior of the organoid, exposing outer cells to higher morphogen concentrations, whereas inner cells are exposed to lower concentrations. To create spatially discrete morphogen sources within organoids and reverse the gradient, previous studies have explored the co-aggregation of micro/nanoparticles during the assembly of early organoid spheres. Using stem cell aggregate fusion techniques, extensive spatial control over morphogen release mediated by microparticles has been achieved in fused aggregates. However, this technique lacks precise and direct spatial or temporal control and is limited to optimizing early organoid assembly, with minimal applicability to mid- and late-stage organoid culture. Therefore, improved methods to initiate and regulate morphogen gradients, as well as novel and broadly applicable morphogen delivery technologies, are required. Afting *et al.*[28] introduced nanostructured DNA microbeads with tunable stiffness that emulate tissue organization to enable spatiotemporal control of morphogen gradients within organoids at any developmental stage. DNA bead technology uses light-sensitive DNA linkers that enable controlled, non-invasive disassembly of the beads when exposed to 405 nm light. These linkers contain photocleavable (PC) groups that break down upon ultraviolet light exposure, causing the DNA beads to lose their fluorescent signal within about 25 – 30 min. This mechanism allows for precise removal of the beads when necessary. Importantly, experiments showed that this light-triggered disassembly does not harm the surrounding cells or organoids. Immunofluorescence staining and confocal microscopy revealed that the presence and breakdown of the DNA beads do not disrupt the normal development or cell-type composition of the organoids, confirming the biocompatibility of the technology. Using retinal organoids (ROs) derived from zebrafish and early embryos, the study demonstrated that DNA microbeads could be integrated into embryos and organoids through microinjection and non-invasively erased using light. This mechanism involves the incorporation of a PC segment at the center of DNA linkers, allowing nearly instantaneous, spatiotemporal disassembly of DNA microbeads upon exposure to 405 nm light (PC-modified DNA microbeads). The light-triggered disassembly of PC-modified DNA microbeads occurs not only in bulk solution but also in injected ROs. Consequently, PC-modified DNA microbeads enable non-invasive removal after tissue integration (Figure 4). By conjugating recombinant Wnt with DNA microbeads, spatiotemporal control over morphogen release at the microinjection site was demonstrated, resulting in the formation of retinal pigment epithelium while preserving neural retina cell types. This study highlights that DNA microbeads, with their cell-sized dimensions and high stiffness adaptability, can be integrated into organoids through microinjection, enabling the non-invasive release of their cargo upon light activation. This technology provides spatial and temporal control in organoid bioengineering, facilitating morphogen delivery from internal sources throughout development. It holds promise to address the demand for implementing morphogen sources in 3D organoid cultures at any stage of development.

3. Breakthrough mechanistic insights based on organoids

Organoids with enhanced functionality have opened new frontiers in exploring disease mechanisms, modeling hereditary disorders, and uncovering developmental processes.[29] By incorporating diverse functional cell types and mimicking complex tissue environments, these advanced organoids provide powerful tools to replicate the pathophysiology of genetic diseases.[30] They enable researchers to identify therapeutic targets by simulating the effects of specific mutations, offering insights into personalized treatments.[31] In addition to disease modeling, functional organoids facilitate the study of developmental mechanisms, revealing critical pathways and cellular dynamics during organ formation.[32] These breakthroughs not only deepen our understanding of human biology but also pave the way for innovative approaches in regenerative medicine and therapeutic discovery.

3.1. Organoids loaded with functional cells for exploring disease mechanisms

Organoids loaded with functional cells for exploring disease mechanisms represent an advanced and innovative approach to biomedical research.[33] By incorporating

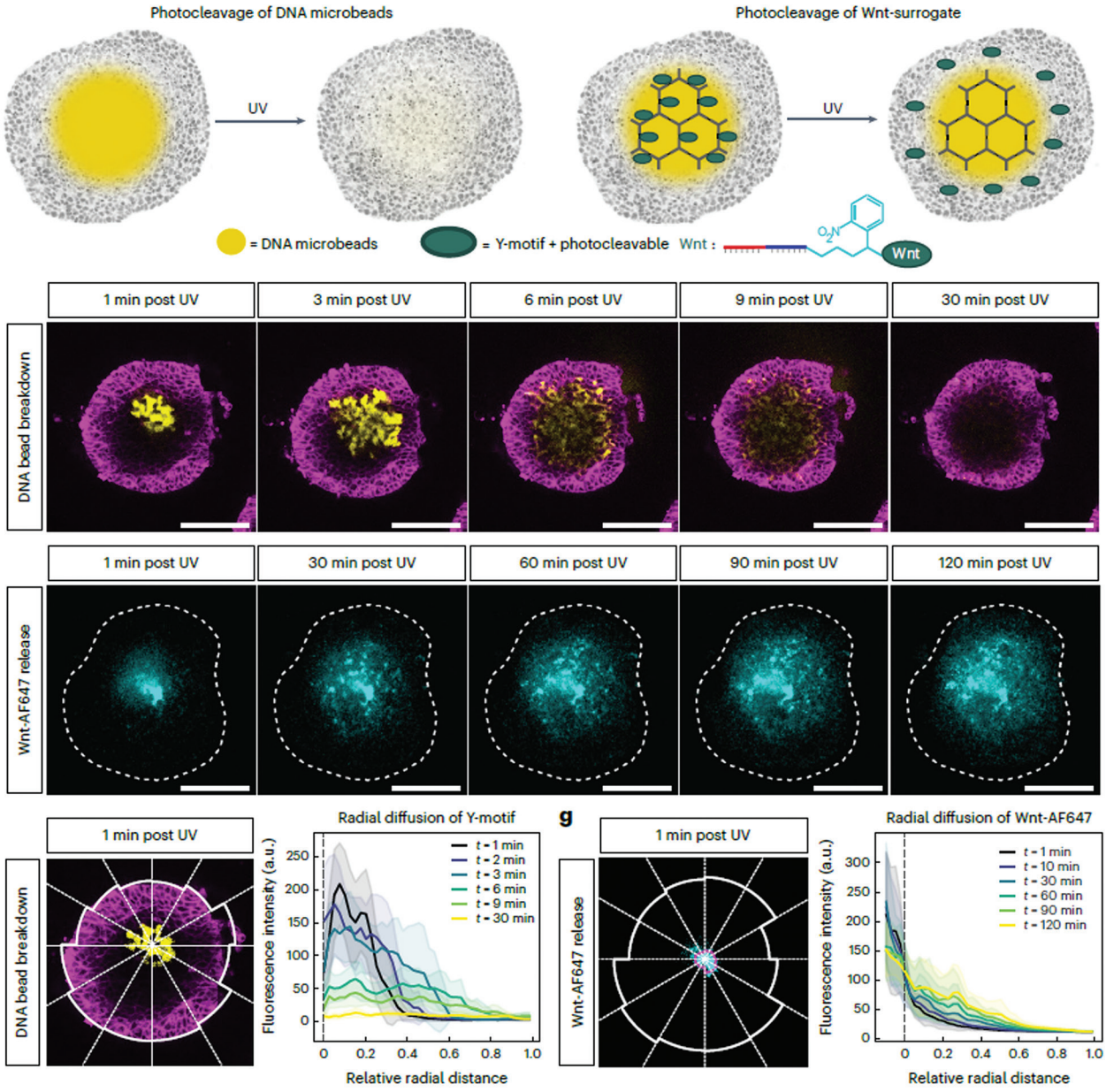

Figure 4. Non-invasive removal of DNA microbeads and local release of Wnt-surrogate in a gradient. Confocal images show the breakdown of PC-modified microbeads upon 405 nm laser illumination, with fluorescence quantification for both PC-modified and non-PC-modified beads. Time-lapse imaging demonstrates the release of Cy3-Y-motif and Wnt-AF647 from microbeads in live small ROs, revealing radial diffusion and gradient formation.[28] Copyright © 2024 The author(s).
Abbreviations: PC: Photocleavable; ROs: Retinal organoids; UV: Ultraviolet.

functional, specialized cells into 3D organoid models, researchers can create more accurate representations of human tissues and organs, allowing for a deeper understanding of how diseases develop at a cellular and molecular level.[2]

Glial cells, particularly astrocytes, are the most abundant cell type in the brain and are responsible for maintaining neuronal health by providing structural and metabolic support, regulating blood flow in the brain, modulating neurotransmission, and actively participating in the repair and protection of neural tissue following injury.[34] However, existing human brain organoid models often lack or inadequately represent astrocytes, limiting their utility in studying neurological diseases, particularly those involving neuroinflammation associated with aging and neurodegenerative disorders such as Alzheimer's disease.[35] To address this gap, Wang *et al.*[36] developed a human brain organoid model enriched with

mature, functional astrocytes using human-induced PSCs (hiPSCs). The team successfully induced astrocyte formation in the organoids by adding specific gliogenic compounds and observed the maturation and functional enhancement of these cells (Figure 5). To better replicate the human brain's microenvironment, they transplanted these organoids into the mouse brain, promoting further astrocyte development. The results showed that the transplanted organoids produced more complex and differentiated astrocyte populations than previous models. This research revealed the significant role of astrocytes in neuroinflammatory processes, particularly in aging and neurodegenerative diseases. Further experiments identified CD38 as a crucial mediator of metabolic and energy stress in these reactive astrocytes, suggesting that CD38 may serve as a potential drug target to mitigate neuroinflammation caused by astrocyte dysfunction. This study provides an advanced *in vitro* model for exploring the role of glial cells, particularly astrocytes, in brain function and disease, offering new insights and experimental platforms for investigating treatments for Alzheimer's disease and other neurological disorders.

3.2. Organoids for investigating therapeutic targets in genetic diseases

By creating organoids derived from patients' own cells or genetically engineered to carry specific mutations, scientists can closely observe how these diseases manifest in human-like tissue.[37] This model is particularly valuable for uncovering previously difficult-to-study genetic pathways and identifying specific biomarkers associated with disease.

Polycystic kidney disease (PKD) is a common genetic disorder characterized by the formation of fluid-filled cysts in the kidneys, leading to kidney failure in many patients.[38] Despite its prevalence, effective treatments for PKD remain limited.[39] In this study, Liu *et al.*[40] used kidney organoid models to investigate new therapeutic strategies for PKD, focusing on the interplay between cilia and autophagy – two critical processes implicated in the

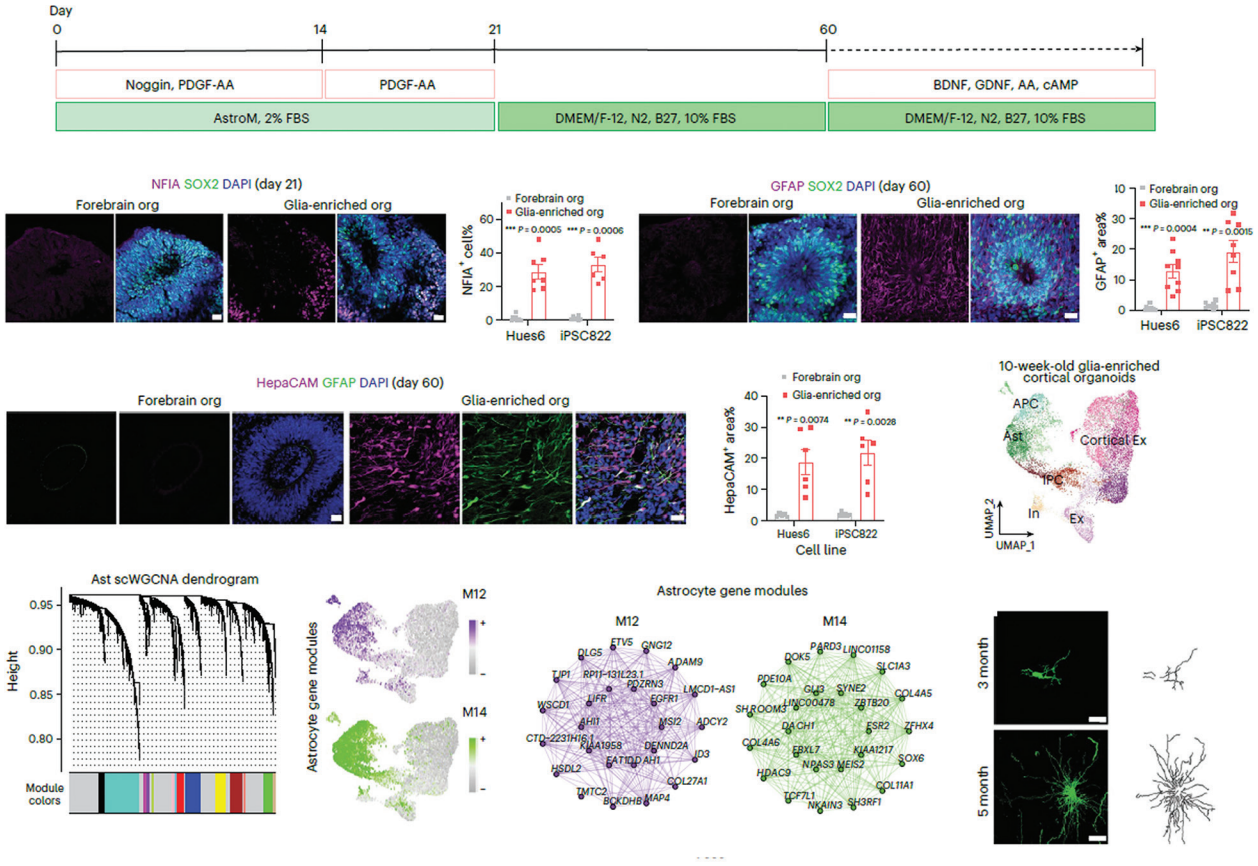

Figure 5. Enhanced astrogliogenesis in glia-enriched cortical organoids. Increased NFIA+ glial progenitors and GFAP+ astrocytes are observed in glia-enriched cortical organoids. UMAP and WGCNA analysis of snRNA-seq data reveal enhanced astrocyte gene expression. Confocal imaging of GFAP::GFP astrocytes shows increased branching in older organoids, confirmed by Sholl analysis and process quantification.[36] Copyright © 2024 The author(s).

Abbreviations: GFAP: Glial fibrillary acidic protein; GFP: Green fluorescent protein; NFIA: Nuclear factor I A; snRNA-seq: Single-nucleus RNA sequencing; UMAP: Uniform manifold approximation and projection; WGCNA: Weighted gene co-expression network analysis.

disease's progression (Figure 6). The team developed both *in vitro* and *in vivo* models using human PSCs genetically engineered to simulate autosomal dominant (ADPKD) and autosomal recessive (ARPKD) forms of the disease. These models displayed key PKD characteristics, such as tubular injury, abnormal activation of the renin-angiotensin-aldosterone system, and spontaneous cyst formation *in vivo*. Through single-cell analysis, the researchers identified significant metabolic disruptions, particularly in autophagy, which played a crucial role in cyst development. The study demonstrated that activating autophagy – either by overexpressing ATG5 (an autophagy regulator) or by disrupting primary cilia – could inhibit cyst formation in both ARPKD and ADPKD organoid models. Furthermore, pharmacological activation of autophagy using minoxidil, an FDA-approved drug, effectively reduced cyst formation *in vivo*, suggesting its potential as a therapeutic option. This research highlights the complex relationship between cilia, autophagy, and metabolism in PKD, positioning the cilium-autophagy metabolic axis as a promising target for new treatments. The organoid models used in this study provide a more accurate reflection of human PKD pathology and offer valuable insights into the disease's mechanisms, potentially accelerating the development of effective therapies for PKD.

3.3. Organoids with immune function

Organoids with immune function represent a major advancement in biomedical research by incorporating immune cells into organoid models to study immune responses in a more realistic and integrated way.[41] Traditionally, organoids have focused on mimicking organ structure and function, but by introducing immune components such as macrophages, dendritic cells, T cells, and B cells, these models can now simulate the complex interactions between the immune system and various tissues.[42] This innovation enhances the ability to investigate immune responses in gut-related diseases

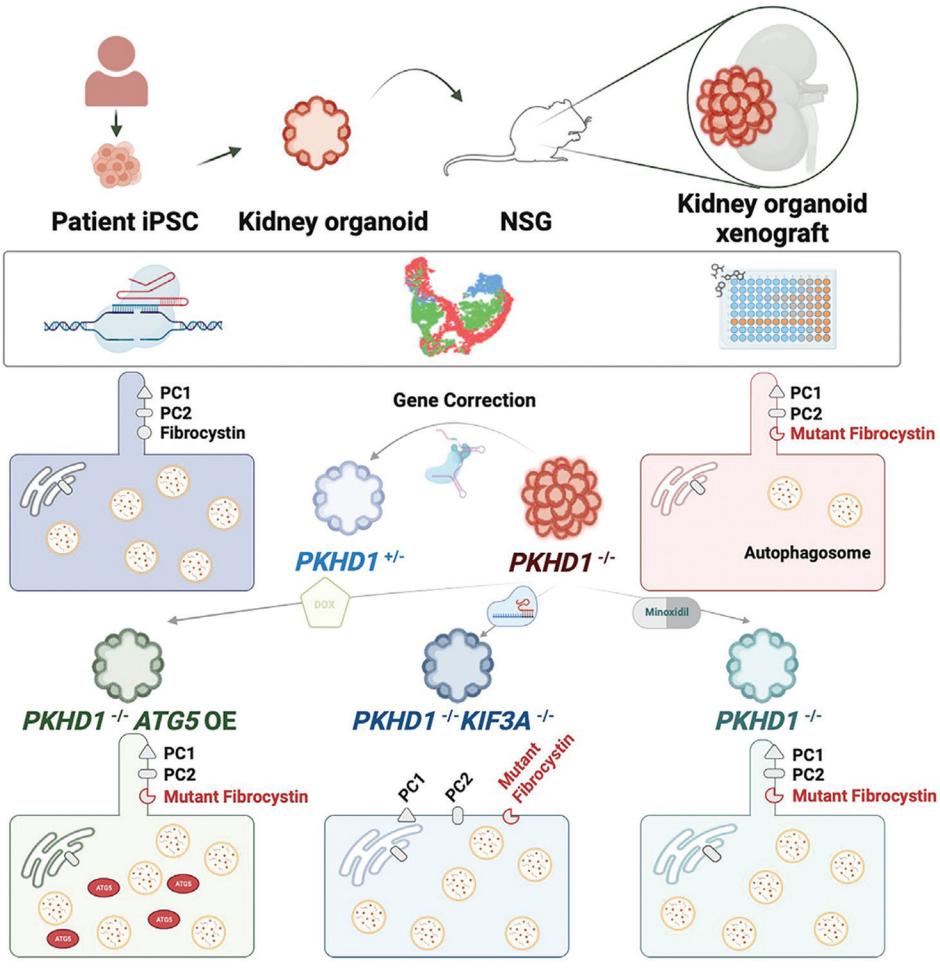

Figure 6. The multiple roles of an organoid xenograft model of PKD replicates key pathophysiological features of human PKD. This *in vivo* model offers a more advanced platform for testing potential drugs and validating genetic pathways for therapeutic targeting.[40] Copyright © 2023 Elsevier Inc. Abbreviations: iPSC: Induced pluripotent stem cell; NSG: NOD.Cg-Prkd^{cscid} IL2rgtm^{1Wjl}/SzJ; PC: Polycystin; PKD: Polycystic kidney disease.

such as inflammatory bowel diseases, where immune cells contribute to chronic inflammation and tissue damage. Furthermore, these immune-competent organoids provide a valuable platform for exploring how pathogens affect the gut, how the immune system responds to infections, and how autoimmune conditions such as celiac disease arise when the immune system mistakenly targets healthy gut tissue.[43] Using patient-specific cells, these models also offer the potential for personalized treatment strategies tailored to individual immune profiles, advancing precision medicine. Overall, gut organoids with resident immune cells provide an essential tool for studying gut immunity and disease mechanisms and for testing therapeutic interventions in a more physiologically relevant context.

The intestinal mucosal immune system is the largest pool of immune cells in the human body and is responsible for maintaining homeostasis between the intestinal barrier and luminal contents. The close interaction between the epithelium and the immune system is essential for maintaining tissue homeostasis, and disruptions in this relationship are associated with autoimmune diseases and cancer.[44] Traditional stem cell-derived organoid models are primarily used to simulate epithelial cell functions but lack tissue-resident immune cells, thus failing to fully capture organ-level physiological and pathological processes.[45] Using an enzyme-free, scaffold-based crawl-out protocol, Recaldin *et al.*[46] isolated large numbers of intestinal immune cells, including tissue-resident memory T cells (TRM) cells, from adult human intestinal tissues. This method retains the tissue-resident characteristics of the cells. Then, they developed human intestinal immuno-organoids (IIOs) by co-culturing intestinal epithelial organoids with autologous TRM cells. These TRM cells integrate into the epithelial layer and continuously monitor the intestinal barrier. Through single-cell RNA sequencing (scRNA-seq), researchers analyzed the transcriptomes of immune and epithelial cells in IIOs, revealing differences in gene expression and function between TRM cells and peripheral blood mononuclear cells (PBMCs) (Figure 7). Compared to PBMCs, TRM cells exhibited higher migratory capacity and ability to integrate with epithelial cells. Within IIOs, they could migrate dynamically and had elongated shapes, resembling the "flossing" behavior observed in mouse intestines. Researchers further used the IIOs to study intestinal inflammation caused by EpCAM-targeting T-cell bispecific antibodies (TCBs) in cancer immunotherapy. The results showed that IIOs could stimulate the inflammatory response observed clinically, characterized by TRM cell-mediated epithelial apoptosis. After TCB treatment, an activated CD8+ T cell population emerged in IIOs, gradually acquiring cytotoxic features. In addition, the CD4+ T cell population showed a trend of shifting from cytokine secretion to cytotoxicity. By inhibiting the Rho pathway, it was found that TRM cell migration and activation could be significantly reduced, thereby alleviating the inflammatory response in IIOs. The IIOs successfully integrated autologous tissue-resident immune cells and could simulate pathological processes such as intestinal inflammation, providing a novel *in vitro* model for studying intestinal immune responses. Inhibiting the Rho pathway could mitigate TRM cell-mediated intestinal inflammation, providing a theoretical basis for developing new immunotherapeutic strategies.

4. Breakthrough disease interventions with organoids

Organoids have ushered in transformative applications across multiple fields, particularly in transplantation therapy and drug discovery.[47] In the realm of transplantation, organoids have shown groundbreaking potential in treating hard tissue disorders, such as regenerating bone or cartilage, where their ability to mimic structural and functional properties of native tissues has led to significant advancements in regenerative medicine.[48] These developments offer new hope for conditions previously deemed untreatable. In drug discovery, organoids have revolutionized high-throughput drug screening and toxicity testing.[49] Their ability to closely replicate human tissue responses has enhanced the accuracy of identifying effective compounds while reducing false positives.[50] AI-assisted platforms integrated with organoid-based models have further accelerated drug screening, enabling the rapid analysis of large datasets to identify optimal therapeutic candidates with reduced side effects.[51] In addition, organoids have been pivotal in recognizing disease-causing factors, including environmental toxins or pathogenic compounds, offering critical insights for preventative and therapeutic strategies.[52] These breakthroughs not only expand the possibilities for personalized medicine but also redefine how diseases are treated and managed, positioning organoids as essential tools in modern biomedical research.[53]

4.1. Bone organoids for hard tissue repair

The skeleton forms a robust structure capable of bearing weight and supporting the body by combining rigid inorganic minerals with an organic matrix that provides flexibility. Bone organoids are a distinct type of organoid technology focused on simulating bone tissue *in vitro*.[54] Multiple research teams have successfully developed various types of bone organoids aimed at promoting bone tissue repair and regeneration. For example, a study utilized microfluidic technology to create a "bone marrow-on-a-chip" device capable of culturing live bone marrow *in vitro,* recreating a functional hematopoietic microenvironment.[55] In 2021, Akiva *et al.*[56] employed silk fibroin as a scaffold material, cultivating human bone marrow mesenchymal stem cells *in vitro* to differentiate into functional, 3D, self-organizing osteoblasts and osteocytes, and successfully

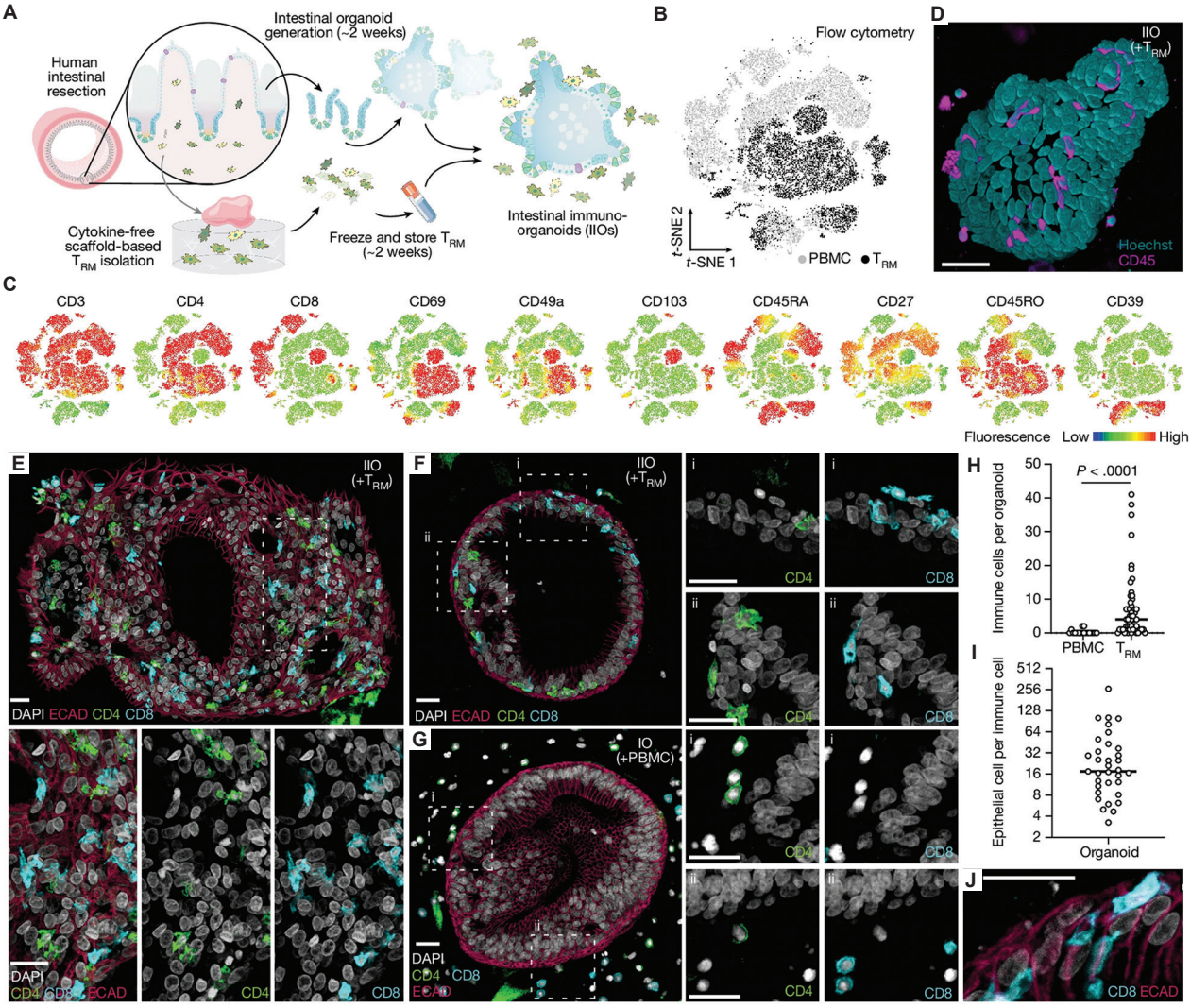

Figure 7. Integration of intestine-derived TRM cells into autologous organoids. t-SNE analysis differentiates gut-derived TRM cells from PBMC T-cells. Fluorescent imaging shows TRM cells integrating within organoids, with PBMCs surrounding them. mIF staining reveals TRM cell incorporation into larger and smaller organoids. Immune cell counts and epithelial-to-immune cell ratios are quantified.[46] Copyright © 2024 The author(s).
Abbreviations: mIF: Multiplex immunofluorescence; PBMC: Peripheral blood mononuclear cell; TRM: Tissue-resident memory T cell; t-SNE: t-distributed stochastic neighbor embedding.

developed bone-weaving organoids that replicate the mineralization process of bone tissue *in vitro*. In 2022, Xie *et al.*[57] constructed bone callus organoids using hydrogel microspheres as stem cell carriers, a novel method that mimics the process of endochondral ossification during bone development. A study demonstrated that 3D multilineage bone marrow organoids generated from hiPSCs could model healthy and perturbed hematopoiesis in an expandable 3D microenvironment, supporting the growth of immortalized cell lines and primary cells and providing a robust *in vitro* model for the study of blood and bone marrow diseases.[58,59] At present, bone organoids are limited in size, reaching only the micron scale, and their cellular composition is simplified compared to natural bone tissue. These organoids also exhibit differences in the ECM components, further distinguishing them from native bone. In addition, their functionality is restricted, as they can only simulate a small portion of the complex functions of bone. Furthermore, bone organoids lack proper spatial characterization and fail to replicate the microstructural organization of bone, limiting their ability to fully mimic both the structure and function of natural bone tissue.

In 2024, Ren *et al.*[60] pioneered the development of an innovative bioink for bone tissue engineering using a "one-pot method." This bioink was utilized to 3D-bioprint bone scaffolds, resulting in the creation of a large-scale bone organoid (Figure 8). This organoid is capable of prolonged *in vitro* and *in vivo* culture, multi-cell differentiation, and self-mineralization and has been

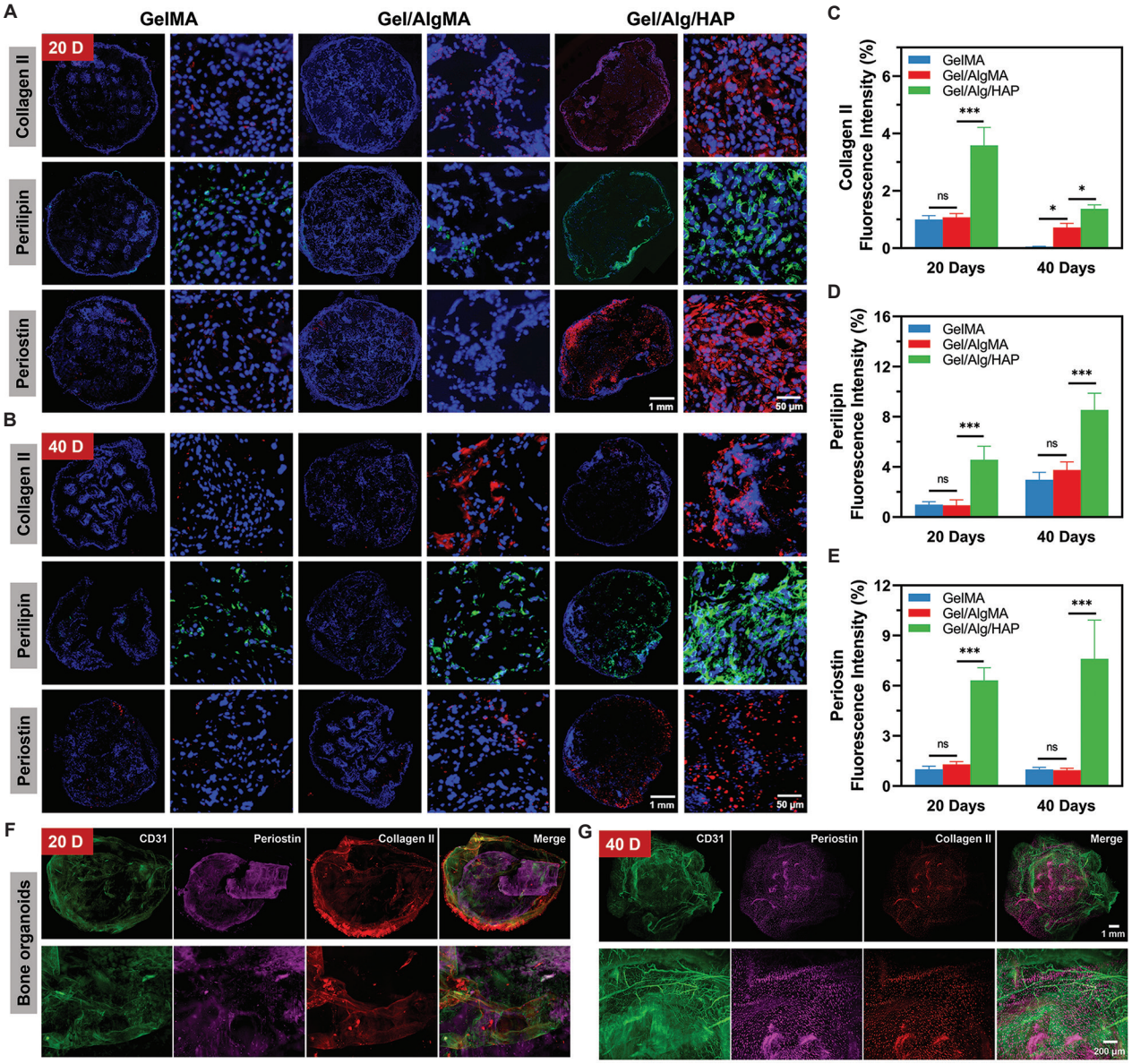

Figure 8. *In vivo* formation and multicellular differentiation of bioprinted bone organoids, highlighted by immunofluorescence staining and 3D imaging for collagen II, perilipin, and periostin after 20 and 40 days, demonstrating tissue-specific differentiation and maturation.[61]
Abbreviations: AlgMA: Alginate methacryloyl; GelMA: Gelatin methacryloyl; HAP: Hydroxyapatite.

applied to the regeneration of large bone defects.[61] To achieve this, the team designed a novel bioink specifically tailored for bone tissue engineering and utilized advanced digital light processing bioprinting technology to precisely replicate the complex microstructures characteristic of bone tissue.[62] The printed scaffolds were cultured under directed conditions for extended periods, enabling the differentiation of various bone marrow cell types, including hematopoietic cells, immune cells, endothelial cells, chondrocytes, osteoblasts, adipocytes, and osteoclasts. The resulting bone organoid exhibited favorable mechanical properties, with Young's modulus in the megapascals range, akin to that of trabecular bone. It also displayed a bone-like spatial topology with porous micro-nano structures and achieved centimeter-scale dimensions. When applied to a bone defect model, the organoid demonstrated remarkable regenerative capabilities, underscoring its potential for therapeutic applications.

4.2. Organoids for drug toxicity assessment

Organoids offer a groundbreaking method for assessing drug toxicity by providing a highly accurate, human-like model that takes individual susceptibility into account.[63] Unlike traditional 2D cell cultures or animal models, organoids are 3D structures that replicate the complexity of human organs, allowing researchers to study how drugs

affect human tissues with a level of precision that reflects individual variations in drug response.[64] This innovation enables the detection of toxic reactions and potential side effects much earlier in the drug development process, highlighting how different individuals may experience varying degrees of toxicity.[65] By simulating real human organ systems, organoids can identify harmful effects based on unique genetic or environmental factors, improving the personalization of drug safety evaluations.[66] This approach not only enhances the precision of toxicity predictions but also allows for more targeted screening of drug candidates to match individual susceptibility, reducing the risk of adverse effects in clinical trials.

Modeling how individual variation in the human brain affects disease susceptibility has long been a challenging task. Different human PSCs lines exhibit inconsistent performance in *in vitro* models, which may be related to differences in reprogramming, epigenetic imprinting, or sensitivity to culture conditions.[67] Genetic variation plays a crucial role in differential susceptibility to disease triggers. However, although individual susceptibility can be detected in *in vitro* systems, exploring its underlying mechanisms has been hindered by the limited availability of experimental models.[68] In July 2024, Antón-Bolaños *et al.*[69] proposed a highly reproducible multidonor human cortical organoid model called Chimeroids. This model is generated by the codevelopment of cells from individual donors within a single organoid, achieved through the reaggregation of cells from multiple single-donor organoids at the neural stem or progenitor cell stage, resulting in a chimeric structure. The team used Chimeroids to investigate individual differences in susceptibility to neurotoxic drugs, such as ethanol and the anticonvulsant valproic acid, which exhibit significant clinical phenotypic variability. Through techniques such as single-cell RNA sequencing and spatial transcriptomics, the results indicated that the human genetic background may be an important mediator of neurotoxin susceptibility. Furthermore, the chimeric model provides a scalable system for high-throughput research into inter-individual differences in brain development and disease processes (Figure 9). The ability to perform large-scale drug response assays using Chimeroids holds the potential for clinically stratifying patients into different treatment response groups based on data-driven insights. Over time, the accumulation of large datasets may propel the development of universal models for predicting drug efficacy before clinical trials.

Despite the immense potential of AI algorithms in drug screening and improvements in efficiency, we currently lack direct, published validation studies with the specific quantitative data required to support these claims. Although AI-assisted methods are widely believed to enhance efficiency, reduce costs, and accelerate drug discovery, specific, data-driven comparisons with traditional methods remain an area that needs further investigation and validation in the literature. Future work will be essential to provide empirical evidence that quantifies these potential efficiency gains.

4.3. AI-assisted organoids for drug screening

AI-assisted organoids for drug screening highlight the transformative role of AI in revolutionizing drug discovery.[70] By combining advanced AI algorithms with human-like 3D organoid models, this approach accelerates drug testing and provides more accurate, personalized results.[71] Organoids replicate the complexity of human organs, offering a superior platform for drug testing compared to traditional cell cultures.[72] AI enhances this process by analyzing vast amounts of data generated from organoid experiments, quickly identifying patterns, and predicting how different drugs interact with human tissues.[73] This not only speeds up the screening of potential drug candidates but also improves the precision of predictions, helping researchers select the most promising therapies.[74] Through AI's ability to process and learn from complex data, this approach minimizes trial and error, reduces the need for animal testing, and enables more effective, targeted drug development.[75] AI is the key to unlocking faster and more accurate drug discovery, making it a game-changer in the pharmaceutical industry.[76]

Venous malformation (VM) is the most common type of vascular malformation, with an incidence rate of 1 – 2/10,000 and a prevalence of 1%.[77] Pan *et al.*[78] developed a novel approach for constructing VM disease models and screening therapeutic drugs using induced PSC (iPSC) technology. The team manipulated cell cycle dynamics and employed the retinoic acid signaling pathway to generate induced venous endothelial cells (iVECs). By introducing the L914F mutation of the *TIE2* gene into the iPSC locus, they found that the mutated iVECs were able to recapitulate the phenotypic features of VM after both *in vitro* and *in vivo* transplantation. These features included vessel dilation, abnormal smooth muscle cell coverage, increased cell proliferation, and enhanced anti-apoptotic ability. Transcriptomic and proteomic analyses revealed potential pathological mechanisms associated with VM. In addition, using AI-based deep learning prediction systems (DLEPS) and digital RNA sequencing (DRUG-seq) technologies for drug screening, the team identified bosutinib as a potential therapeutic agent. Bosutinib was found to reverse the VM phenotype by inhibiting endothelial-to-mesenchymal transition (EndoMT), restoring cell function, and alleviating VM symptoms. The iPSC-derived VM model and drug screening approach established in this study offer a groundbreaking strategy for therapeutic research in vascular malformations (Figure 10).

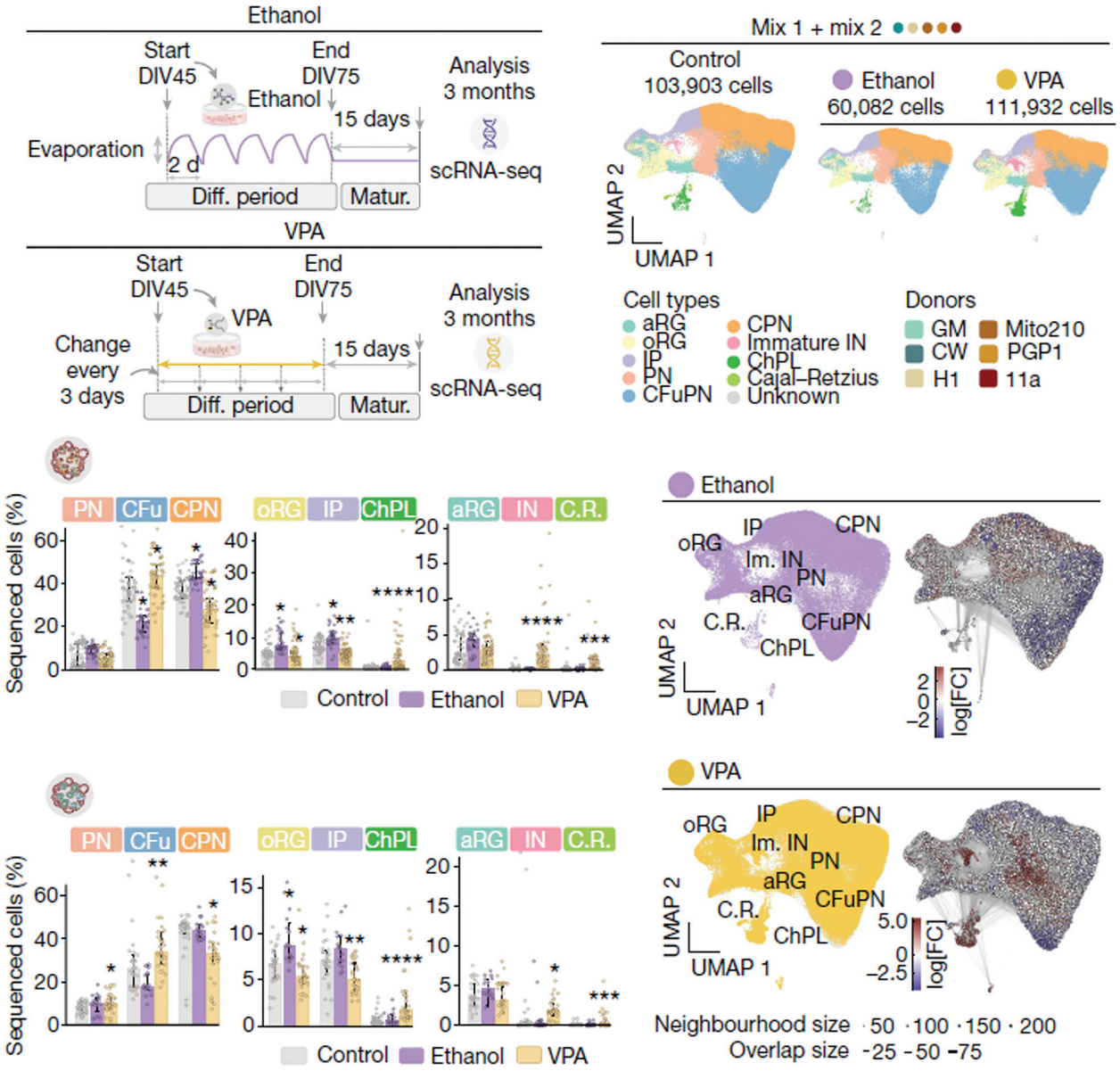

Figure 9. Treatment-specific changes in NSC-Chimeroids. UMAPs show cell type distribution in control and treated Chimeroids. Proportions of MD-NSC and SD-NSC-Chimeroids are compared across ethanol and VPA treatments. Neighborhood shifts in response to treatments are shown in UMAPs and beeswarm plots.[69] Copyright © 2024 The author(s).

Abbreviations: ND: Neurodevelopment; NSC: Neural stem cells; scRNA-seq: Single-cell RNA sequencing; SD: Standard deviation; UMAPs: Uniform manifold approximation and projection; VPA: Valproic acid.

4.4. Organoid models for the identification of carcinogenic factors

Organoid models have become a crucial tool in identifying carcinogenic factors, offering a more accurate and physiologically relevant system for studying cancer development.[79] Researchers can expose organoid models derived from patient tissues or genetically engineered cells to various carcinogenic agents such as chemicals, radiation, or viruses and monitor cellular changes indicative of cancer, including abnormal cell proliferation, genetic mutations, and altered signaling pathways.[80] These models allow for a detailed understanding of how different carcinogens affect specific organs, such as the lung, liver, or colon. Furthermore, organoid models can be used to identify early-stage cancer biomarkers and screen for chemopreventive agents or targeted therapies, making them valuable tools for cancer prevention, early detection, and drug discovery.[81] Ultimately, organoids not only enhance our understanding of carcinogenesis but also provide a more effective platform for developing personalized cancer therapies.[82]

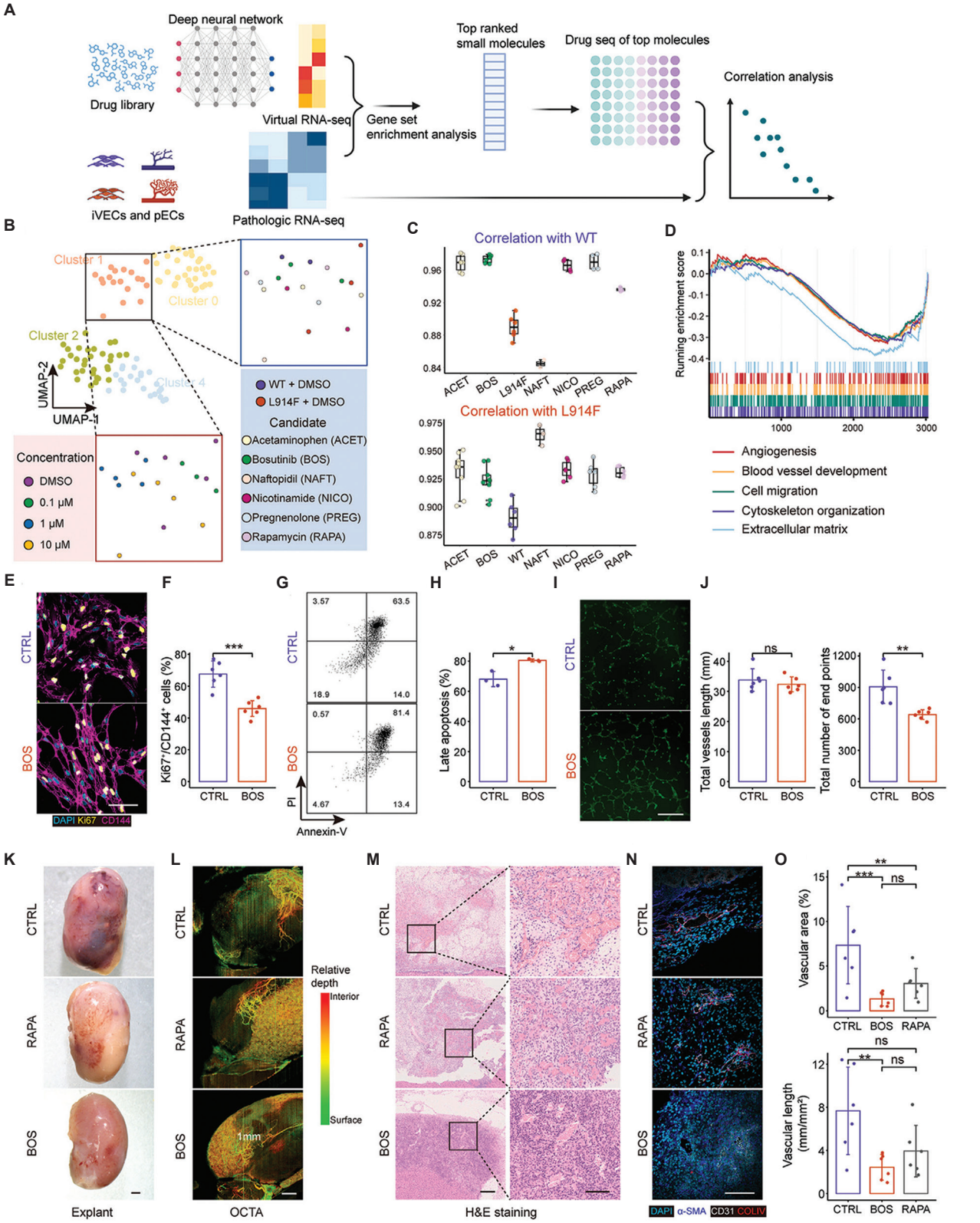

Figure 10. AI-supported discovery of bosutinib for alleviating venous malformations (VMs), showcasing drug prediction and screening workflow, transcriptomic and functional analyses of L914F-iVECs, immunofluorescence, flow cytometry, tube formation assays, *in vivo* vascular imaging, and histological and quantitative evaluation of vascular structure and function under various treatments.[78] Copyright © 2024 The author(s).
Abbreviations: iVECs: Induced venous endothelial cells; pECs: Primary endothelial cells; RNA-seq: RNA sequencing.

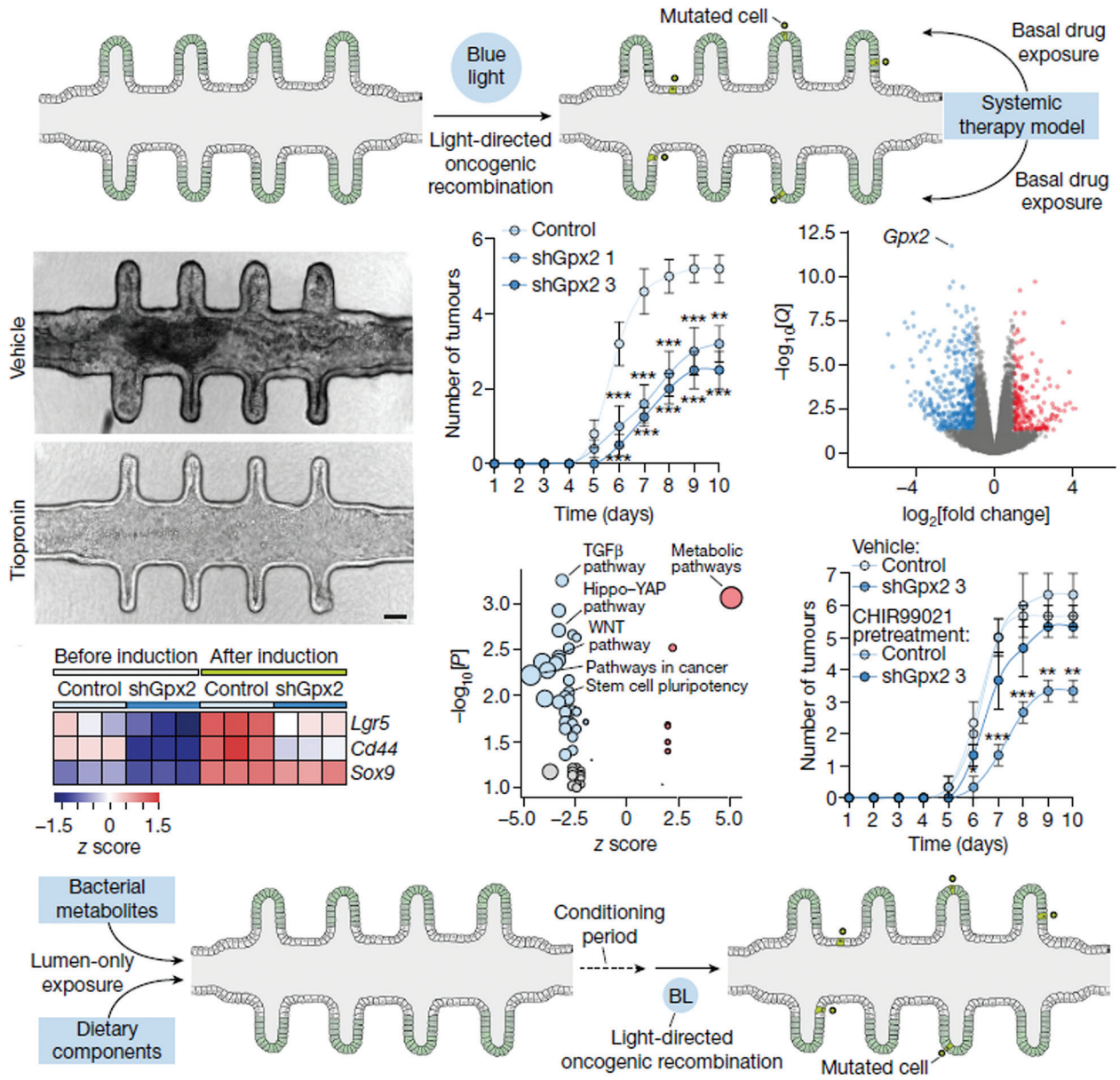

Figure 11. Mini-colons for screening tumorigenic factors. Experimental workflows for systemic therapy and microbiota/dietary pattern modeling are shown. Bright-field images of mini-colons treated with vehicle or tiopronin, and tumor multiplicity in different genotypes and pretreatments are quantified. Gene expression changes and enriched functional terms after Gpx2 knockdown in AKP tumor cells are presented. Tumor multiplicity following metabolite treatments is also quantified.[85] Copyright © 2024 The author(s).
Abbreviation: AKP: *Apc*$^{fl/fl}$*Kras*$^{LSL\text{-}G12D/+}$*Trp53*$^{fl/fl}$.

Traditional 2D and 3D *in vitro* cell culture models, while capable of mimicking certain simple cancer cell behaviors, have limitations in simulating complex tissue structures and the process of cancer development.[83] Current organoid models, although considered a bridge between *in vitro* and *in vivo* models, are limited by their closed structure, short lifespan, lack of stable tissue architecture, and inability to generate hybrid tissues composed of both healthy and cancer cells.[84] These limitations mean that cancer research still relies heavily on animal models, which cannot provide high-resolution analyses of cellular dynamics in terms of temporal and spatial resolution and are also costly in terms of resources. Lorenzo-Martín *et al.*[85] developed a "mini-colon" that can undergo tumorigenesis *in vitro* by integrating microfabrication, optogenetics, and tissue engineering approaches (Figure 11). Using blue light exposure to control oncogene activation, they achieved spatiotemporal control of tumorigenesis and were able to track tumor development at single-cell resolution in real-time without disrupting the culture. Compared to

conventional organoid cultures, tumors in the mini-colon developed more complex structures, evolving from polyp-like formations to fully developed tumors. Through single-cell transcriptomic analysis, researchers discovered that tumor cells in the mini-colon exhibited high heterogeneity, including differences in proliferation, stem cell properties, and differentiation markers. Moreover, they found that different tumor clones had distinct transcriptomic profiles, which correlated with subtypes of human colorectal cancer. Using this mini-colon model, they further investigated the effects of gut microbiota metabolites and dietary patterns on tumorigenesis. It was found that specific metabolites, such as deoxycholate, promoted tumor development, whereas others, such as butyrate, had inhibitory effects. In addition, calorie-restricted dietary patterns reduced tumor burden. Mini-colon offers a powerful tool for cancer research and helps reduce the reliance on animal models. The researchers pointed out that the model is not only applicable to the study of colorectal cancer but could also be extended to the research of other epithelial cancers, such as lung, breast, or prostate cancer.

5. Conclusion and perspectives

The year 2024 saw remarkable breakthroughs in organoid technology, which contributed to significant advancements in biomedical research, particularly in the fields of disease modeling, drug discovery, and regenerative medicine. The development of organoids with anatomical region specificity, such as brain organoids mimicking distinct brain regions, and the introduction of AF-based organoids for prenatal diagnostics have opened new doors for understanding complex diseases and disorders. In addition, the integration of cutting-edge technologies such as CRISPR/Cas9, single-cell RNA sequencing, and AI has enhanced the precision and efficiency of organoid research, allowing for more accurate disease modeling and personalized therapeutic strategies. Organoids have demonstrated their potential in a variety of applications, including identifying carcinogenic factors, uncovering therapeutic targets for genetic diseases, and advancing regenerative medicine. These advances have led to the creation of organoids that better replicate human organ systems, offering unprecedented opportunities for high-throughput drug screening, understanding disease mechanisms at the cellular level, and testing therapeutic interventions in a more relevant human model. The ability to utilize AI for data analysis and drug screening, combined with advancements in multiorgan models and organ-on-a-chip platforms, further solidifies the role of organoids in reshaping personalized medicine and therapeutic discovery. Despite these advancements, challenges remain, particularly in scalability, reproducibility, and the need for improved vascularization in organoid models. Recent research has shown promising advances in addressing this issue, including the 3D bioprinting of vascular networks and the co-culture of endothelial cells with organoids.[86] These approaches have demonstrated success in creating vascularized organoids, with 3D bioprinting enabling the formation of functional vascular networks that mimic natural blood vessels. Co-culturing endothelial cells with organoid cultures has also facilitated the development of blood vessel-like structures, improving nutrient and oxygen supply within the organoid. Biomimetic scaffold design appears to hold significant potential. The development of scaffolds that more accurately mimic the ECM and native tissue environment could promote the efficient growth of vascular structures within organoids. Advances in materials such as hydrogels and bioinks for 3D bioprinting are expected to further enhance these strategies, supporting the growth and integration of endothelial cells with other cell types within organoids. In addition, ongoing research and the development of new techniques, such as DNA microbeads for spatiotemporal regulation and bioengineering of functional tissues, will continue to address these limitations and pave the way for more complex and clinically relevant models. As these innovations mature, organoid technologies hold great promise for accelerating the development of novel treatments, improving patient outcomes, and providing more precise, personalized approaches to medicine.

Looking ahead, the future of organoid research will likely involve further integration with emerging technologies, such as AI-driven drug discovery platforms, advanced biomaterials for enhanced tissue complexity, and multiorgan models that simulate the intricate interactions between different organs. With continued progress in these areas, organoids have the potential to become indispensable tools in both pre-clinical and clinical settings, revolutionizing how we study disease, develop drugs, and create personalized therapies for a wide range of conditions.

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Conflict of interest

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ACCSCIENCE PUBLISHING

OR

REVIEW ARTICLE

Application of cancer organoids: The forefront of personalized oncology and preclinical testing

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Abstract

Cancer remains one of the most pressing medical problems in the world. Recent years have seen a gradual rise in utilization of "organoids," a novel *in vitro* three-dimensional culture technology, in cancer research. Organoids are multicellular structures derived from human stem cells, and cancer organoids can replicate the characteristics, morphology, and functionality of the original tumor in the human body. At present, organoid technology has been widely used in various oncologic contexts, including colorectal, liver, lung, pancreatic, and breast cancers, providing considerable assistance in patient-specific drug testing, precision medicine, and the development of personalized medical strategies. Therefore, this preclinical model contributes to significantly accelerating the translation from basic cancer research to clinical therapeutics. This review discusses the preparation of cancer organoids and their recent progress in multiple cancer research fields. Finally, the challenges of organoid technology in current clinical practice and future development prospects are discussed.

Keywords: Tumor organoids; Cancer; Precision medicine; Preclinical model; Personalized oncology

1. Introduction

Cancer poses a formidable challenge to global healthcare systems in the United States and worldwide.[1,2] As of 2024, the American Cancer Society estimates approximately 2 million new cancer cases and 610,000 deaths in the U.S., with lung, breast, prostate, and colorectal cancers (CRCs) being the top causes of death.[2] In addition, according to the World Health Organization's statistics on cancer incidence/mortality in 185 countries or regions in 2022, the number of new cancer cases globally is predicted to exceed 35 million by 2050, a 77% rise over the current number of cases.[1] Cancer continues to threaten the health and social development of the population, according to these statistics. Therefore, investigating tumor pathogenesis, identifying potential biomarkers, and enhancing treatment outcomes should be the main goals of cancer research.

To date, treatment models used in cancer research include cancer cell lines for 2D cell culture, murine models, and primary patient-derived xenografts (PDXs).[3-7] Traditional tumor models have significantly advanced cancer research. However, many factors have limited the experimental outcomes of these models, and they have not shown satisfactory results after entering the clinical trials.[8,9] For example, since cancer cell lines grow adherently in culture medium, this growth modality fails to effectively simulate the 3D structure of tumors and the corresponding microenvironment within the human body. In addition, during numerous passaging *in vitro*, these tumor cells are prone to unpredictable gene mutations or genetic drift, resulting in the inability to replicate the genetic heterogeneity of the original tumors.[5,10,11] As mice grow, tumor cells in PDX models may undergo genetic drift

and specific tumor evolution.[6,7,12-14] Thus, neither cancer cell lines grown in culture media nor tumor cells grown in mouse models can accurately and perfectly replicate and simulate the diversity and complexity of tumors in the human body (Figure 1).

Organoids are three-dimensional (3D) miniaturized *in vitro* organic models derived from human stem cells, organ-specific progenitor cells, or disassociated tumor tissues developed in a specific 3D culture system.[15] Cancer organoids possess self-renewal and self-proliferation capacities *in vitro* and can simulate the structure and function of primary tissues.[15,16] Its advantage lies in its ability to retain histopathological characteristics, genetic characteristics, mutations, and even response to drug treatment.[17] Therefore, organoids are an excellent tool for studying tumorigenesis and cancer progression *in vitro* and show outstanding research potential in clinical applications. As organoids can accurately maintain the genetic diversity and phenotypic heterogeneity of tumors *in vivo*, they provide powerful experimental assistance in simulating tumor development, predicting drug sensitivity *in vivo*, evaluating drug efficacy and toxicity, and personalized treatment.[17,18] Until recently, organoids for multiple cancer types have been successfully established, including colorectal, liver, pancreatic, breast, and gastric cancers.[16] In this review, we summarize and outline the latest developments in the application of organoid technology and clinical cancer research.

2. An overview of organoids

The development of cancer organoid culture technology has become one of the key breakthroughs in the field of cancer research. Organoids can be obtained from embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), and adult stem cells (ASCs).[19] Among them, ESCs can differentiate into all cell types, while ASCs have the ability to differentiate into specific organs, maintain homeostasis and regenerate.[20] These stem cells provide a diverse starting point for the development of organoids. However, there is currently no standardized experimental procedure for the establishment of cancer organoids. In brief, the experimental procedure mainly includes selecting the optimal tumor sample from the tumor's edge with the lowest necrosis rate, dissociating the tumor sample into cell clusters or single cells, and then cultivating it in the hydrogel containing extracellular matrix components under appropriate culture conditions (typically Matrigel).[19,20] Sample processing methods consist of mechanical dissociation and chemical

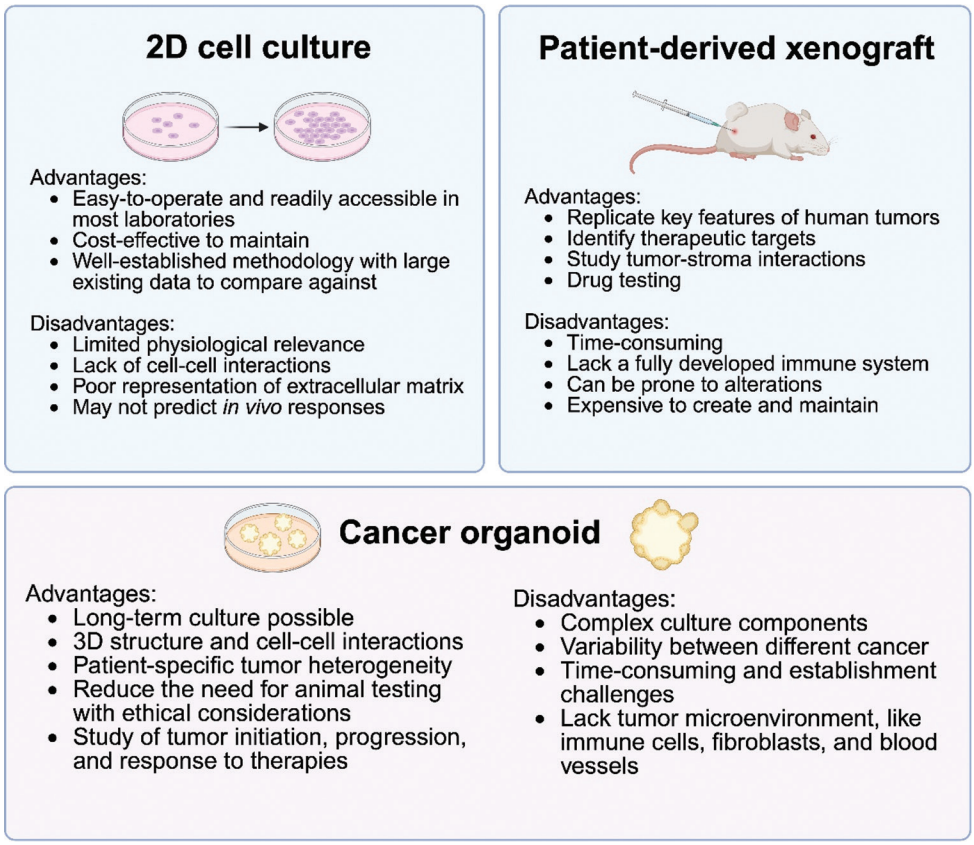

Figure 1. Comparison of the advantages and disadvantages of preclinical cancer models, including immortalized cell lines, patient-derived xenografts, and organoids. Created with BioRender. Cao, K. (2025) https://BioRender.com/48hmj45.

digestion.[15] During the culture process, the 3D culture system of organoids can achieve stable expansion, and the passage is carried out every 1 – 2 weeks.[15] The composition of the culture medium varies depending on the tissue origin. Growth factors and inhibitors are necessary to facilitate the growth and differentiation of organoid tissue[19] (Figure 2). Patient-derived organoids (PDO) can retain patient-specific morphological characteristics and cellular structure, and their histological characteristics resemble the microscopic characteristics of patient tissues and tumor subtypes.[21] Furthermore, depending on the degree of necrosis and viability of the original tumor tissue, the development rate and volume size of different organoids present considerable variations.[21] This characteristic suggests that cancer organoids can accurately represent the original tumor's heterogeneity, offering a valuable platform for researching the biological characteristics and pharmacological responses of tumors unique to individual patients. Through cancer organoid culture technology, researchers can explore the biological mechanisms of cancer more deeply while providing a reliable experimental basis for preclinical drug screening and the development of personalized treatment options.[21]

With the development of cancer organoids development technology, cancer organoids are gradually being used in various cancer fields (Figure 3). Cancer organoids are primarily used in preclinical drug screening and personalized medicine to simulate patients' initial drug response.[22] At present, the most pertinent research direction is large-scale drug screening based on cancer organoids derived from multiple patients.[22] Organoid research aids in the analysis of drug sensitivity and provides a potential basis for optimizing personalized treatment strategies.[23] In high-throughput drug screening, cancer organoids have shown broad applicability, which can be applied to multiple tumor types. They are instrumental in target identification, demonstrating great potential in discovering effective drugs against rare cancer types.[23] In terms of chemotherapy research, cancer organoids provide a more accurate evaluation of the direct impact of chemotherapy on cancer cells.[20] This property provides the basis for improving existing chemotherapy regimens and designing new drugs. In addition, in the field of immunotherapy, organoid models help reveal potential mechanisms for complex interactions between tumors and immune cells.[24] For example, organoid models can be applied to evaluate tumor responses to immune checkpoint blockade (ICB) therapy, yielding important insights for improving immunotherapy.[25] At the same time, organoid immunoassay provides an experimental platform for the development of combination treatment strategies for multiple ICB therapies. It provides a scientific basis for the joint application of ICB with targeted therapies, including MEK or BRAF inhibitors.[25] In terms of genomic screening, 3D organoid culture provides a more accurate representation of oncogenes and tumor suppressor genes in genomic screening than traditional two-dimensional cell culture.[26] 3D organoids closely simulate the tumor xenograft environment, thereby more genuinely reflecting the complex biological characteristics of tumors.[26] This feature gives organoid

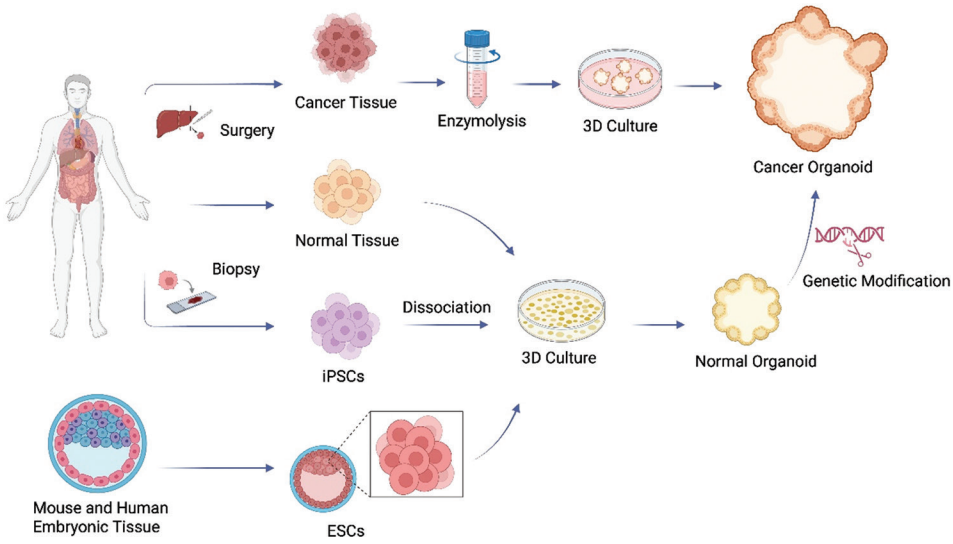

Figure 2. The process of cancer organoid formation and culture. Cancer organoids are usually derived from patient tumor biopsies and surgical resections. Enzymatic digestion is performed using enzymes to break down the extracellular matrix (ECM). The isolated cancer cells or tumor fragments are mixed with an ECM. The mixture is plated in droplets or spread in culture wells to allow the cells to self-organize into 3D organoids. Created with BioRender. Cao, K. (2025) https:// BioRender.com/lfsqfti.
Abbreviations: ESCs: Embryonic stem cells; iPSCs: Induced pluripotent stem cells.

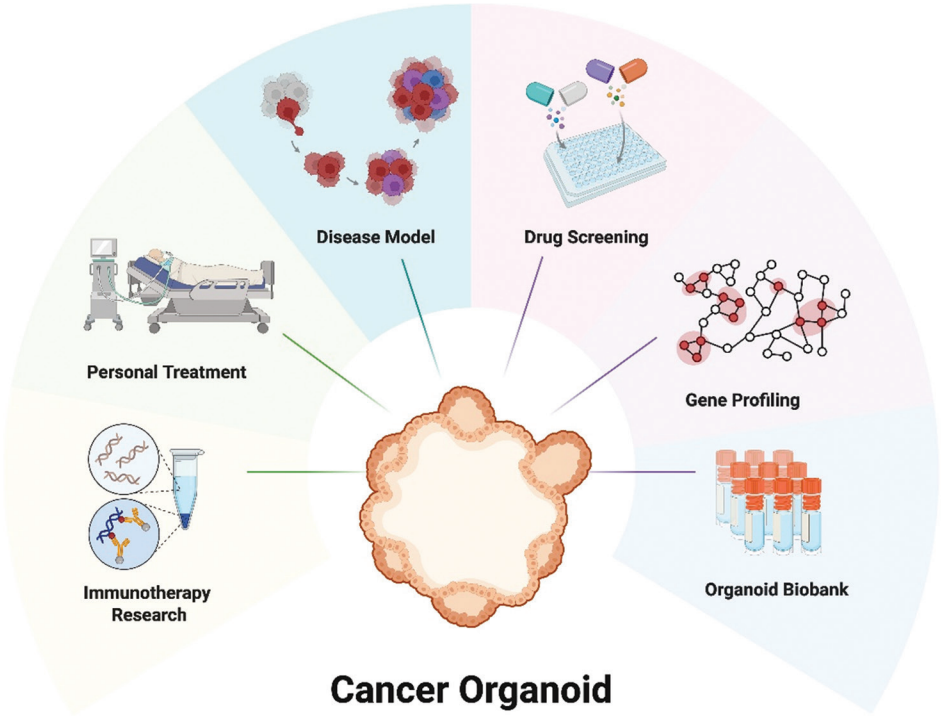

Figure 3. Potential applications of cancer organoids to improve treatment prediction and clinical applicability. Cancer organoid models provide unlimited possibilities for high-throughput drug screening, disease modeling, personalized therapy, gene editing, immunotherapy, and so on. Created with BioRender. Cao, K. (2025) https://BioRender.com/gv6xwxf.

models unique value in studying the mechanism of action of oncogenes during tumor evolution. In addition, the tumor microbiome affects the occurrence and development of tumors at multiple levels. Organoid models have been widely used to study the mechanism of inflammatory responses and pathogen interactions.[27] For example, after injecting *Helicobacter pylori* into gastric organoids, researchers observed its ability to induce the release of interleukin (IL)-8 and other inflammatory cytokines.[27] This simulation allows researchers to investigate the complex causal relationship between microorganisms and cancer. To sum up, cancer organoids serve as a significant research tool, offering extensive opportunities across various fields, including chemotherapy, immunotherapy, genomics, and microbiome studies, thereby advancing the prospects of personalized medicine.

3. Applications of organoids in cancer research

Our current understanding of tumor pathogenesis and treatment response remains superficial and limited, mainly due to the heterogeneity of tumors and the complexity of gene mutations. With the development and improvement of organoid technology, tumor organoid models have been widely used in biomedical research, especially cancer research (Table 1).

3.1. CRC

Organoids are usually extracted from normal human epithelial cells, harboring potential for gene mutations in various stages of cancer *in vitro*.[28] Therefore, for CRC, organoids can be routinely obtained from cancer tissue at different clinical stages. For early malignancies, PDOs can be utilized to identify molecular changes that may serve as biomarkers and prevention targets.[28] Matano *et al.*[29] employed human intestinal organoids as a platform to simulate CRC using CRISPR-Cas9 genome editing technology. Researchers employed the CRISPR-Cas9 technology to introduce mutations in key genes associated with CRC, such as *APC*, *TP53*, *KRAS*, *SMAD4*, and *PIK3CA*.[29] These mutations were introduced in sequence, replicating the progressive genetic alterations observed in human CRC, thus successfully replicating the progression from normal epithelium to advanced malignant tumors in the organoid system.[29] This study provides a controllable, scalable model for CRC biology to study the interactions between specific gene mutations and CRC development. Ooft *et al.*[30] conducted a prospective clinical study based on PDO from CRC metastatic tumors to assess whether PDO could effectively simulate a patient's specific response to chemotherapy. Conventional chemotherapy drugs (*e.g.*, 5-fluorouracil, irinotecan, oxaliplatin) were utilized for PDO. The results demonstrated that the PDO test accurately

Table 1. Overview of the application of different cancer organoids

Year	Cancer organoid	Origin	Results	References
2015	Colorectal cancer	ISCs	Using CRISPR-Cas9–mediated engineering of human intestinal organoids	29
2019		CRC-PDO	A clinical study to evaluate sensitivity to chemotherapy of PDO	30
2020		CRC-PDO, CAFs	Established a hydrogel CRC PDO-CAF model to evaluate standard-of-care drugs	31
2024	Liver cancer	Liver cancer tissues	Established a biobank of 65 human liver cancer organoids for clinical drug screening and pharmacoproteomic analysis	34
2021		Huh7 cells, iPSC-EC, iPSC-MC	Generated functional, 3D sheet-like human HCC organoids *in vitro*	35
2025	Pancreatic cancer	WT, KC, KPC	Developed pancreatic organoids containing PDAC driver mutations to screen for KRAS mutation inhibitors	37
2023		KPC	Development of a T cell-integrated pancreatic tumor organoid model to establish a high-throughput drug screening platform	38
2022		PDO	Feasibility trials of personalized treatments to test their drug sensitivity and clinical outcomes	39
2024	Breast cancer	PDO	60 organoids were established from 75 breast samples	41
2024		PDO	PDO established in tissues collected before (O-PRE) and after (O-POST) treatment	42
2020	NSCLC	NSCLC primary patient tissue, PDX	Establishment of NSCLC organoids from primary lung cancer patient and PDX tumor tissue	46
2023	Metastatic lung cancer	Human malignant serous effusions	Generated 214 cancer organoids from 107 patients	47
2024	Gastric cancer	PDO	57 gastric cancer PDOs were established for personalized drug screening	51
2021		PDO	Three independent oxaliplatin-resistant gastric cancer organoids were established, demonstrating that *MYOF* is a promising marker gene	54
2021	Prostate cancer	PDO	Generated a novel organoid model derived from hormone-naïve lung metastases that showed alterations in the PI3K/Akt and Wnt/β-catenin pathways in response to androgen deprivation	59
2021		PDO	Established *ex vivo* primary PDCO cultures from prostatectomy specimens of patients with locally advanced prostate cancer	60
2019	Bladder cancer	Bladder epithelium (urothelium)	Created a living biobank of organoids grown from more than 50 patient samples	64
2020	Ovarian cancer	EOC	Establishing organoid cultures from high-grade serous ovarian cancer to decipher the role of NRG1/ERBB	69

Abbreviations: CAFs: Cancer-associated fibroblasts; CRC: Colorectal cancer; EOC: Epithelial ovarian cancer; HCC: Hepatocellular carcinoma; iPSC-EC: iPSC-derived endothelial cells; iPSC-MC: iPSC-derived mesenchymal cells; ISCs: Intestinal stem cells; KC: *Kras* mice; KPC: *TP53* mice; KRAS: Kirsten rat sarcoma viral oncogene homolog; NSCLC: Non-small cell lung cancer; PDAC: Pancreatic ductal adenocarcinoma; PDCO: Patient-derived cancer organoid; PDO: Patient-derived organoid; PDX: Patient-derived xenograft; WT: Wildtype.

predicted the treatment response in over 80% of patients using irinotecan, with no instances of misclassification for those who could benefit from the treatment.[28,30] The study highlights the potential of PDO as a preclinical model to help make personalized treatment decisions and improve treatment outcomes in patients with metastatic CRC. This approach bridges the gap between laboratory research and clinical application, providing a reliable, patient-specific model for predicting chemotherapy response.[30] Luo *et al.*[31] established hydrogel-based CRC organoid co-culture models to enable CRC PDO co-culturing with patient-derived cancer-associated fibroblasts (CAFs). Scientists have successfully replicated the CRC tumor microenvironment by establishing the hydrogel-based co-culture model, a significant improvement compared to traditional organoid cultures, making it highly relevant for precision medicine and drug discovery.[31]

3.2. Liver cancer

Liver cancer is characterized by a high incidence rate, poor clinical prognosis, and high mortality worldwide.[32] Broutier *et al.*[33] were the first to establish primary liver cancer organoids, including the three main types: Hepatocellular carcinoma (HCC), cholangiocarcinoma (CC), and combined HCC (CHCC). Organoid cultures faithfully preserved the histological, transcriptomic, and

genomic landscape of their parent tumors.[33] Ji *et al.*[34] used liver cancer organoids in a drug-proteomic framework to advance precise oncology. Scientists have established a patient-derived liver cancer organoids biobank (LICOB) that comprehensively represents the histological and molecular characteristics of various liver cancer types determined through multi-group analysis.[34] Proteomic analysis of LICOB identified proliferative and metabolic subtypes in liver cancer pertinent to patient prognosis. High-throughput drug screening of 76 drugs with LICOB revealed different response patterns for each cancer subtype.[34] Therefore, this study provides a comprehensive view of drug responses, actionable targets, and resistance mechanisms in drug proteomics methodologies. In addition, some studies integrate organoids with animal models to enhance their relevance. Qiu *et al.*[35] developed a novel approach for creating a mouse model of HCC that incorporates human tumors and simulate the tumor microenvironment of clinical patients. Scientists used luciferase-expressing Huh7 cells, human iPSC-derived endothelial cells (iPSC-EC), and human iPSC-derived mesenchymal cells (iPSC-MC) to generate 3D sheet-like human HCC organoids *in vitro* and implant them into the fragmented livers of immunodeficient mice.[35] Traditional methods for establishing liver cancer xenograft models have limitations such as low implantation rates, uncontrolled tumor sizes, and a propensity for off-target tumor development. This novel animal model has the advantages of a high implantation rate, controllable tumor size, and practical preservation of the original liver microenvironment. The tumor microenvironment in HCC organoids can be modified by adjusting the ratio of iPSC-EC and iPSC-MC.[35]

3.3. Pancreatic cancer

Pancreatic ductal adenocarcinoma (PDAC) accounts for 90% of pancreatic cancers, mainly due to *KRAS* mutations.[36] Mutated *KRAS* genes stimulate tumor cell proliferation, migration, transformation, survival; facilitate escape from tumor immune surveillance; and reprogram cell metabolism during cancer development.[36] A recent study by Duan *et al.*[37] developed a group of homologous mouse pancreatic organoids containing common PDAC-driven mutations, including *KRAS G12D*, *TP53 R172H*, and *SMAD4* deletions. According to a report, out of the approximately 6000 compounds screened through PDAC organoids, perhexiline maleate was found to selectively inhibit the growth of PDAC organoids carrying the *KRAS* G12D mutation both *in vivo* and *in vitro*.[37] Chemical and genetic perturbations of the *SREBP2* gene can also attenuate the growth of pancreatic organoids carrying the *KRAS* G12D mutation both *in vivo* and *in vitro*.[37] Therefore, this study based on high-throughput chemical screening of homologous pancreatic cancer organoids has significant potential for translational research and drug discovery. At the same time, PDAC has limited efficacy on immune checkpoint blocking therapy due to the inhibition of effector T cell function. To combat this immunosuppressive tumor type, scientists established a new model of T cell-integrated pancreatic tumor organoid.[38] The organoids incorporated with T cells effectively represent the characteristics observed in primitive tumors. In a drug screening utilizing PDA organoids, scientists evaluated the epigenetic inhibitors ITF2357 and I-BET151, which showed significantly enhanced anti-tumor effects when combined with anti-PD-1-based therapies.[38] This study dramatically demonstrates the research prospects of combining organoids and tumor immunotherapy. In addition, the HOPE trial is a pilot feasibility trial of personalized treatment using organoids to test drug sensitivity and its correlation with clinical outcomes.[39] This prospective trial assessed the feasibility of generating PDO in real time from PDAC patients using a limited number of biopsy samples collected during routine clinical practice.[39] In an attempt to evaluate PDO impacts on donors' responses to traditional anti-cancer drugs and develop a model to predict disease control, Grossman *et al.*[39] found that PDO can aid in drug sensitivity profiling and personalization of treatment options within 12 – 16 weeks after biopsy.[39]

3.4. Breast cancer

Breast cancer is and will be one of the important diseases threatening the health of women around the world.[40] At present, breast cancer is classified and treated clinically based on the differences in the expression of estrogen receptor (ER), progesterone receptor, and human epidermal growth factor receptor 2 (HER2).[40] Common models utilized in breast cancer research include cancer cell lines and PDXs. The methods exhibit significant limitations due to their high demands for the quantity of primary tumor cells, rendering them inappropriate for high-throughput drug screening.[40] One study established a PDO biobank using samples from breast cancer patients that faithfully preserved the histological and genetic characteristics of their parental tissues.[41] To assess the model's feasibility in predicting patient drug responses within clinical practice, scientists conducted an *in vitro* drug screening of breast cancer PDO. PDO successfully predicts drug responses in patients, and drug sensitivity testing results of PDO are consistent with actual clinical responses in matched patients in most cases.[41] Therefore, PDO can serve as a valuable platform for evaluating treatment efficacy to support and guide drug therapy for individual patients. In addition, another study utilized PDO developed from tissue samples collected before treatment (O-PRE) and after treatment (O-POST) to study tumor evolution before and after neoadjuvant chemotherapy and surgery.[42] Both PDO cultures reproduced the histological and

molecular characteristics of the original tissue, effectively demonstrating typical breast tissue. These PDOs have 3D-ordered structure, and large amount of cell debris extruded at the junction of the two organoid cells indicates high secretory activity and cell turnover ratio.[42] In addition, the study also assessed the expression of HER-2 and EGFR as cell-surface-related tumor biomarkers. O-POST is highly correlated with increased EGFR expression and an increased percentage of EGFR-positive cells, indicating that neoadjuvant chemotherapy upregulates EGFR expression.[42] Therefore, breast cancer PDO could serve as an excellent 3D model for assessing disease evolution.

3.5. Lung cancer

Lung cancer is a very complex disease that shows phenotypic and genotypic diversity in different patients, posing considerable challenges to precision medicine. To date, growing evidence shows the feasibility and superiority of lung cancer organoids.[43-45] Non-small cell lung cancer (NSCLC) is the most widely studied subtype of lung cancer.[46] A study established organoid cultures of NSCLC from surgically resected primary patient tissue and a patient-derived xenograft model.[46] Short-term and long-term established NSCLC organoids, that are cultivated *in vitro* and xenografted, not only reproduce the histological characteristics and tumorigenicity of their matching tumor tissues but also retain the sensitivity of the parent tumors to targeted therapy, rendering them ideal for the discovery and validation of biomarker-drug combinations.[46] Whole-exome sequencing and RNA sequencing have shown that long-term NSCLC organoids retain the mutation, copy number, and gene expression profiles of their parent tumors despite multiple subcultures in an *in vitro* environment.[46] In addition, Wang *et al.*[47] generated 212 lung cancer organoids (LCOs) from malignant serous effusion (MSE) obtained from 107 patients. MSE primarily consists of tumor cells and can be obtained in a relatively minimally invasive procedure.[47] As a result, LCOs established through MSE are characterized by their purity as tumor organoids, making these models highly suitable for drug sensitivity tests (DST). LCO faithfully reflects the pathological and molecular characteristics of the original tumor.[47] Among them, the results of LCO-based DSTs (LCO-DSTs) are highly consistent with clinical treatment responses. For example, the LCO-DST results of one patient reflected that dual-targeting drugs showed high tumor control rates, which were confirmed in the subsequent actual clinical treatment.[47] Therefore, LCO-DST is expected to become an important predictive tool for formulating personalized treatment plans for lung cancer in personalized medicine.

3.6. Gastrointestinal cancer

Gastric cancer currently ranks fifth in the global cancer incidence rate and is also the fourth most prominent cause of cancer-related death.[48] The main reason for the poor prognosis of gastrointestinal cancer is that the early symptoms of the tumor are not obvious, resulting in the tumor progressing to advanced stages at the time of diagnosis and drug resistance that often occurs during drug treatment.[49] Therefore, numerous studies have effectively established gastrointestinal cancer organoids for investigating drug resistance mechanisms and predicting patient responses.[50-56] Among them, Vlachogiannis *et al.*[50] first described that a PDO model of metastatic gastrointestinal cancer can predict and reproduce a patient's treatment response during clinical treatment. In this study, scientists used gastric cancer PDO, which has a highly similar genotype and phenotype to patients' tumors, to test the anticancer drugs commonly used in clinical practice.[50] The results showed that PDO demonstrated effectiveness comparable to 100% in predicting patient sensitivity to chemotherapy and targeted therapy, achieving a positive predictive value (that is, predicting that a drug is effective) of 88% and a negative predictive value (that is, predicting that a drug is ineffective) of 100%.[50] In another study, scientists established 57 organoids derived from gastric cancer patients. Using different chemotherapy drugs and verifying them in PDOs-based xenograft mice, it was found that the drug response results were entirely consistent with the actual clinical response of 91.7% of patients.[51] Overall, these studies have shown that gastrointestinal cancer organoids have an apparent predictive effect on the clinical response of chemotherapeutic drugs, which provides a powerful help in screening drugs for gastrointestinal cancer patients and personalized medicine. In addition, Ukai *et al.* and Harada *et al.* established gastric cancer organoids exhibiting resistance to 5-FU and oxaliplatin, respectively, and successfully explored potential therapeutic targets and biomarkers associated with chemotherapy-resistant gastric cancer.[53,54] In addition, research by Seidlitz *et al.*[52] proved that organoids derived from human gastric cancer can retain identical phenotypes and histological characteristics as parent tissues after 1 year of culture. These findings provide strong evidence and confidence for establishing a biobank of gastrointestinal cancer organoids in the future.

3.7. Prostate cancer

Prostate cancer is currently one of the most common types of cancer in men around the world, and the mortality rate among men is only lower than that of lung cancer.[57] In addition, the global incidence of prostate cancer is gradually increasing, with 2.9 million new cases expected by 2040.[57] Therefore, the development of prostate cancer organoids is imminent. Gao *et al.*[58] pioneered the establishment of a prostate cancer organoid line from prostate cancer patients. These organoid lines exhibit several prostate cancer-specific gene mutations, such as *FOXA1* mutations, *ETS* translocation, *CHD1* loss, and *SPOP* mutations.[58] The

development of these organoids provides a solid basis for the development of more prostate cancer organoids in the future. Due to the low success rate of establishing prostate cancer organoid lines, Servant *et al.*[59] established an organoid bank from 81 different prostate cancer pathological specimens and evaluated factors that affected the success rate of establishing prostate cancer. Scientists have found that due to the lineage-specific nature of prostate cancer cells, using tissue aggregates instead of traditional cell suspensions can effectively improve the success rate of establishing prostate cancer organoids.[59] However, there are other endogenous factors that require further exploration. In addition, Heninger *et al.*[60] established organoids from patients with locally advanced prostate cancer and verified that these organoids effectively preserved the tumor microenvironment complexity of prostate cancer through the integration of novel biotechnologies (such as orthogonal flow cytometry analysis and microfluidic rare-event screening protocols). In general, the development of prostate cancer organoids still faces many challenges, and further optimization of development methods and culture technologies is needed.

3.8. Bladder cancer

Bladder cancer is an urothelial cancer originating from the lining of the bladder and is currently the second most common malignant tumor among urinary system diseases.[61] Since the high mutation rate of bladder cancer leads to poor prognosis and a high risk of recurrence, it is imperative to establish an effective bladder cancer research model.[62,63] Mullenders *et al.*[64] established an *in vitro* biobank of bladder cancer organoids from 53 bladder cancer patient samples. Experimental results show that the organoid generation efficiency reaches 60 – 70%, surpassing traditional cell line generation technology while preserving original shape and function after long-term passage.[64] In addition, these organoid lines also retain common genetic mutations in bladder cancer, such as *TP53* and *FGFR3* mutations, providing a good model basis for screening new chemotherapeutic drugs for bladder cancer.[64] The study by Kong *et al.*[65] utilized a machine learning framework to analyze and successfully predict the drug response of 77 bladder cancer patients treated with cisplatin using a bladder cancer organoid model. These studies suggest that bladder cancer organoids may effectively predict treatment response. In terms of precision medicine, the bladder cancer organoid model established by Lee *et al.*[66] effectively preserves the tumor heterogeneity and genomic alterations characteristic of primary cancer. This means that treatment practice can be guided and adjusted based on the responses of organoid models to current drugs.

3.9. Ovarian cancer

Ovarian cancer is one of the most difficult to treat among gynecologic cancers, and the lack of adequate research models is the main reason currently limiting research on ovarian cancer.[67-69] Kopper *et al.*[68] established 56 organoid lines from ovarian cancer patients, including all common subtypes of ovarian cancer, such as serous BT (SBT), mucinous BT (MBT), mucinous BT (MC), endometrioid (END), clear cell carcinomas (CCC), and high-grade serous (HGS) tumors. In addition, Maenhoudt *et al.*[69] established organoid cultures from HGS ovarian cancer, identifying neuregulin-1 (NRG1) as a crucial factor for cultivating ovarian cancer organoids. The establishment of these ovarian cancer organoid lines has laid a solid foundation for future basic research on ovarian cancer.

4. Current limitations and future perspectives

Compared with traditional *in vivo* and *in vitro* research methods, organoids have shown extraordinary potential due to their "more organ-like" characteristics. Organoids preserve the histological and biological characteristics of the original tumors, enabling scientists to dynamically observe tumor growth.[23,70] In addition, the costs associated with organoid maintenance and cultivation are minimal, and the use of experimental animals is unnecessary, thus aligning with ethical standards regarding animal welfare.[23] Therefore, organoid models provide unlimited possibilities in high-throughput drug screening, anti-cancer drug development, overcoming cancer drug resistance, clinical efficacy evaluation, and personalized and precision medicine, thereby accelerating the transformation of drugs from laboratory to clinical practice.[23,70] Over the past few decades, there has been a gradual rise in the utilization of PDO models in multiple cancer research areas, including colorectal, lung, breast, gastrointestinal, prostate, liver, pancreatic, bladder and ovarian cancers.[10,15,18,19,70]

Despite their surprisingly unexpected applications, many organoid models are replete with limitations that cannot be ignored. Firstly, initial culture of organoid lines takes a long time and is costly, and the success rate varies depending on tumor types.[70-72] Second, The existing tumor organoid lines are predominantly derived from adenocarcinoma, but for tumors of non-epithelial origin, the application of organoids may be greatly limited.[73,74] Third, organoid culture necessitates the use of costly specialized media containing various growth factors. Some commonly used media supplements may affect the response of drugs targeting the same signaling pathway to a certain extent, an important factor worthy of consideration by scientists when monitoring and evaluating drug responses.[75-77] In addition, one of the important limitations of organoid culture is the lack of key tumor microenvironment and cellular components, including blood vessels, stromal cells, fibroblasts, and immune cells.[23,70] There have been many studies attempting to mitigate this deficiency by

establishing and developing co-cultivation systems. These co-culture systems integrate some components of the tumor microenvironment (such as stromal cells and immune cells), which will be an important development direction for cancer organoids in the future.[78-83]

Given the limitations of organoids, Organ-on-Chip is emerging as an innovative platform to optimize cancer organoids. Cancer organoids are 3D cell cultures derived from patient tumors that retain key genetic and histological features of the original cancer. Thus, organoids are self-assembling and rely on the extracellular matrix to maintain the 3D structure. Complex microenvironment interactions and the absence of dynamic fluid flow are significant shortcomings of organoids, but they can be addressed with the organ-on-a-chip technology. By integrating organoids into microfluidic chips, researchers have created "organoid-on-chip" systems.[84] This system can deliver nutrients and oxygen, demonstrate realistic tumor-stroma interactions, and improve drug screening platforms to simulate real-world pharmacokinetics.[85] The combination of the two products, cancer cells and organoid-on-chip, creates cancer-on-chips (CoC), which is an advanced tumor model capable of simulating cell interactions, dynamic drug concentrations and drug effects, and angiogenesis or mechanical stimulation of cells.

The combination of the two products, cancer cells, and organoid-on-chip, creates CoC, which is an advanced tumor model capable of simulating cell interactions, dynamic drug concentrations and drug effects, and angiogenesis or mechanical stimulation of cells.[84,85] This advancement will bridge the gap in drug responses observed in *in vitro* studies and real patients and enable a more accurate simulation of the tumor microenvironment.[86] Different microfluidic organ chips can be connected to each other to construct a human chip model capable of simulating multi-organ interactions. These microphysiological systems can be used to study cancer multi-organ metastasis.[87] Shirure *et al.*[88] reported the design of a vascularized tumor chip model to simulate physiological material transport at the arterial end of capillaries within the tumor microenvironment, and demonstrated that the effective delivery of nutrients or drugs to tumor tissue through the vascular network helps to maintain the physiological activity of tumor organoids for a long time. In the organoid model on a vascular chip, tumor organoids were cultured in a central chamber. Adjacent chambers were connected to the central chamber, and endothelial cells were cultured with fibroblasts in hydrogel. It has been demonstrated that perfusion of tumor organoid cultures with the vascular system can simulate angiogenesis[88,89] (Figure 4). Notable features of this vascular tumor chip model include the simulation of dynamic tumor evolution through cell proliferation, angiogenesis, migration, and intravascular infiltration.[88,89] Therefore, the integration of the two technologies is expected to become a new technology for studying the mechanism of

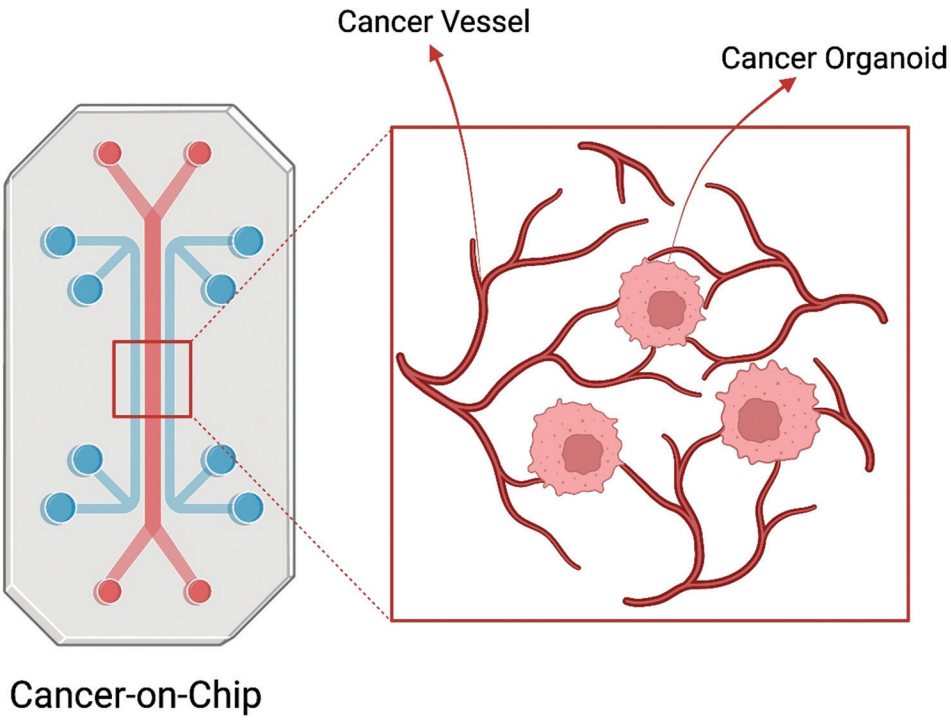

Figure 4. Cancer-on-chip and microfluidic devices. Tumor organoids are cultured in a central chamber. Tumor organoid cultures are perfused with a vascular system to mimic angiogenesis. Created with BioRender. Cao, K. (2025) https://BioRender.com/12vphvf.

tumor evolution, screening anti-cancer drugs and cancer therapies, and achieving precision medicine.

5. Conclusion

Despite many limitations, the development of organoid models is of great significance in the field of cancer research. Variations in patients' drug sensitivity have an impact on the clinical transformation after drug screening with organoids. Therefore, there is an urgent need to develop new technologies and platforms based on patient-derived samples in the future to overcome the limitations of organoid culture while improving the accuracy of drug screening. In addition to precision medicine and personalized treatment, the high similarity between organoids and primitive tumors can also provide great help for basic cancer research. In the future, organoid models can also be applied to research in the fields of infectious diseases, autoimmune diseases, proteomics analysis, and immunotherapy. Organoid technology can significantly promote the development of new drugs, thereby improving patient survival and prognosis.

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Conflict of interest

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Author contributions

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ACCSCIENCE PUBLISHING

ORIGINAL RESEARCH ARTICLE

A trabeculae-like biomimetic bone-filling material as a potential organoid for bone defect treatment

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Abstract

Bone-filling materials are critical tools for the treatment of bone defects. However, existing materials require improvements in tissue compatibility and drug-loading capacity. In this study, we designed and synthesized a novel trabeculae-like biomimetic bone-filling material (TBM) that mimics the composition and structure of natural bone trabecular tissue. This TBM demonstrated high mechanical strength and excellent biocompatibility. It effectively embedded osteogenic cells and potentially functioned as an organoid. We demonstrated that the TBM exhibited therapeutic efficacy in treating various bone defects and fractures by filling defect regions and enabling sustained release of small-molecule and nucleic acid drugs. Based on these findings, we propose TBM as a promising candidate for the treatment of bone defects and provide innovative insights for the development of bone-filling materials.

Keywords: Bone-filling material; Organoid; Bone defect; Tissue engineering

1. Introduction

Bone defects can arise from fractures and various bone diseases, including bone tumors and osteomyelitis.[1,2] Critical-sized bone defects, in particular, cannot self-heal through intrinsic repair mechanisms.[3,4] These defects are characterized by prolonged rehabilitation periods, high disability rates, and significant post-operative treatment costs, severely impairing patients' quality of life.[5] Consequently, the accurate and efficient treatment of bone defects remains a major challenge in orthopedic research.

Bone-filling materials are essential tools for addressing bone defects, as they fill defect regions, provide mechanical support, and promote bone formation.[6-8] While autologous or allogeneic bone transplantation is considered the optimal

treatment, its application is often limited by donor scarcity, surgical site pain, and infection risks.[9,10] To overcome these limitations, synthetic bone-filling materials, such as metals, inorganic salts, organic polymers, and composite materials, have been developed.[11] For example, composite scaffolds based on sodium alginate hydrogel and calcium phosphate ceramics are widely used due to their excellent osteoconductivity and bioabsorbability.[12,13] However, certain synthetic bone-filling materials still require improvement. For instance, most metallic bone-filling materials lack natural degradability, potentially leading to stress-shielding effects.[14]

Recent advancements in bone tissue engineering and organoid technology have led to the development of novel composite bone-filling materials. These materials, fabricated using tissue engineering approaches, exhibit high biocompatibility, mechanical robustness, and a composition similar to natural bone tissue. Enabled by 3D printing and rapid prototyping techniques, they allow for rapid mass production and customization to fit specific bone defect regions.[15-17] These characteristics have made them highly significant in the field of bone therapeutics.[18]

In this study, we developed a novel trabeculae-like biomimetic bone-filling material (TBM) with a similar composition and porous structure closely resembling natural bone trabecular tissues. The TBM demonstrates high biocompatibility, sufficient mechanical strength, and the ability to carry osteogenic cells, functioning as a potential organoid. In addition, it enables the sustained release of small-molecule and nucleic acid drugs tailored to the primary disease, thereby accelerating bone repair processes. This study highlights the TBM's potential as a functional organoid mimicking natural trabecular bone, demonstrates its efficacy in treating bone defects, and provides a robust framework for the development of bone-filling materials.

2. Materials and methods

2.1. Stem cells, nucleic acid delivery system, and animals

Human mesenchymal stem cells (hMSCs) were purchased from Procell (CP-H166, China). Mouse adipose-derived mesenchymal stem cells (ADSCs) were primary cells isolated from mice. Professor Deng Xudong from Northwestern Polytechnical University, China, provided the polyvinylamine (PVAm) nucleic acid delivery system. C57BL/6 male mice were purchased from Huafukang Bioscience Co., Ltd. (SCXK 2009-0008, China). All animal protocols received approval from our local Ethics Committee (Reference: 2023078, Date: September 05, 2023). In this study, a total of 188 mice were used, and animal suffering was minimized.

2.2. Preparation of TBM

First, 7% silk protein (S26299, Yuanye, China), 2% chitosan (C8320, Solarbio, and matrigel (354480, Corning, USA) were mixed. The mixture was then immediately supplemented with an equal quantity of biphasic calcium phosphate (BCP; DULY, China) powder, thoroughly stirred at 65°C, frozen at −80°C, and subsequently lyophilized. Next, the mixture was gently soaked in water and lyophilized again without freezing to form a core pillar structure (referred to as the Core). This structure served as a scaffold and was coated with the same mixture (supplemented with 0.1% acrylated arginine-glycine-aspartate [RGD] peptide [EFL-Pep-RGDfkAC, engineering for life, Spain]) on the peripheral surface. After stirring, heating to 65°C, freezing, lyophilizing, and soaking, the material underwent a final lyophilization step following 30 min of freezing at −80°C, resulting in a trabeculae-like porous skeletal structure (referred to as the Porous). A hyaluronic acid methacrylate (HAMA; H398345, Aladdin, China) hydrogel containing either a water-soluble drug (dissolved in HAMA solution) or an organic-soluble drug (dissolved in a dimethyl sulfoxide [DMSO; BioFroxx, China] solution of photoinitiator I2959 [HY-W013508, MedChemExpress LLC, China]) was then overlaid onto the porous structure and crosslinked through ultraviolet light to construct the drug-loaded TBM.

2.3. Characterization of TBM

The micromorphology of the TBM was examined using scanning electron microscopy (Gemini 300, ZEISS, Germany) at 3 kV. Elemental composition analysis was conducted using energy-dispersive spectroscopy (EMX, HORIBA, Japan). Meanwhile, the chemical residues of the TBM were analyzed using Fourier-transform infrared spectroscopy (FTIR; Nicolet iS10, Thermo Fisher Scientific, USA), with a spectral range of 400 – 4,000 cm^{-1}, a resolution of 4 cm^{-1}, and a signal-to-noise ratio of 50,000:1.[19] The rheological properties of the TBM were analyzed using an MCR92 rheometer (Anton Paar, Austria) at 25°C. Measurements were performed within a shear strain range of 0.1 – 10%.[19] The water content of the TBM was calculated using the following equation:

$$\text{Water content (\%)} = (W_w\text{-}W_d)/W_w \times 100\% \quad \text{(I)}$$

Where W_w is the wet weight of the material, and W_d is the freeze-dried weight.[19]

The swelling ratio was calculated using the following equation:

$$\text{Swelling ratio (\%)} = (W_N\text{-}W_0)/W_0 \times 100\% \quad \text{(II)}$$

Where W_0 is the initial weight of the material, and W_N is the weight after water soaking.[20]

Degradation ratios were calculated using the following equation:

Degradation ratio (%) = $(W_0-W_N)/W_0 \times 100\%$ (III)

Where W_0 is the initial weight of the material, and W_N is the weight after treatment with collagenase II (BS033B, BioSharp, China).[20]

2.4. Preparation of PVAm

Aqueous PVAm solution (molecular weight = 1,100, concentration = 10%) was dialyzed using a membrane with a molecular weight cutoff of <10,000 and passed through a syringe with a pore size of 5 μm. The purified PVAm was then lyophilized and subsequently re-dissolved in distilled water at a concentration of 0.9 mg/mL.[21]

2.5. Slow-release studies

The TBM loaded with rhodamine B (5 μg/mL; R104960, Aladdin, China), fluorescein amidite (FAM)-modified siRNA-negative control (FAM-NC) (100 μM; GenePharma, China), or PVAm-FAM-NC (1:1 by v/v) was thoroughly washed in deionized water for 30 min. For slow-release studies, the washed TBM was then immersed in 1 mL deionized water at 4°C (rhodamine B was tested at room temperature) for slow-release studies. The medium was collected at specific time intervals, and the TBM was re-immersed in fresh 1 mL deionized water to continue the slow-release process. Fluorescence measurements were performed using the SpectraMax Paradigm microplate reader (Molecular Devices, USA).

2.6. Cell culture

The hMSCs and ADSCs were cultured in high-glucose Dulbecco's modified Eagle medium (DMEM; KGM12800-500, KeyGEN BioTECH, China) supplemented with 10% fetal bovine serum (FBS; BS-1101, OPCEL, China) and 1% streptomycin (0242, Amresco, USA) and 1% penicillin (0382, Amresco, USA). To induce osteogenic differentiation, cells (100% density) were cultured in osteogenic media consisting of DMEM, 10% FBS, 1% penicillin/streptomycin, 1% β-glycerophosphate (G9422, Sigma-Aldrich, USA), 1% ascorbic acid (A7631, Sigma-Aldrich, USA), and 1% L-glutamine (G8540, Sigma, USA). Cells were maintained at 37°C with 5% CO_2.

2.7. Cytotoxicity studies

Cell viability mediated by the TBM was assessed using the Cell Counting Kit-8 (CCK-8; C0038, Beyotime Biotechnology Co., Ltd., China) assay. Both hMSCs and ADSCs were seeded in 96-well plates and treated with either HAMA or TBM for 2 d. The cells were then incubated in DMEM supplemented with 10% CCK-8 for 4 h, after which 100 μL of the solution was transferred to measure absorbance at 490 nm using the Sunrise™ microplate reader (TECAN, Switzerland).

Cell cycle activity in hMSCs was analyzed using flow cytometry. Cells were cultured with TBM for 2 d, digested using trypsin (Gibco, USA), fixed in 70% chilled ethanol, washed, and stained with 0.1 mg/mL propidium iodide (P422887, Aladdin, China). Cell cycle distribution was determined using the fluorescence-activated cell sorting Calibur flow cytometer (BD Biosciences, USA).[22]

2.8. Hemolysis studies

Anticoagulated blood from 3-month-old C57BL/6 male mice was processed to isolate pure erythrocytes, which were then suspended in phosphate-buffered saline (PBS). The TBM was added to the erythrocyte suspensions, and the mixture was incubated at 37°C for 180 min. Following incubation, the supernatants were photographed, and absorbance values were recorded as previously described.[23]

2.9. Osteogenic differentiation methods

To evaluate osteogenic differentiation in hMSC or ADSC, reverse transcription-polymerase chain reaction (RT-PCR), alkaline phosphatase (ALP) staining, and alizarin red S (ARS) staining were employed. ALP staining was conducted using a BCIP/NBT ALP coloWr development kit (C3206, Beyotime Biotechnology, China), whereas ARS staining was conducted using 0.5% ARS (pH = 4.2; A5533, Sigma-Aldrich, USA).[24] For RT-PCR, total RNA was extracted using the E.Z.N.A.® Total RNA Kit I (R6834-02, Omega Bio-TEK, USA) and reverse-transcribed into complementary DNA (cDNA) using the HiScript® II 1st Strand cDNA synthesis kit (R211-02, Vazyme, China). RT-PCR was then performed using the ChamQ Universal SYBR quantitative PCR Master Mix (Q711-02-AA, Vazyme, China). Primers were purchased from Tsingke, Inc. (China), and their sequences can be found in Table 1.

Table 1. The primer sequences for quantitative reverse transcription-polymerase chain reaction

Target gene	Sequences (5'→3')
Human *ALP*-Forward	GGCCATTGGCACCTGCCTTA
Human *ALP*-Reverse	ACCCATCCCATCTCCCAGGAA
Human *RUNX2*-Forward	TCTGGCCTTCCACTCTCAGT
Human *RUNX2*-Reverse	GACTGGCGGGGTGTAAGTAA
Human *GAPDH*-Forward	CATGGAGAAGGCTGGGGCTC
Human *GAPDH*-Reverse	CACTGACACGTTGGCAGTGG
Human miR-138-5p	CTTGAGCTGGTGTTGTGAATCAG
Mouse *Alp*-Forward	GTTGCCAAGCTGGGAAGAACAC
Mouse *Alp*-Reverse	CCCACCCCGCTATTCCAAAC
Mouse *Run×2*-Forward	CGCCCCTCCCTGAACTCT
Mouse *Run×2*-Reverse	TGCCTGCCTGGGATCTGTA
Mouse *Gapdh*-Forward	TGCACCACCAACTGCTTAG
Mouse *Gapdh*-Reverse	GGATGCAGGGATGATGTTC

2.10. RNA-sequencing (RNA-seq)

To investigate the molecular mechanisms underlying the effects of the TBM embedded with bergamottin (B757586, Aladdin, China) or miR-138-5p antagonist (RQCON Biological Technology Co., Ltd., China), mRNA-sequencing was performed on TBM-treated hMSCs. Total RNA was extracted using TRIzol reagent (15596018, Invitrogen, USA), and RNA quantity and quality data were assessed using the NanoDrop OneC spectrophotometer (Thermo Fisher Scientific, USA). After confirming RNA integrity, RNA-seq was conducted by Annoroad Gene Technology Co. Ltd. (China). Raw sequencing data were processed using the Fastp software (v.0.19.11; https://github.com/OpenGene/fastp). Fold changes in differentially expressed RNAs were calculated as log2 values (normalized intensity of the treatment group/normalized intensity of the control group).

2.11. Enrichment analysis

Gene ontology (GO) enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were conducted to investigate the differentially expressed genes (DEGs) between blank TBM and TBM loaded with bergamottin, as well as between TBM loaded with an empty recombinant RNA (MSA) and TBM loaded with recombinant miR-138-5p antagonist. Gene set enrichment analysis was conducted using the "clusterProfiler" package from the Bioconductor project (https://bioconductor.org/packages/release/bioc/html/clusterProfiler.html). A $p<0.05$ was set as the significance threshold.

2.12. Cell embedding in the TBM

A total of 25 8-month-old C57BL/6 male mice (37 ± 1.4 g) were used in the cell embedding study. The mice were anesthetized using CO_2, and inguinal fat tissue was collected and washed. The tissue was cut into pieces and digested in 0.25% collagenase I (BS032B, Biosharp, China) at 37°C for 30 min. Primary ADSCs were collected by centrifugation and resuspended in DMEM.[25]

Cultured ADSCs were detached using trypsin and suspended in a culture medium. HAMA was dissolved in the cell suspension with a density of $6 \times 10^6/cm^3$, then crosslinked and overlaid onto the Porous to construct an ADSC-embedded TBM. These constructs were either cultured in a microfluidic system or subcutaneously implanted over the calvarial surface of mice, with each mouse receiving an autologous ADSC-embedded TBM. The samples were then ready for CCK-8 assays.

2.13. Cell viability assay for TBM organoids

Cell viability within the TBM was qualitatively assessed using the Calcein AM/PI (live and dead cell staining) test kit (KGA9501-1000, KeyGEN BioTECH, China) following the manufacturer's protocol. Briefly, the TBM was rinsed with PBS and then incubated in 2×10^{-6} M Calcein AM and 4×10^{-6} M PI for 30 min at room temperature in the dark. Stained microspheroids were visualized using a confocal microscope (FV3000, Olympus, Japan) with 4 μm thick slices.

2.14. 5-Ethynyl-2'-deoxyuridine labeling cell proliferation assay for TBM organoids

To assess the effects of treatment on cell proliferation, 5-ethynyl-2'-deoxyuridine (EdU; RQCON Biological Technology Co., Ltd., China) labeling was conducted using the EdU labeling/detection kit (C10310, RiboBio, China). The TBM-embedded primary ADSCs were cultured in 24-well plates. Then, 50 μM of EdU was added to each well, and the cells were further incubated for 12 h at 37°C. Following incubation, the TBM was fixed with 4% paraformaldehyde (F1635, Sigma-Aldrich, USA/BL539A, BioSharp, China) for 30 min at 37°C and treated with 0.5% Triton X – 100 (T9284, Sigma-Aldrich, USA) for 30 min at 37°C. After three washes with PBS, 100 μL of 1 × Apollo reaction cocktail (C10310-3, RiboBio, China [in the EdU labeling/detection kit]) was added to each well, and the cells were incubated for 45 min at 37°C. Then, the cells were stained with 100 μl of Hoechst 33,342 (C10310, RiboBio, China) for 30 min at 37°C. The cells were visualized using a confocal microscope (FV3000, Olympus, Japan). All cells were gamma-adjusted, merged with Hoechst staining, and analyzed using Image-Pro Plus 6.0 software (Media Cybernetics, USA). The EdU incorporation rate was calculated as the ratio of EdU-positive cells to total Hoechst-positive cells. All experiments were performed in triplicate and repeated independently 3 times.

2.15. Immunofluorescence

The TBM samples (2 mm^3 ^{sec}tions) were subjected to immunofluorescence staining in 96-well plates.[26] Samples were first incubated with primary antibodies at 4°C overnight, followed by incubation with fluorescent-labeled secondary antibodies at room temperature for 1.5 h. The primary antibodies used were ALP rabbit polyclonal antibody (pAb) (1:20; ET1601-21, HUABIO, China, RRID: AB_3069604), runt-related transcription factor 2 (RUNX2) rabbit pAb (1:25; ET1612-47, HUABIO, China, RRID: AB_2924311), ACTIN rabbit pAb (1:40; BL005B, Biosharp, China), cluster of differentiation (CD)31 (1:20; AG2849, Beyotime, China). A fluorescent-labeled secondary antibody CoraLite 488-conjugated goat anti-rabbit (1:100; 20000259, Proteintech, USA, RRID: AB_2797132) was used for detection. Stained cells were visualized using laser scanning confocal microscopy (FV3000, Olympus, Japan) with excitation wavelengths of 488 nm and 405 nm.

2.16. Western blotting

For western blotting, TBM-embedded ADSCs were lysed using a cell lysis buffer (P0013, Beyotime, China). The lysates were electrophoresed, and proteins were transferred onto nitrocellulose membranes (66485, Pall, USA). The membranes were blocked with 5% skimmed milk and incubated overnight at 4°C with primary antibodies. After washing, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies. Protein bands were visualized using enhanced chemiluminescence (BL520B, Biosharp, China) and exposed using an imager (Fusion FX, Vilber Lourmat, France). The following antibodies were supplied by HUABIO (China) and used at a dilution of 1:2000: ALP Rabbit pAb (ET1601-21), RUNX2 Rabbit pAb (ET1612-47), and GAPDH Rabbit pAb (ET1601-4, RRID: AB_3069615). HRP-conjugated anti-immunoglobulin G antibodies (1:2000; CW0102-RRID-AB_2814710 and CW0103-RRID-AB_2814709, CWBIO, China) were used for detection.

2.17. Bone defect and fracture modeling

A total of 120 2-month-old C57BL/6 male mice (25 ± 0.5 g) were used in bone defect and fracture models. For the bone defect model, 1.2 mm boreholes were created in the tibiae or calvariae using a dentist's drill,[27] followed by the insertion of TBM. Mice were humanely euthanized at 3 weeks post-surgery using CO_2. For the fracture model, tibial fractures were induced, and TBM was implanted into the fracture sites. Mice were humanely euthanized at 4 weeks post-surgery.

2.18. *In vivo* hydrogel biocompatibility

Blood samples were collected, and supernatants were isolated for alanine transaminase, aspartate aminotransferase, blood urea nitrogen, and creatinine blood analyzes using the ADVIA 2400 Chemistry System (Siemens Healthcare Diagnostics, USA). In addition, tissue samples from the liver, spleen, lung, kidney, and heart were collected, fixed, and embedded in paraffin. Sections were prepared and stained with hematoxylin and eosin (HE) for histological evaluation under optical microscopy.

2.19. Bone histomorphometry

The effects of the TBM on bone repair were evaluated using double calcein labeling and micro-computed tomography (micro-CT) imaging (v.6.5, Viva CT40, SCANCO Medical, Switzerland), as previously described.[28,29] To assess bone regeneration and bone resorption, paraffin sections were prepared for Masson's trichrome staining, Goldner's trichrome staining, and tartrate-resistant acid phosphatase (TRAP) staining.[30,31]

Bone microstructures were analyzed using micro-CT, with a focus on the bone defect regions. Images were reconstructed and calibrated at an isotropic voxel size of 10.5 mm (70 kVp, 114 mA, 200 ms integration time, 260 thresholds, and 1200 mg HA/cm^3). Using SCANCO software, regions of interest within the bone defect areas were manually contoured for three-dimensional reconstruction (sigma = 1.2, supports = 2, and threshold = 200). Parameters such as bone mineral density (BMD), bone volume to tissue volume (BV/TV), trabecular number (Tb.N), and bone mineral content (BMC) were calculated to evaluate bone microarchitecture.[31]

2.20. Immunohistochemistry

All antibodies were purchased from Santa Cruz Biotech (USA). Inflammatory marker protein levels were evaluated using CD3 mouse monoclonal antibody (mAb) (1:100; sc-20047, RRID: AB_627014) and CD68 mouse mAb (1:100; sc-20060, RRID: AB_627158). Bone-specific markers were osteocalcin (OCN) mouse mAb (1:200; sc-73464, RRID: AB_1126894) and RUNX2 mouse mAb (1:200; sc-390351, RRID: AB_2892645). The incubation with primary antibodies was at 4°C overnight, and for secondary antibodies was at room temperature for 1.5 h.[28]

2.21. Statistical analyzes

All experiments were independently repeated at least 3 times, with each experiment performed in triplicate. The statistical analyzes were conducted using GraphPad Prism version 10.1.2 software (GraphPad Software, USA; www.graphpad.com). For comparison involving three or more groups, ordinary one-way analysis of variance with Bonferroni *post hoc* analysis was used to assess variance. Differences between the two groups were evaluated using Student's t-test. Data are presented as mean ± standard deviation. A $p<0.05$ was considered statistically significant for all comparisons.

3. Results

3.1. Construction and characterization of the TBM

The TBM was prepared using a combination of silk protein, chitosan, matrigel, BCP, and HAMA. An organic collagen-like network was first formed by mixing silk protein, chitosan, and matrigel. BCP was added to the organic network, mixed thoroughly, and immediately lyophilized to form the Core, which provides mechanical support. The core was then coated with the organic network-BCP mixture. The resulting construct underwent rapid freezing and lyophilization to generate the Porous. Finally, the structure was coated with a HAMA hydrogel to form the TBM (Figure 1A).

The TBM exhibited a microstructure resembling trabecular bone, with a relatively uniform pore size range of 50 – 250 μm. The water content of the TBM was 40.81%

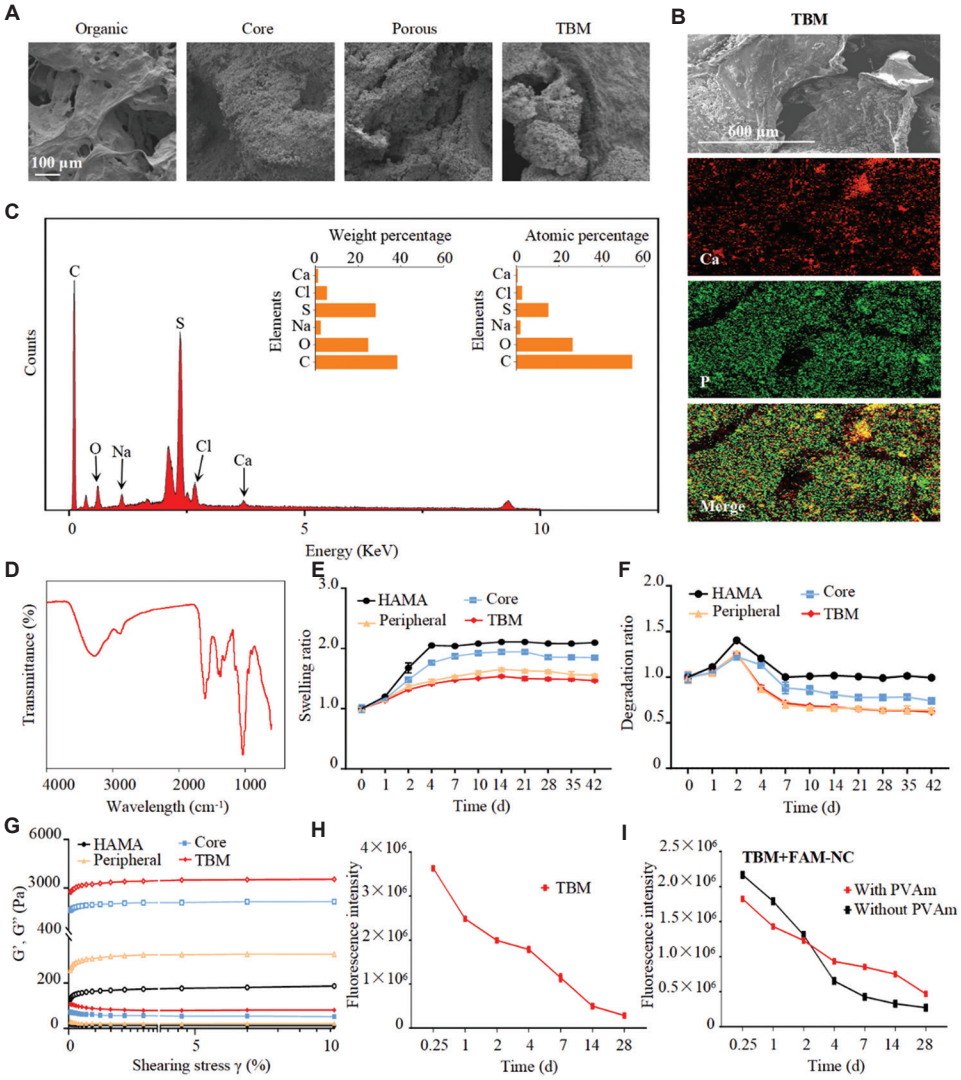

Figure 1. Characterization of the trabeculae-like biomimetic bone-filling material (TBM). (A) Scanning electron microscopy imaging of the organic network (Organic), the core pillar of the TBM (Core), the porous framework of the TBM (Porous), and the constructed TBM (TBM; scale bar: 100 μm; magnification: ×50). (B) Energy-dispersive spectroscopy mapping of the TBM (scale bar: 600 μm; magnification: ×40). (C) Energy-dispersive spectroscopy analysis of the TBM elements, including calcium (Ca), chloride (Cl), sulfur (S), sodium (Na), oxygen (O), and carbon (C). (D) Fourier-transform infrared spectrometry spectra of the TBM. (E) Swelling ratios of the hyaluronic acid methacryloyl (HAMA), the HAMA-embedded core pillar (Core), the HAMA-embedded peripheral porous framework (Peripheral), and the constructed TBM (TBM). (F) Degradation curve of the HAMA, the Core, the Peripheral, and the TBM in pure phosphate-buffered saline (PBS) or collagenase II-containing PBS. (G) Rheological behavior of the HAMA, the Core, the Peripheral, and the TBM. Hollow indicates G". Solid indicates G'. (H) Verification of the slow release of small-molecule drugs by TBM. (I) Verification of the slow release of nucleic acids by TBM in the presence or absence of polyvinylamine (PVAm).
Note: Organic: Organic network formed by silk protein, chitosan, and matrigel.

(Figure S1A). Energy dispersive spectrometry analysis identified the elemental components of the TBM, including calcium, chlorine, sulfur, sodium, oxygen, and carbon. Key elements, such as calcium, phosphorus, and sulfur, were uniformly distributed throughout the TBM (Figures 1B and C, S1B), whereas FTIR spectroscopy revealed no unusual chemical groups in the TBM (Figure 1D).

In general, swelling can severely compromise material structures and mechanical properties. Here, the swelling and degradation rates of the HAMA hydrogel, the HAMA-embedded core, the HAMA-embedded peripheral porous framework, and the TBM were measured. All materials demonstrated stable swelling ratios and maintained a dissolution equilibrium state for 42 d (Figure 1E). In contrast, in the presence of collagenase (100 U/mL), the TBM degraded faster compared to the HAMA hydrogel group (Figure 1F). These results indicate that the TBM maintains structural stability over extended periods while exhibiting faster degradation rates than the HAMA hydrogel alone, making it suitable for prolonged bone-

filling and drug delivery applications, as well as facilitating bone regeneration at defect regions.

The elastic modulus of the materials was also measured. We observed that the core markedly enhanced the crush resistance of the HAMA hydrogel, whereas the TBM exhibited superior elasticity and material strength compared to the other groups, addressing the mechanical load-bearing requirements for a potential bone-filling material (Figure 1G).

The TBM also demonstrated the ability to carry and slow-release drugs. To evaluate small-molecule slow-release properties, we used rhodamine B and demonstrated that the TBM slow-released this drug within 4 days (Figure 1H). In addition, the sustained release of nucleic acid drugs was investigated using FAM-NC. Importantly, the TBM effectively loaded and slow-released nucleic acid drugs within 4 days. When combined with the nucleic acid delivery vector PVAm,[21] the TBM's slow-release capability was markedly enhanced, with high fluorescence signals persisting until day 14 (Figure 1I). These TBM's slow-release properties findings highlight its potential as a bone-filling material for the repair of bone defects.

3.2. Effect of the TBM on *in vitro* osteogenic differentiation

We evaluated the *in vitro* cytocompatibility of the TBM by immersing it in cell culture media and assessing its effects on cell activity, the cell cycle, and cell adhesion (Figure 2A). CCK-8 results showed that the activity of hMSCs remained largely unchanged over 72 h in the presence of the TBM compared to the untreated group (Figure 2B). Cell cycle analysis further confirmed that the TBM did not alter the distribution of cell cycle phase in hMSCs compared to untreated cells or HAMA-treated cells (Figure S2A). In addition, hemolysis assays demonstrated that the TBM caused no hemolysis (Figure S2B and S2C). Collectively, these results indicate that the TBM exhibits no cytotoxic effects.

To assess the TBM's potential as an osteogenic drug carrier, we selected the small-molecule drug bergamottin and the nucleic acid drug recombinant miR-138-5p antagonist, both of which have been previously shown to significantly promote osteogenic differentiation.[32,33] Blank TBM with DMSO was used as the control group for bergamottin, whereas MSA served as the control for the recombinant miR-138-5p antagonist. The drugs were loaded into the TBM, and their effects on osteogenic differentiation levels in hMSCs were evaluated. ALP and ARS staining demonstrated that the TBM loaded with either bergamottin or the miR-138-5p antagonist markedly increased ALP activity and mineralization nodule formation rates in hMSCs (Figure 2C). RT-PCR analysis further confirmed that both drug-loaded TBMs significantly promoted osteogenic differentiation in hMSCs, as evidenced by the increased expression of osteogenic marker genes (*ALP* and *RUNX2*) (Figure 2D and E). In addition, RNA-seq was performed on ADSCs treated with drug-loaded TBM (Figure 2F). After log transformation, DEGs induced by bergamottin or the recombinant miR-138-5p antagonist were identified (Figure 2G). GO and KEGG pathway enrichment analyzes revealed that these DEGs are mainly involved in extracellular matrix organization, ossification, and biomineralization processes (Figure 2H and I).

Based on this drug-carrying capacity, we also investigated the long-term slow-release effects of the TBM for osteogenic drugs. Drug-loaded TBM was immersed in high-glucose DMEM to monitor its release effects. The medium was collected on days 7, 14, 21, and 28 to treat hMSCs. We observed that the TBM loaded with the miR-138-5p antagonist consistently inhibited miR-138-5p expression in hMSCs for up to 28 d (Figure 3A). Subsequent RT-PCR, ALP, and ARS staining results demonstrated that the TBM loaded with bergamottin or the miR-138-5p antagonist promoted osteogenic differentiation over a long period. Specifically, the TBM loaded with bergamottin maintained osteogenic promotional effects for 21 d, while the TBM loaded with the miR-138-5p antagonist maintained these effects for 28 d (Figure 3B-E). These findings indicate that the TBM can slowly release drugs and exert long-term regulatory effects on osteogenic differentiation.

3.3. Cell-embedded TBM as a potential organoid

We further investigated TBM's potential as an organoid by embedding cells into its matrix. ADSCs were used to minimize the risk of immunological rejection. ADSCs were embedded into both the TBM and HAMA hydrogel and cultured in a flowing cultural medium (Figure 4A). CCK-8 assays demonstrated that ADSCs embedded in the TBM remained highly active after 72 h (Figure 4B). EdU staining revealed that embedded ADSCs maintained a high proliferation rate of over 50% for 2 weeks, whereas calcein AM/PI double staining confirmed cell viability remained at approximately 85% during the same period (Figure 4C and D). When ADSCs were cultured in an osteogenic medium for 72 h, alizarin red-alcian blue staining and safranin O staining showed that cells were distributed both within the hydrogel layers and on the Porous (Figure 4E and F) of the TBM, suggesting that the TBM supports osteogenic cell growth in skeletal structures to facilitate potential trabecular bone development. Micro-CT imaging revealed the formation of a trabecular-like structure in the ADSCs-embedded TBM after 1 week of culture. The trabecular density increased markedly over 3 weeks of culture (Figure 4G), implying that ADSCs could form trabecular-like bone within the TBM. We also embedded ADSCs along with osteogenic drugs

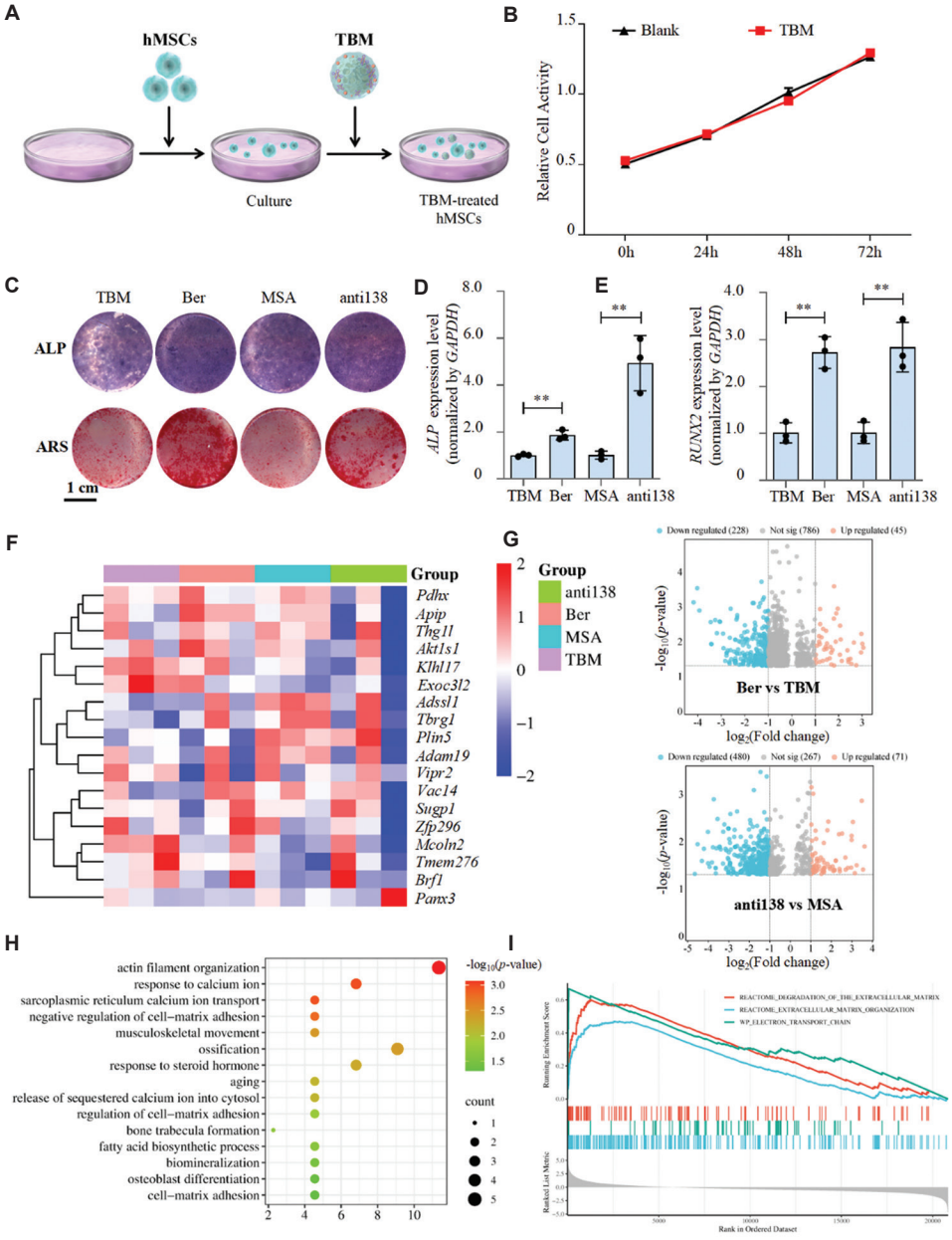

Figure 2. Effects of drug-loaded trabeculae-like biomimetic bone-filling material (TBM) on the regulation of osteogenic differentiation *in vitro*. (A) Schematic diagram of TBM-induced osteogenic differentiation *in vitro* in human mesenchymal stem cells (hMSCs). (B) Cytocompatibility of the TBM was detected by Cell Counting Kit-8. (C) Alkaline phosphatase (ALP) and alizarin red S (ARS) staining of the hMSCs treated with TBM loaded with either bergamottin (Ber; compare with blank TBM [TBM]) or recombinant miR-138-5p antagonist (anti138; compare with TBM loaded with empty recombinant tRNA [MSA]) (scale bar: 1 cm; magnification: ×1.4). (D and E) The *Alp* and *Runx2* expression levels in hMSCs of the TBM, Ber, MSA, and anti138 groups were detected by reverse transcription-polymerase chain reaction (data represented as mean ± SD, $n = 3$). (F) Selected area of the RNA-sequencing heatmap in mouse adipose-derived mesenchymal stem cells (ADSCs) in the TBM, Ber, MSA, and anti138 groups. (G) Volcano plot of differentially expressed genes (DEGs) between TBM and Ber groups (up), or between MSA and anti138 groups (below). (H and I) Top osteogenic Kyoto Encyclopedia of Genes and Genomes pathways (H) and gene set enrichment analysis (I) of DEGs between MSA and anti138 groups.
Note: $^{**}p<0.01$.

into the TBM. After 72 h of *in vitro* culture, the protein and mRNA expression levels of ALP and RUNX2 were assessed using immunofluorescence, western blotting, and RT-PCR. Both ALP and RUNX2 levels in either bergamottin or miR-138-5p antagonist-loaded TBM were markedly increased, indicating that osteogenic cells embedded in the TBM could undergo differentiation similarly to conventionally cultured cells (Figure 4H-K, Figure S6).

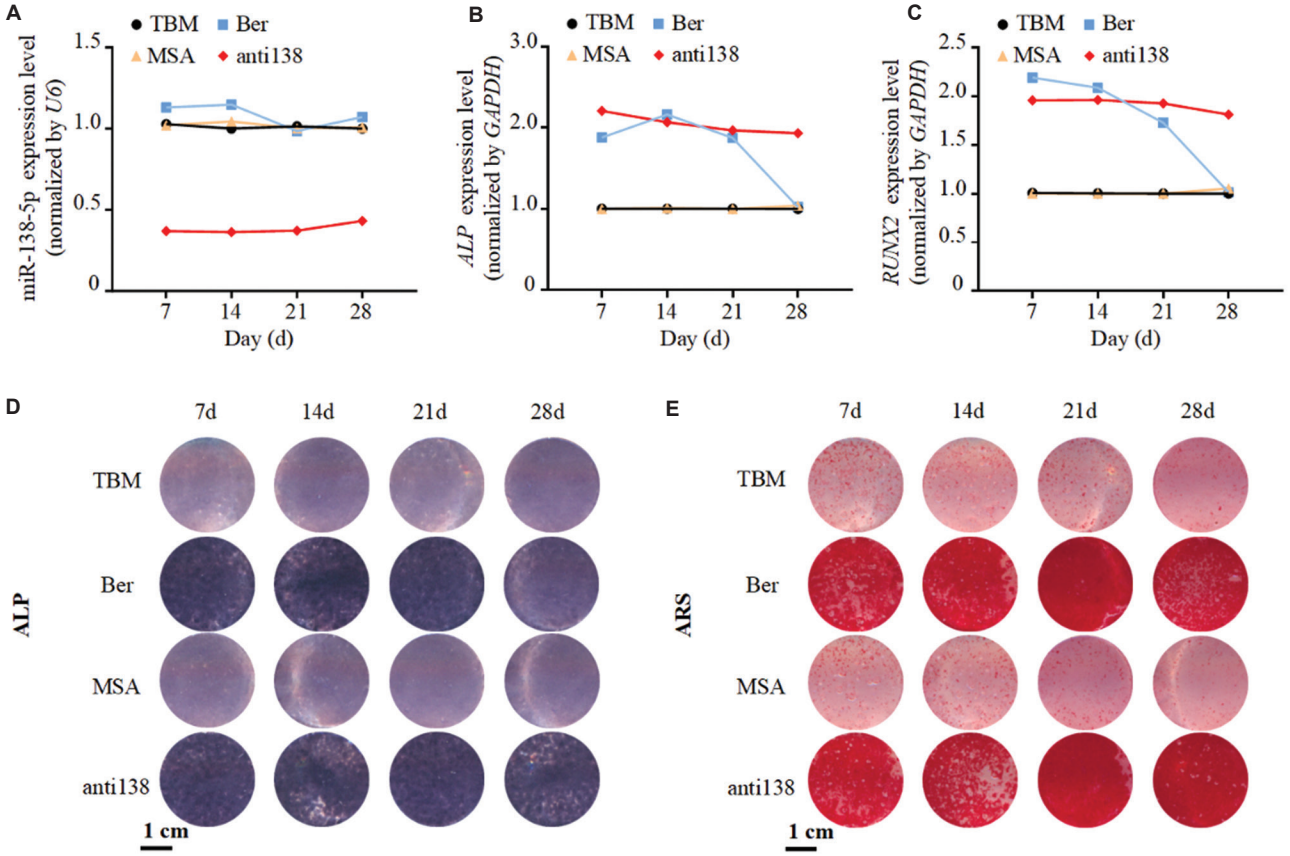

Figure 3. Slow-release effect of the trabeculae-like biomimetic bone-filling material (TBM) *in vitro.* (A-C) The expression levels of miR-138-5p (A), *Alp* (B), and *Runx2* (C) in human mesenchymal stem cells treated with slow-release solution from TBM loaded with either bergamottin (Ber; compare with blank TBM [TBM]) or recombinant miR-138-5p antagonist (anti138; compare with TBM loaded with empty recombinant tRNA [MSA]) were detected by transcription-polymerase chain reaction ($n = 3$). (D and E) Alkaline phosphatase (ALP; D) and alizarin red S (ARS; E) staining of hMSCs in the TBM, Ber, MSA, and anti138 groups at different time points (scale bar: 1 cm; magnification: ×2).

3.4. Reparative effect of the TBM organoid on bone defects

Based on these findings, we conducted *in vivo* analyzes by subcutaneously implanting ADSC-embedded TBM over the calvarial surface of cell-donor mice (Figure 5A). Mice were sacrificed on day 10, and TBM samples were collected for cell activity assays. The *in vivo* implantation showed no cytotoxicity effects on the embedded ADSCs (Figure 5B). In addition, the TBM implantation exerted no hepatorenal toxicity in mice, as evidenced by no significant changes in the levels of CD3 and CD68 inflammatory factors in attached bone tissue (Figure 5C-E). Meanwhile, immunofluorescence imaging of ACTIN demonstrated that the activity of embedded ADSCs *in vivo* was comparable to that of embedded cells cultured *in vitro* (Figure S3).

To further investigate the osteogenic potential of the TBM *in vivo*, ADSCs were transfected with the recombinant miR-138-5p antagonist and embedded into TBM. The control group included ADSCs transfected with MSA. Both TBM organoids were implanted over the calvarial surface of mice for 10 d respectively. Immunofluorescence imaging of ALP, RUNX2, and CD31 revealed that the recombinant miR-138-5p antagonist-transfected ADSCs exhibited markedly enhanced osteogenic differentiation, while no differences in angiogenesis were observed (Figure 5F). Moreover, TBM containing recombinant miR-138-5p antagonist-treated ADSCs was implanted into the bone defect regions of either tibial or calvarial defection mice model. Micro-CT imaging demonstrated that the recombinant miR-138-5p antagonist markedly promoted bone regeneration in both defect models (Figure 5G and H). The BMD, BV/TV, and BMC values at bone defect regions were significantly increased (Figure 5I-N). These results indicate that the TBM supports the viability and functionality of embedded cells without adversely affecting the embedded cells and host physiological properties. Therefore, the TBM shows high potential as an organoid for bone defect therapy.

3.5. The therapeutic effects of the drug-loaded TBM in bone defect mouse model

Based on our observation that the TBM showed high biocompatibility *in vivo*, we investigated its therapeutic

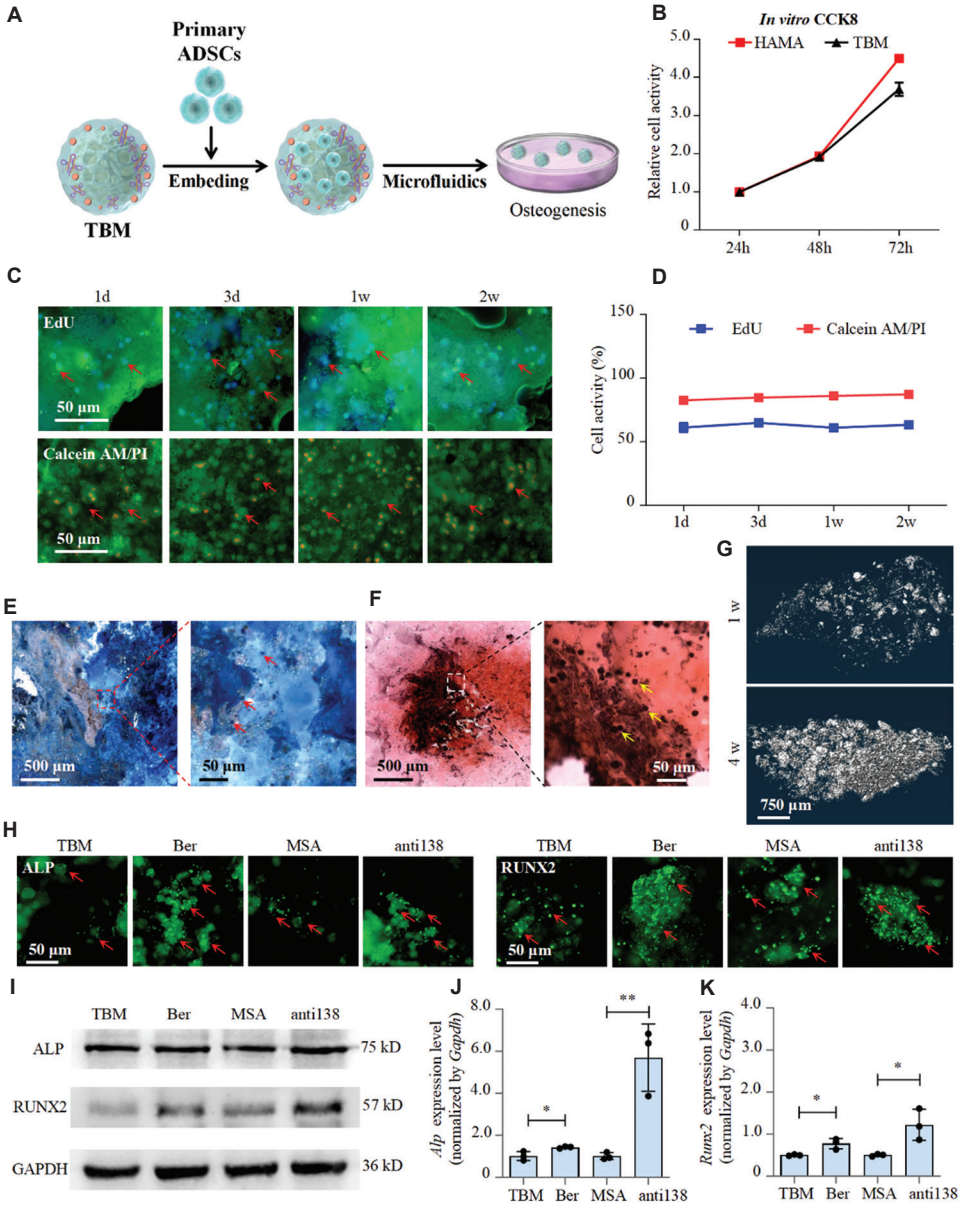

Figure 4. The potential of trabeculae-like biomimetic bone-filling material (TBM) as an organoid. (A) Schematic diagram of TBM embedding mouse adipose-derived mesenchymal stem cells (ADSCs) *in vitro*. (B) Cell viability of ADSCs embedded in HAMA hydrogel or TBM cultured *in vitro*, as detected by Cell Counting Kit-8. (C and D) Cell proliferation and viability of ADSCs embedded in HAMA hydrogel or TBM cultured *in vitro*, as detected by 5-ethynyl-2'-deoxyuridine (EdU) staining and Calcein AM/PI staining (C: scale bar: 50 μm; magnification: ×23). Red arrows indicate live and dead cells in the first and second rows, respectively. (E and F) Distribution of ADSCs in TBM, as detected by alizarin red-alcian blue staining (E; Scale bar: 50μm; Magnification: ×17 and ×126 for left images and right images, respectively) and safranin O staining (F; scale bar: 50μm; magnification: ×126). Red and yellow arrows indicate the ADSCs in the hydrogel layer or upon the porous skeletal structure. (G) Micro-computed tomography reconstructed images of the ADSCs embedded in TBM and cultured *in vitro* for 1 week (up) and 4 weeks (below) (scale bar: 750 μm; magnification: ×12). (H and I) Protein expression of alkaline phosphatase (ALP) and runt-related transcription factor 2 (RUNX2) in ADSCs cultured in TBM, as detected by immunofluorescence staining (H) and western blotting (I). Red arrows indicate ALP and RUNX2 expression in cells. (J and K) Expression levels of *Alp* and *Runx2* in ADSCs cultured in TBM, as detected by transcription-polymerase chain reaction (data represented as mean ± SD, $n = 3$).
Notes: TBM: Blank TBM; Ber: TBM loaded with bergamottin; MSA: TBM loaded with empty recombinant tRNA; anti138: TBM loaded with the recombinant miR-138-5p antagonist; *$p<0.05$; **$p<0.01$.

effects in bone defect mouse models. Tibial and calvarial bone defect mouse models were constructed, and the TBM loaded with either bergamottin or miR-138-5p antagonist was implanted into the defect regions. After 3 weeks, mice were humanely sacrificed and the TBM's therapeutic effects on bone defects were evaluated (Figures 6A and 7A). The TBM exhibited no hepatorenal toxicity in mice and did not affect the morphology of tissues in several organs,

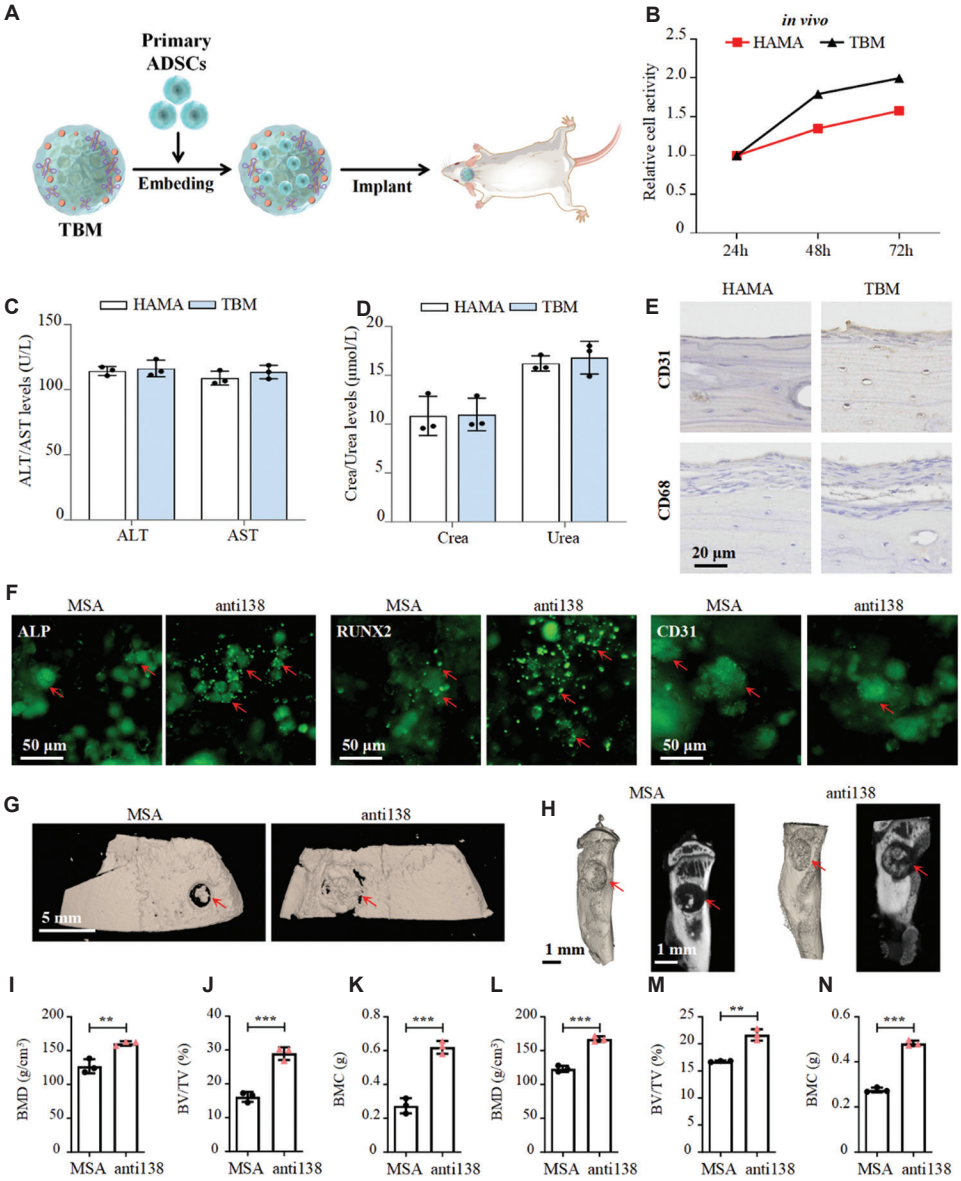

Figure 5. Reparative effect of the trabeculae-like biomimetic bone-filling material (TBM) organoid on bone defection. (A) Schematic diagram of the TBM embedding mouse adipose-derived mesenchymal stem cells (ADSCs) *in vivo*. (B) Cell viability of ADSCs embedded in organoid-implanted mice, as detected by Cell Counting Kit-8. (C and D) Alanine transaminase (ALT), aspartate aminotransferase (AST), creatinine (Crea), and blood urea nitrogen (Urea) levels in blood serum of organoid-implanted mice ($n = 3$). (E) Images of immunohistochemical staining of inflammatory factors cluster of differentiation CD31 and CD68 in calvaria of organoid-implanted mice (scale bar: 20 μm; magnification: ×350). (F) Expression of alkaline phosphatase (ALP), runt-related transcription factor 2 (RUNX2), and CD31 in organoid-implanted mice *in vivo* for 10 d, as detected by immunofluorescence staining (scale bar: 50 μm; magnification: ×182). Red arrows indicate the ALP, RUNX2, and CD31 expression in cells. (G and H) Micro-computed tomography reconstructed images of organoid-implanted calvarial (G; scale bar: 5 mm; magnification: ×2.4) or tibial (H; scale bar: 1 mm; magnification: ×3.9 and ×5 for the image on left and right, respectively) defect mouse models after 3 weeks. Red arrows indicate bone defect areas. (I-N) Quantitative analyzes of the bone mineral density (BMD; I and L), bone volume to tissue volume (BV/TV; J and M), and bone mineral content (BMC; K and N) in (G) and (H) (data represented as mean ± SD, $n = 3$). Notes: MSA: TBM embedding ADSCs treated by empty recombinant tRNA; anti138: TBM embedding ADSCs treated by the recombinant miR-138-5p antagonist; $^{**}p<0.01$; $^{***}p<0.001$.

including the heart, liver, spleen, lung, and kidney (Figure S4), confirming its excellent biocompatibility in mice. Furthermore, we examined the pharmacokinetics of the TBM in tibial defect mice implanted with the TBM carrying the miR-138-5p antagonist. Analysis revealed that the inhibitory effects of miR-138-5p were maintained for 28 d in the tibia, with no significant changes in other organs (Figure S5), indicating that the TBM effects were specific to the tibia.

Both the HE staining and micro-CT imaging showed that the TBM implantation filled the bone defect regions,

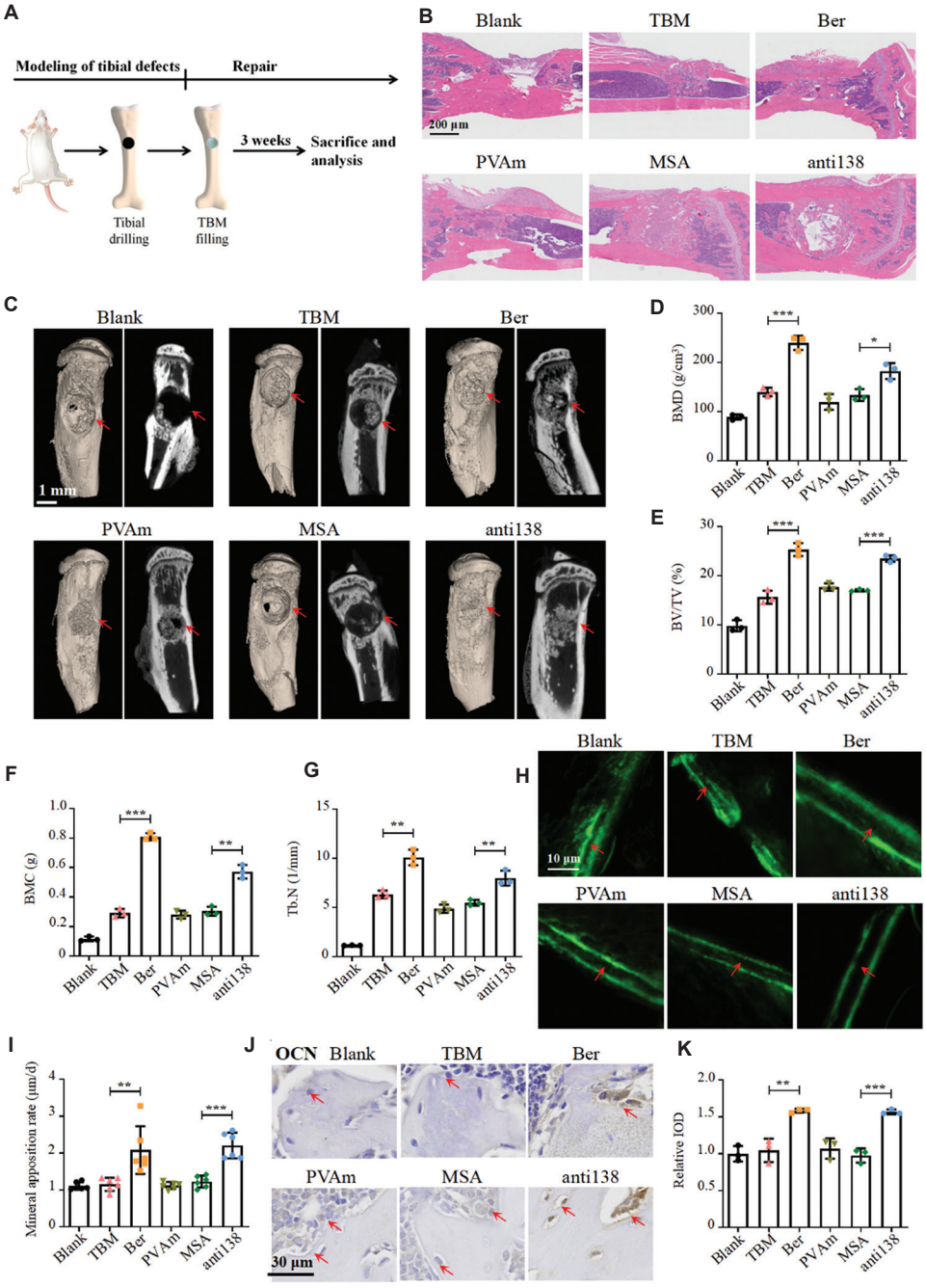

Figure 6. Effect of the trabeculae-like biomimetic bone-filling material (TBM) on i*n situ* bone regeneration in tibial defect mice. (A) Schematic diagram of TBM treatment on tibial defect mice. (B) Representative hematoxylin and eosin staining images of mice tibial defect region (Scale bar: 200 μm; Magnification: ×32.5). (C) Micro-computing tomography reconstructed images of tibial defect after 3 weeks (scale bar: 1 mm; magnification: ×4.5). Red arrows indicate bone defect areas. (D-G) Quantitative analyzes of the bone mineral density (BMD; D), bone volume to tissue volume (BV/TV; E), trabecular number (Tb.N; F), and bone mineral content (BMC; G) in (C). (H and I) Representative images show the mineral apposition rate of mice tibial defect regions (H; scale bar: 10 μm; magnification: ×700) and quantitative analysis of the mineral apposition rate (I). Red arrows indicate the done formation line distance labeled with calcein. (J and K) Osteocalcin (OCN) staining images of tibial defect region after 3 weeks as detected by immunohistochemical staining (J; scale bar: 30 μm; Magnification: ×350) and quantification of related integrated optical density (IOD) values (K). Red arrows indicate OCN-positive cells.

Notes: All data in bar graphs are represented as mean ± SD, n = 3; Blank: No treatment for the bone defect region; TBM: TBM filling the bone defect region; Ber: TBM loaded with bergamottin filling the bone defect region; Polyvinylamine (PVAm): TBM loaded with PVAm filling the bone defect region; MSA: TBM loaded with empty recombinant tRNA (loaded by PVAm) filling the bone defect region; anti138: TBM loaded with recombinant miR-138-5p antagonist (loaded by PVAm) filling the bone defect region; *p<0.05; **p<0.01; ***p<0.001.

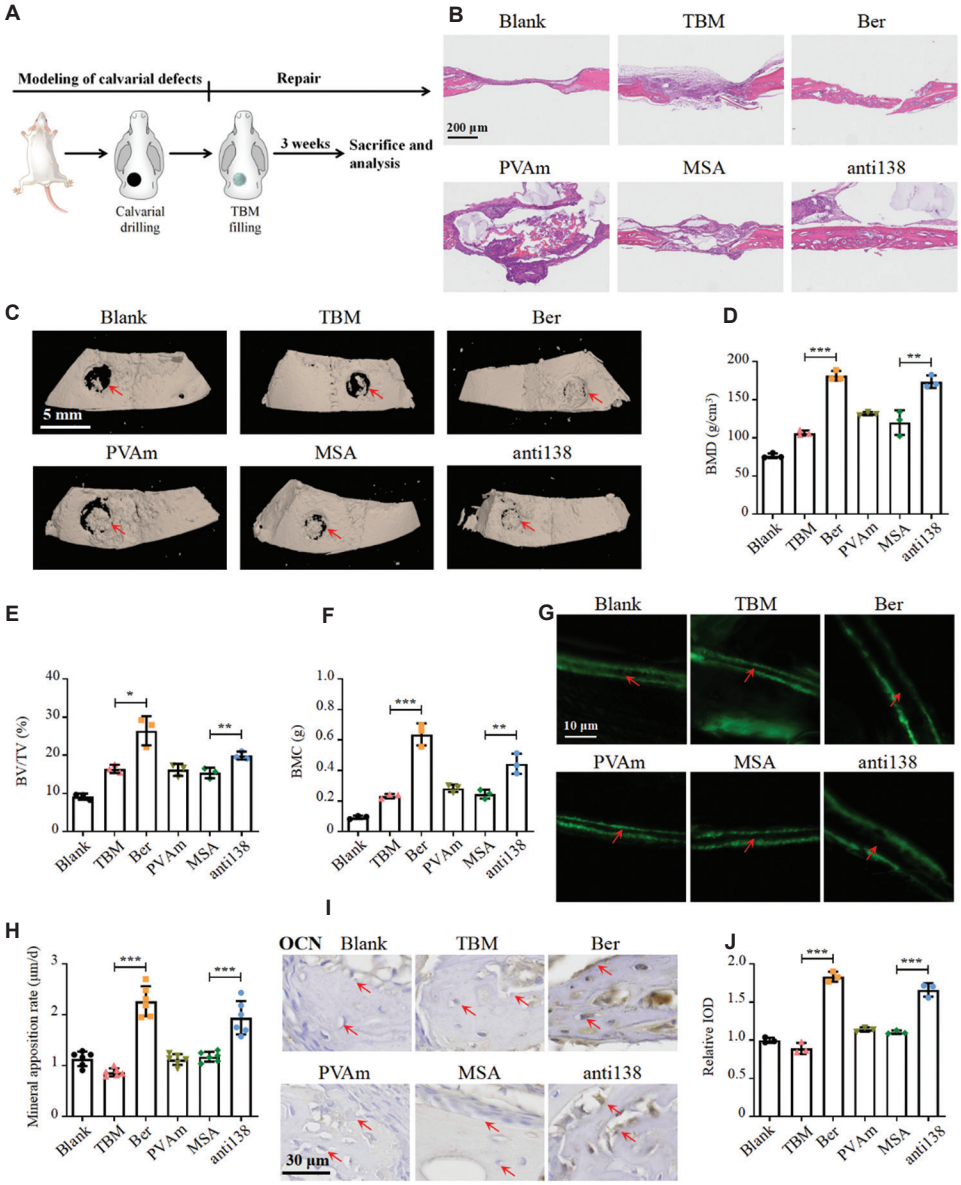

Figure 7. Effects of the trabeculae-like biomimetic bone-filling material (TBM) on *in situ* bone regeneration in calvarial defect mice. (A) Schematic diagram of the TBM treatment on calvarial defect mice. (B) Representative hematoxylin and eosin staining images of mice calvarial defect region (scale bar: 200 μm; magnification: ×32.5). (C) Microcomputing tomography reconstructed images of calvaria defect after 3 weeks (scale bar: 5 mm; magnification: ×2.16). Red arrows indicate a bone defect area. (D-F) Quantitative analyzes of bone mineral density (BMD; D), bone volume to tissue volume (BV/TV; E), and bone mineral content (BMC; F) in (C). (G and H) Representative images show the mineral apposition rate of mice's calvarial defection region (G; scale bar: 10 μm; magnification: ×700) and quantitative analysis of the mineral apposition rate (H). Red arrows indicate bone formation line distance labeled with calcein. (I and J) Osteocalcin (OCN) staining images after 3 weeks as detected by immunohistochemical staining (I; scale bar: 30 μm; magnification: ×350) and quantification of related integrated optical density (IOD) values (J; data represented as mean ± SD, $n = 3$). Red arrows indicate OCN-positive cells.

Notes: All data in bar graphs are represented as mean ± SD, n = 3; Blank: No treatment for the bone defect region; TBM: TBM filling the bone defect region; Ber: TBM loaded with bergamottin filling the bone defect region; Polyvinylamine (PVAm): TBM loaded with PVAm filling the bone defect region; MSA: TBM loaded with empty recombinant tRNA (loaded by PVAm) filling the bone defect region; anti138: TBM loaded with recombinant miR-138-5p antagonist (loaded by PVAm) filling the bone defect region; *$p<0.05$; **$p<0.01$; ***$p<0.001$.

while osteogenic drug-loaded TBM markedly promoted bone repair (Figures 6B and C, 7B and C). The values of bone apposition rates, BMD, BV/TV, BMC, and tibial Tb.N at bone defect regions were significantly increased (Figures 6D-I and 7D-H). Moreover, immunohistochemical staining for OCN in osteoblast demonstrated significantly higher intensity in osteoblast within the defected regions treated with either bergamottin or miR-138-5p antagonist-loaded

TBM (Figures 6J and K, 7I and J), indicating effective bone formation.

Masson's trichrome and Goldner's trichrome staining further confirmed that more new bone formation occurred in defect regions treated with either bergamottin or miR-138-5p antagonist-loaded TBM (Figures 8A and B, 9A and B). The CD3 and CD68 immunohistochemical staining and TRAP staining revealed no significant changes in inflammation and bone resorption in the defect areas (Figures 8C-H, 9C-H). Meanwhile, RUNX2 immunohistochemical staining showed that either bergamottin or miR-138-5p antagonist-loaded TBM significantly enhanced osteogenic differentiation in bone defect regions (Figures 8I and J, 9I and J). Collectively, these results indicate that the TBM served as an excellent bone-filling material.

3.6. The therapeutic effects of the drug-loaded TBM in fracture mouse model

To expand the potential applications of the TBM, we constructed a tibial fracture mouse model and implanted the TBM at the fracture sites. After 4 weeks, the mice were humanely euthanized to evaluate the TBM's therapeutic effects on fractures (Figure 10A). Micro-CT and calcein AM/PI double staining revealed that the implantation of TBM loaded with either bergamottin or the miR-138-5p antagonist significantly enhanced fracture repair, as well as increased BMD, BV/TV, BMC, and bone apposition rates at the fracture regions (Figures 10B-G). These results demonstrate that the TBM can serve as a potential therapeutic platform for fracture repair, mediated through the slow release of osteogenic drugs.

4. Discussion

At present, the treatment of bone defects mainly relies on the surgical implantation of bone-filling materials and bone healing via intrinsic repair capability.[34] Ideal bone substitutes for defect repair must not only exhibit degradability and biocompatibility but also possess sufficient mechanical strength to provide structural support. In addition, they should promote osteogenic differentiation and angiogenesis to accelerate bone formation and repair processes. Although autologous bone transplantation is the current gold standard for bone defect treatment, it is not widely used due to donor scarcity.[6,7] In this study, we designed and synthesized a bone-filling material, termed TBM, which closely mimics the structure and composition of natural trabecular bone.

The TBM is a composite material consisting of a central porous framework and a peripheral hydrogel. The porous framework is composed of chitosan, collagen, BCP, and silk fibroin. Apart from the silk fibroin, the other components are naturally present in bone tissue, which minimizes the risk of foreign body reactions and enhances the TBM's biocompatibility and biodegradability.[35] Moreover, BCP, a mixture of hydroxyapatite and tricalcium phosphate in skeleton structures, combines the advantages of both calcium salts. As an inorganic component in bone tissue, BCP provides excellent biocompatibility and mechanical strength.[36,37]

A random porous bone tissue material formed by freeze-drying cannot guarantee mechanical strength comparable to that of natural trabecular bone.[38] To address this limitation, we adopted a two-stage fabrication strategy during the design of TBM. In the first stage, the material was lyophilized within a limited volume, forming a core skeleton (diameter = 0.7 – 3.0 mm) with enhanced mechanical strength due to water crystallization. In the second stage, rapid freezing and lyophilization generated a multilayer pore structure (50 – 250 μm pore size), closely resembling natural trabecular bone. This design significantly improved the TBM's mechanical support compared to a single-stage freeze-dried porous material. Thus, the TBM demonstrated sufficient strength derived from covalent bonding between proteins in the material, eliminating the need for crosslinking agents that might compromise biosafety. In addition, the number, size, and distribution of micropores on the surfaces of bone-filling material significantly affect bone tissue metabolism and proliferation. In general, pore sizes of 150 – 800 μm promote nutrient transport and metabolic waste excretion, whereas smaller pore sizes of 40 – 100 μm support non-mineralized tissue growth.[11] As the size of the micropores on the TBM's surfaces falls within the range of 50 – 250 μm, it demonstrates great potential to promote cell growth and tissue regeneration while withstanding stress (Figure 1).

Hydrogels are highly favored in tissue engineering due to their excellent plasticity, biocompatibility, and drug-loading capacity.[39,40] The application of hydrogels to bone-filling materials may enhance their toughness, biocompatibility, and drug delivery capacity. HAMA hydrogels, in particular, are widely used for cell and drug encapsulation due to their spatial structures, which make them suitable for cell growth and provide excellent biocompatibility and degradability characteristics.[41,42] HAMA synthesis involves crosslinking water-soluble HAMA with the DMSO-soluble photoinitiator I2959, enabling the simultaneous encapsulation of both water-soluble and organic-soluble drugs for modular drug delivery.[43] In our study, HAMA was used to encapsulate the TBM's porous skeletons and load either the organic-soluble bergamottin or the water-soluble miR-138-5p antagonist.[32,33] This design endowed the TBM with long-term, slow-release drug properties, promoting osteogenic differentiation and demonstrating the potential for treating various bone defects (Figures 2, 4-6).

To further enhance the TBM's therapeutic efficacy in bone defect repair, we explored its potential as an organoid.

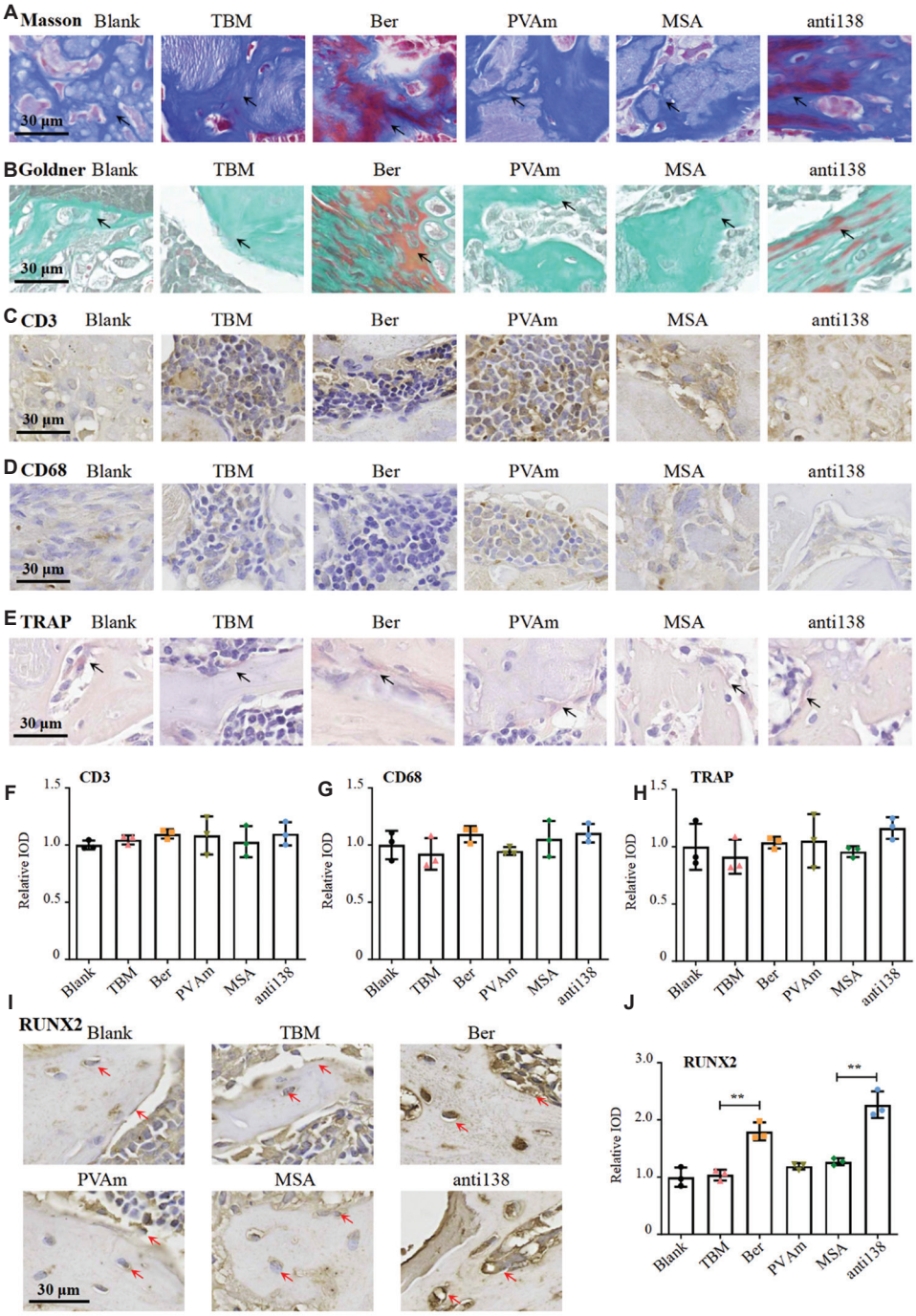

Figure 8. Effects of the trabeculae-like biomimetic bone-filling material (TBM) on *in situ* bone regeneration, inflammation, resorption, and osteogenic differentiation in tibial defect mice. (A) Masson's trichrome staining of tibial defect regions (Scale bar: 30 μm; Magnification: ×400). Black arrows indicate a new bone regeneration region. (B) Goldner's trichrome staining of tibial defect regions (Scale bar: 30 μm; Magnification: ×400). Black arrows indicate a new bone regeneration region. (C and D) Immunohistochemical images of inflammatory factors cluster of differentiation CD3 (C) and CD68 (D) in tibial defect regions (scale bar: 30 μm; magnification: ×400). (E) Representative images of tartrate-resistant acid phosphatase (TRAP) staining in tibial defect regions (scale bar: 30 μm; magnification: ×400). Black arrows indicate bone resorption area. (F-H) Quantification of corresponding integrated optical density (IOD) of immunohistochemical staining of CD3 (F), CD68 (G), and TRAP staining (H). (I and J) Runt-related transcription factor 2 (RUNX2) staining images of tibial defect region as detected by immunohistochemical staining (I; scale bar: 30 μm; magnification: ×450) and quantification of corresponding IOD (J). Red arrows indicate RUNX2 positive cells.

Notes: All data in bar graphs are represented as mean ± SD, n = 3; Blank: No treatment for the bone defect region; TBM: TBM filling the bone defect region; Ber: TBM loaded with bergamottin filling the bone defect region; Polyvinylamine (PVAm): TBM loaded with PVAm filling the bone defect region; MSA: TBM loaded with empty recombinant tRNA (loaded by PVAm) filling the bone defect region; anti138: TBM loaded with recombinant miR-138-5p antagonist (loaded by PVAm) filling the bone defect region; $^{**}p<0.01$.

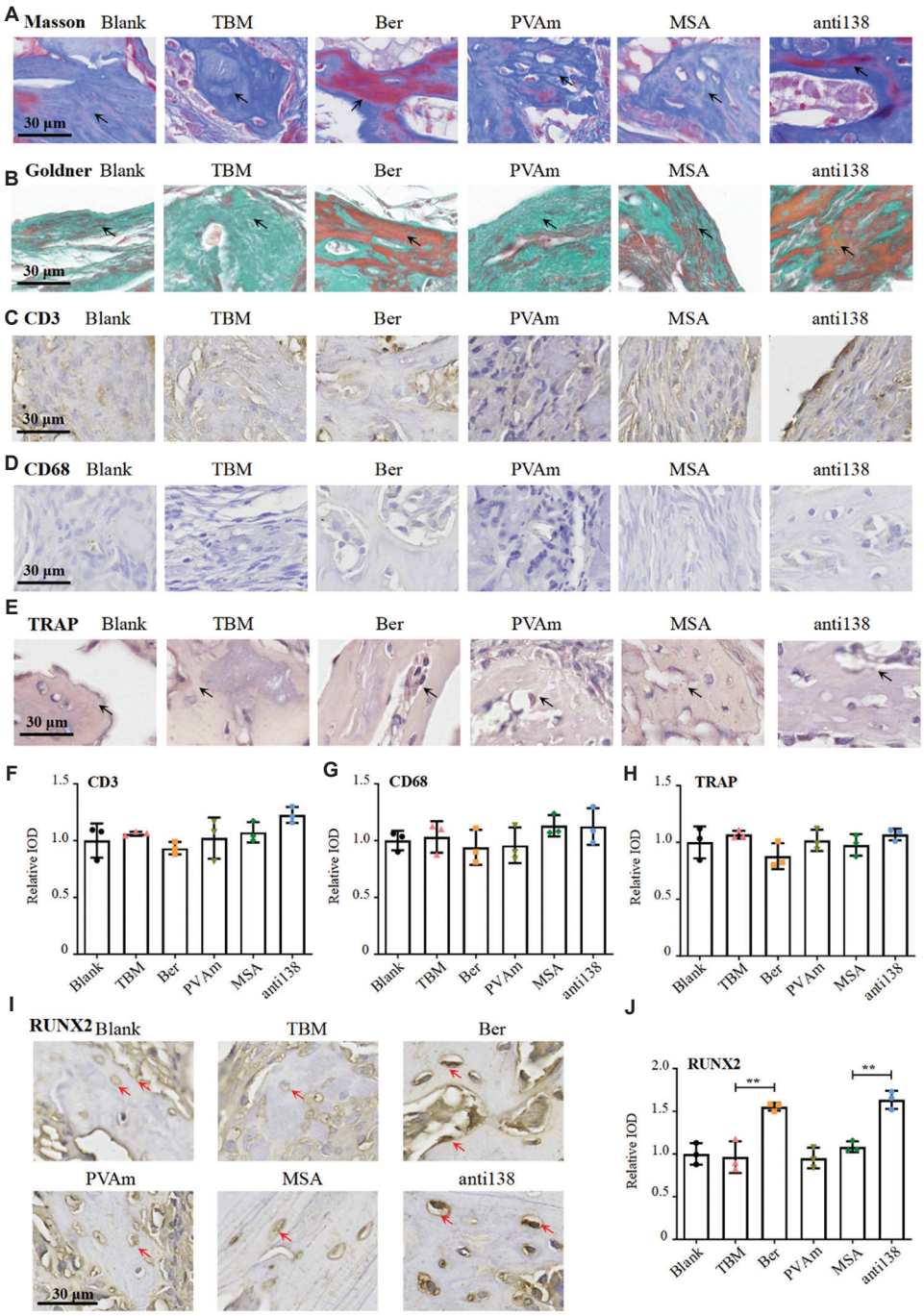

Figure 9. Effects of the trabeculae-like biomimetic bone-filling material (TBM) on *in situ* bone regeneration, inflammation, resorption, and osteogenic differentiation in calvarial defect mice. (A) Masson's trichrome staining of calvarial defect regions (scale bar: 30 μm; magnification: ×400). Black arrows indicate a new bone regeneration region. (B) Goldner's trichrome staining of calvarial defect regions (Scale bar: 30 μm; Magnification: ×400). Black arrows indicate a new bone regeneration region. (C and D) Immunohistochemical images of inflammatory factors cluster of differentiation CD3 and CD68 in calvarial defect regions (scale bar: 30 μm; magnification: ×400). (E) Representative images of tartrate-resistant acid phosphatase (TRAP) staining in calvarial defect region (scale bar: 30 μm; magnification: ×400). Black arrows indicate bone resorption area. (F-H) Quantification of corresponding integrated optical density (IOD) values of immunohistochemical staining of CD3 (F), CD68 (G), and TRAP staining (H). (I and J) Runt-related transcription factor 2 (RUNX2) staining images of calvarial defect regions as detected by immunohistochemical staining (I; scale bar: 30 μm; magnification: ×450) and quantification of related IOD (J). Red arrows indicate RUNX2 positive cells.

Notes: All data in bar graphs are represented as mean ± SD, n = 3; Blank: No treatment for the bone defect region; TBM: TBM filling the bone defect region; Ber: TBM loaded with bergamottin filling the bone defect region; Polyvinylamine (PVAm): TBM loaded with PVAm filling the bone defect region; MSA: TBM loaded with empty recombinant tRNA (loaded by PVAm) filling the bone defect region; anti138: TBM loaded with recombinant miR-138-5p antagonist (loaded by PVAm) filling the bone defect region; $^{**}p<0.01$.

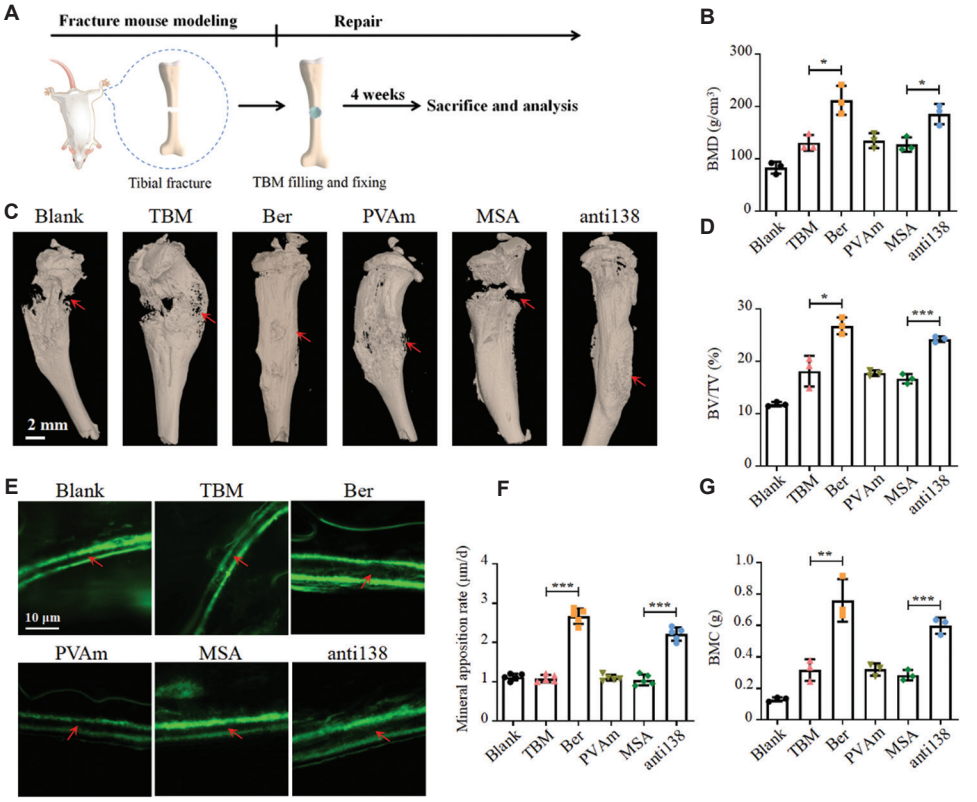

Figure 10. Reparative effect of the trabeculae-like biomimetic bone-filling material (TBM) in a mouse fracture model. (A) Schematic diagram of the TBM treatment on fracture mice. (B-D, G) Microcomputing tomography reconstructed images of fractured mice tibia after 4 weeks (C; scale bar: 2 mm; magnification: ×2) and quantitative analyzes of bone mineral density (BMD; B), bone volume to tissue volume (BV/TV; D), and bone mineral content (BMC; G) in (C). Red arrows indicate bone defect area caused by fracture. (E and F) Representative images show the mineral apposition rate of mice tibial fracture region (E; scale bar: 10 μm; magnification: ×700) and quantitative analysis of the mineral apposition rate (F). Red arrows indicate bone formation line distance labeled with calcein.

Notes: All data in bar graphs are represented as mean ± SD, n = 3; Blank: No treatment for the fracture region; TBM: TBM filling the fracture region; Ber: TBM loaded with bergamottin filling the fracture region; Polyvinylamine (PVAm): TBM loaded with PVAm filling the fracture region; MSA: TBM loaded with empty recombinant tRNA (loaded by PVAm) filling the fracture region; anti138: TBM loaded with recombinant miR-138-5p antagonist (loaded by PVAm) filling the fracture region; *p<0.05; **p<0.01; ***p<0.001.

The incorporation of RGD peptides facilitates osteogenic cell attachment to inorganic layers.[44] In this study, we used acrylate RGD peptides to not only improve cell attachment to the porous frameworks but also enhance the binding interactions between HAMA and the porous frameworks. Autologous stem cell therapy is a rapidly developing field in tissue engineering.[45] In this study, primary mouse ADSCs demonstrated an excellent osteogenic potential, with an easy extraction technique and minimal damage to the host. We also showed that the TBM supported cell attachment through both HAMA and porous framework surfaces. The embedded cells proliferated, migrated, and differentiated normally, effectively repairing bone defects (Figure 3), highlighting the potential of the TBM as an organoid for bone tissue engineering.

Despite its advantages, some imitations still restrict the application of the TBM. The slow degradation rate of HAMA hydrogel may hinder bone defect recovery, necessitating the development of novel hydrogels with improved mechanical properties and faster degradation rates. Additionally, targeted drug delivery by the TBM remains unfulfilled. Future studies will focus on integrating targeted delivery systems, such as exosomes or labeled vesicles, into the TBM. Moreover, we aim to develop cell transdifferentiation techniques to facilitate rapid *in situ* bone repair using autologous stem cells in patients, making the TBM a more efficient bone tissue organ for treating bone defects. Optimization of TBM size and hydrogel proportions will also facilitate its use as an organoid for *in vitro* osteogenic factor detection and screening.

5. Conclusion

In summary, we developed a TBM, a composite bone-filling material that closely mimics the composition and structure of natural trabecular bone. The TBM exhibits excellent mechanical properties, high biocompatibility, and the ability to slowly release small-molecule and nucleic acid drugs. It also supports the embedding of osteogenic cells,

demonstrating its potential as an organoid for bone tissue engineering. This study presents a promising approach to bone defect treatment and provides new insights for advancing bone tissue engineering research.

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Conflict of interest

The authors declare they have no competing interests.

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Ethics approval and consent to participate

All animal protocols received approval from our local Ethics Committee (Reference: 2023078, Date: September 05, 2023).

Consent for publication

Not applicable.

Availability of data

The data supporting the findings of this study are available upon reasonable request from the corresponding author, Chong Yin.

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OR

COMMENTARY

Bone marrow organoids: Decoding the three-dimensional code of hematopoietic niches

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Abstract

The bone marrow serves not only as a "factory" for hematopoiesis but also as a dynamic ecological "repository" regulating immune responses, metabolic processes, and disease progression. Its complexity stems from the three-dimensional interplay of vascular networks, mesenchymal stromal cells, hematopoietic stem cells, and immune cells - a spatial dynamism poorly captured by traditional models. Recently, the first functional human bone marrow organoids were constructed *in vitro* through multilineage differentiation and self-organization of induced pluripotent stem cells. This model accurately captures the key functional and structural characteristics of the human bone marrow hematopoietic niche, marking a significant milestone in advancing research on hematopoietic development and bone marrow diseases.

Keywords: Bone marrow organoids; Human induced pluripotent stem cells; Hematopoietic microenvironment

1. Introduction

Conventional bone marrow models often rely on single exogenous cytokines (e.g., granulocyte colony-stimulating factor) to induce hematopoiesis.[1,2] In a previous study, researchers adopted a multi-signal strategy to recapitulate developmental dynamics.[3] Specifically, they induced pluripotent stem cells through stepwise differentiation within 3 weeks to generate complex organoids composed of hematopoietic cells, mesenchymal cells, and endothelial cells. Following embryoid body formation (Day 3), mesoderm was induced using the Wnt agonist CHIR99021, bone morphogenetic protein 4, and vascular endothelial growth factor (Day 0).[4,5] Subsequently, mesodermal patterning and hemogenic endothelial induction were achieved using the activin/nodal pathway inhibitor SB431542, basic fibroblast growth factor, stem cell factor, and vascular endothelial growth factor (Day 2) (Figure 1A). This endogenous signal-driven approach not only avoids interference from exogenous factors but also mirrors the molecular logic of definitive hematopoiesis during embryogenesis.

To promote organoid self-assembly, researchers embedded differentiating embryoid bodies in a collagen I/Matrigel matrix on Day 4 to guide cellular self-organization through biomechanical microenvironments. Three-dimensional imaging revealed that vascular ($CD31^+$) networks were enveloped by perivascular platelet-derived growth factor beta, forming functional lumens. Nestin stromal cells extended protrusions that closely neighbored hematopoietic cells ($CD45^+$), recapitulating the structural features of the *in vivo* perivascular niche. This "bottom-up" assembly approach overcomes the spatial limitations of traditional co-culture systems.[6,7]

Single-cell RNA sequencing (scRNA-seq) unveiled the molecular diversity of bone marrow organoids (BMOs), including hematopoietic populations (encompassing lymphoid progenitors [interleukin-7 $receptor^+$],

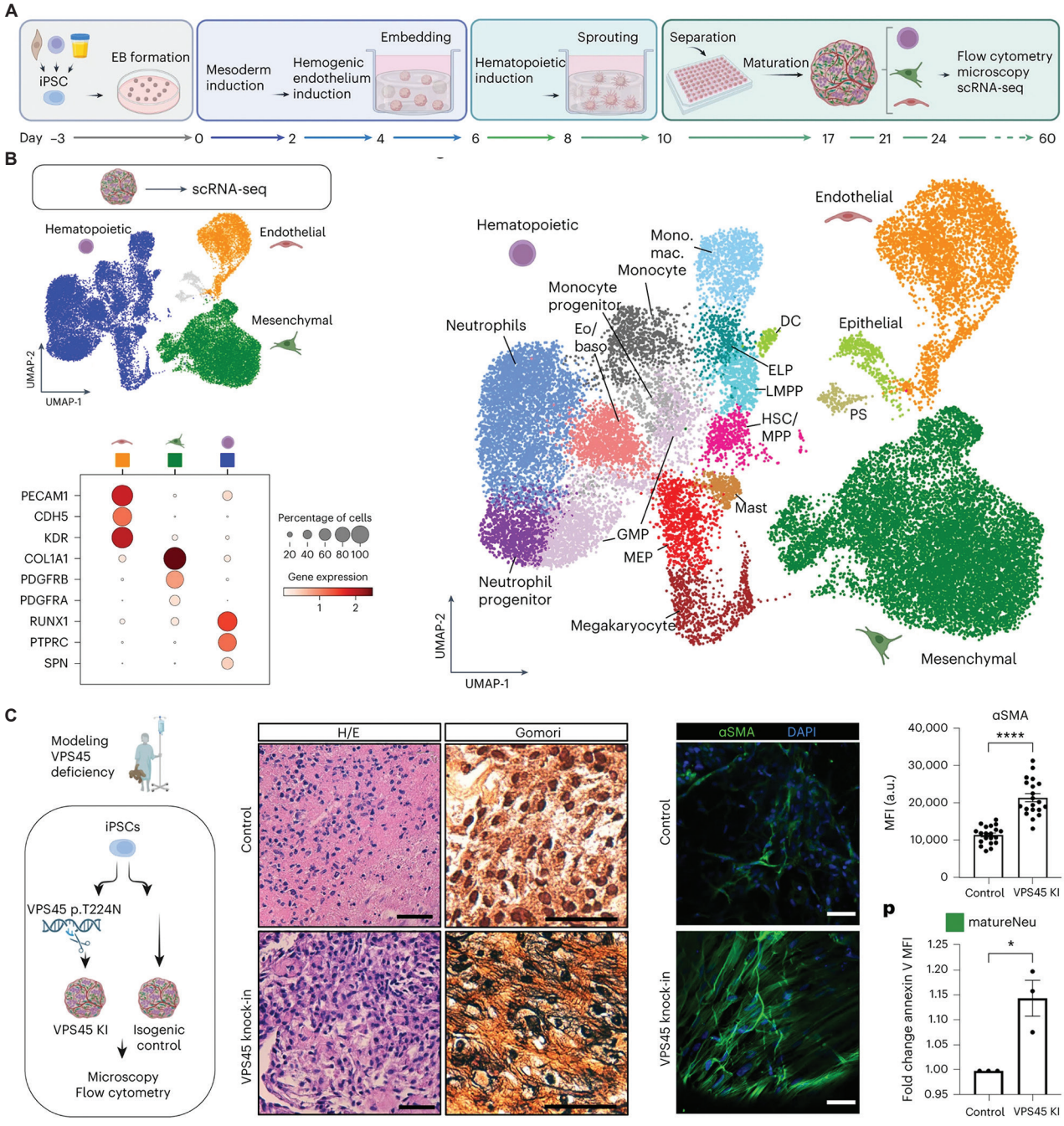

Figure 1. Strategies and representative results of constructing complex bone marrow organoids (BMOs) from human induced pluripotent stem cells. Image adapted from Frenz-Wiessner *et al.*[3] (A) Schematic illustration of the workflow for BMO generation. (B) Coarse-grained clustering of single-cell RNA sequencing data reveals three main populations comprising endothelial, hematopoietic, and mesenchymal cells. (C) Modeling of *VPS45* deficiency in BMOs and follow-up analysis. Schematic overview of gene editing and experimental setup to create an isogenic *VPS45* mutant induced pluripotent stem cell (iPSC) line. Histological comparison of control and *VPS45* mutant BMOs using hematoxylin and eosin (H/E) and Gomori stain reveals reticulin fibrosis in *VPS45* mutant BMOs; $n = 8$ organoids for each condition of two batches. Alpha smooth muscle actin (α-SMA) expression in control and *VPS45*$^-$ mutant BMOs analyzed using immunofluorescence; quantification of mean fluorescence intensity (MFI) of SMA expression; four different regions of $n = 5$ organoids per condition of two batches. Statistical significance determined at $^{****}p < 0.0001$, unpaired two-tailed *t*-test.
Abbreviations: KI: Knock-in; Neu: Neural cells.

granulocyte precursors [CCAAT/enhancer binding protein epsilon$^+$], megakaryocytes [pro-platelet basic protein$^+$], and hematopoietic stem cells [HSCs]), endothelial populations (arterial-type endothelial cells [Ephrin B2$^+$] and pre-hematopoietic endothelial cells [delta-like canonical Notch ligand 4$^+$neurogenic

locus notch homolog protein 1[+]], suggesting endothelial-to-hematopoietic transition), and mesenchymal populations (pericytes [angiopoietin 1[+]], vascular smooth muscle cells [actin alpha 2[+]], and osteochondral precursors [paired related homeobox 1[+]]). This high-resolution molecular map provides a foundation for analyzing cellular interactions within the niche (Figure 1B).

2. Paradigm shift: From static models to dynamic simulation

Unlike previous organoids modeling yolk sac hematopoiesis,[8] the more recently developed BMOs replicated key features of the aorta-gonad-mesonephros region via arterial endothelial differentiation. scRNA-seq showed HSCs expressing fetal liver-stage markers (e.g., selectin L, Musashi RNA binding protein 2), indicating their transitional state toward mature HSCs. This discovery offers insights into human HSC development and differentiation trajectories.

Upon lipopolysaccharide stimulation, BMOs secreted interleukin (IL)-6/IL-8 within 4 h, with pre-neural populations expanding 2.5-fold by 24 h. This rapid response mirrors *in vivo* "emergency hematopoiesis" and demonstrates niche-autonomous regulation of granulocyte differentiation without exogenous granulocyte colony-stimulating factor. This model enables dynamic studies of post-infection or chemotherapy-induced bone marrow regeneration.

Clustered regularly interspaced short palindromic repeats-engineered BMOs with *VPS45* mutations exhibited increased reticulin fiber deposition, expansion of alpha-smooth muscle actin[+] myofibroblasts, and collagen accumulation, leading to hematopoietic defects and elevated apoptosis of mature neutrophils, consistent with bone marrow biopsy phenotypes in *VPS45*-deficient patients (Figure 1C). Such "genotype-phenotype linkage models" may offer biopsy-free solutions for studying rare hematologic disorders.[9-11]

3. Expansion of applications: From basic research to clinical translation

Leukemia cells rely on the bone marrow microenvironment to acquire drug resistance. By integrating patient-derived induced pluripotent stem cells, BMOs can be engineered into "leukemia niche organoids" to investigate stroma-mediated drug resistance mechanisms (e.g., the role of the C-X-C motif chemokine ligand 12-C-X-C chemokine receptor type 4 axis in minimal residual disease) and evaluate immune cell interactions (e.g., infiltration and cytotoxic efficacy of chimeric antigen receptor T-cell therapy cells within organoids).

On April 10, 2025, the United States Food and Drug Administration announced a phased elimination of animal experiments in drug development, with a strategic shift toward "new approach methodologies," such as artificial intelligence-based predictive models and organoid toxicity testing. Organoids, as a core technology in this transition, exhibit distinct advantages in precision, efficiency, and ethical compliance, with demonstrated applications across multiple research and development scenarios. The high-throughput potential of BMOs (e.g., 96-well plate culture) supports diverse toxicity assessment studies, including hematotoxicity screening, evaluation of synergistic damaging effects of chemotherapeutic agents on HSCs and stromal cells, and validation of targeted therapies (e.g., testing the therapeutic efficacy of Janus kinase inhibitors in myelofibrosis-modeled organoids).

By integrating gene editing with organoid technology, BMOs enable HSC expansion optimization through supplementation with retinoic acid or Hedgehog pathway agonists, thereby enhancing transplantation efficiency. Furthermore, constructing mutation-specific organoids based on individual genetic profiles allows screening of personalized therapeutic regimens, making patient-specific treatments feasible.[12]

4. Conclusion

The *in vitro* reconstruction of the bone marrow niche represents not only a technical achievement but also a paradigm shift from "reductionism" to "systems biology." Integrating spatial transcriptomics and live-cell imaging, BMOs may decode spatiotemporal cellular crosstalk within niches, bridging molecular mechanisms to clinical translation. Future BMOs could serve as "*ex vivo* testbeds" for personalized medicine, offering novel strategies for the design of drug screening models, *in vivo* regeneration, and precision therapies.

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Conflict of interest

The authors declare they have no competing interests.

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Formal analysis: Yifan Xia
Investigation: All authors
Methodology: Liangyu Guo, Yifan Xia

Writing – original draft: Liangyu Guo
Writing – review & editing: Panpan Pan, Jingdi Chen

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data

Not applicable.

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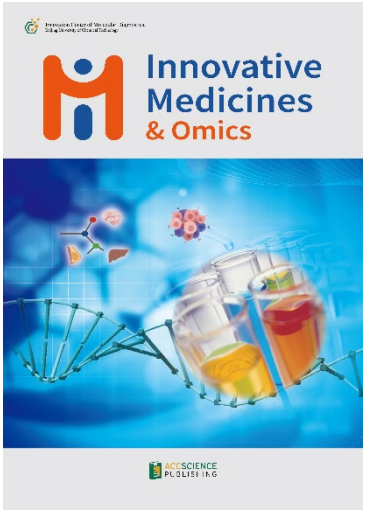

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