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Advancing musculoskeletal organoids research: Overcoming barriers to reduce animal model dependency

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Abstract

Musculoskeletal (MSK) disorders represent a leading cause of disability worldwide, with their incidence increasing steadily each year. While animal models have been instrumental in replicating various aspects of MSK pathologies, they face significant limitations, including interspecies variations, ethical concerns, and prolonged modeling timelines. The emergence of MSK organoids presents promising complementary models for pathophysiological research, disease modeling, drug screening, and regenerative medicine. As a valuable adjunct for traditional two-dimensional cultures and animal experiments, organoids provide novel mechanistic insights into MSK biology in a more physiologically relevant context. This review provides a comprehensive overview of current modeling strategies for MSK diseases and highlights the potential of organoids to reduce reliance on animal models. We critically assess the advantages and limitations of MSK organoids in disease recapitulation, identify key challenges in their development, and propose potential strategies for refinement. Finally, future directions and opportunities in this rapidly evolving field have been discussed.

Keywords: Musculoskeletal system; Organoid; Animal model; Standardization

1. Introduction

Musculoskeletal (MSK) system diseases refer to pathological conditions affecting MSK structures, including bones, joints, muscles, and ligaments.[1,2] These disorders are generally categorized into degenerative, traumatic, metabolic, or inflammatory diseases.[3-5] They may also include systemic issues, such as diabetes, hypertension, and even cardiac conditions.[6] The global burden of these conditions has risen substantially due to demographic aging and increasing unhealthy lifestyles. For example, osteoarthritis (OA) affected approximately 7.6% of the global population in 2024, and the number of OA cases in the working-age population represented a growth rate of 116.16% from 1990 to 2021.[7,8] Moreover, as of 2024, the total number of osteoporosis (OP) patients worldwide has surpassed 200 million, with a global prevalence rate of 18.3%.[9] From a socioeconomic perspective, MSK system diseases impose staggering healthcare costs, with annual expenditures for OA and OP management alone surpassing billions of dollars worldwide.[10] Furthermore, these conditions contribute significantly to workforce productivity losses and long-term care burdens, thereby emerging as critical public health challenges.

Animal models remain indispensable in MSK system disease research due to their unique ability to replicate human pathophysiology, providing invaluable insight into medical knowledge and alleviation of human suffering.[11] Through precise model establishment, including surgically induced OA or genetically modified muscular dystrophy models, researchers have successfully replicated the complex physiological environment of the human MSK system.[12,13] With the advanced technologies, animal models not only facilitate longitudinal monitoring of disease progression but also enable comprehensive functional assessment through motor behavior analysis. This allows researchers to systematically investigate disease mechanisms and progression. In therapeutic development, animal studies serve as a critical platform for validating novel biomaterials, pharmacological agents, and surgical interventions.[14-18] The pharmacokinetic, biocompatibility, and safety data derived from these preclinical studies are fundamental for clinical translation. Up to now, notable successes have been achieved in animal models, which include neural regeneration strategies developed through spinal cord injury (SCI) models and prosthetic joint designs optimized through large-animal biomechanical testing.[19,20]

Organoids, three-dimensional (3D) structures derived from stem cells or tissue-specific progenitors, are miniature and simplified *in vitro* model systems that mimic the structure and function of organs.[21-23] These miniature organ analogs replicate key functional and structural characteristics of native tissues, including myofiber contraction, bone matrix mineralization, and cartilage mechanical properties.[24,25] Compared to animal models, organoid systems offer distinct advantages, including high-throughput screening capacity, precise experimental control, and reduced ethical concerns.[21,26] These features make organoids particularly valuable for preliminary mechanistic studies, drug toxicity assessments, and genetic manipulation experiments. However, current organoid technologies face significant limitations, most notably the lack of vascular networks, neural innervation, hormone regulation, mechanical stimulation, and immune components, all of which are essential to MSK physiology and pathology.

Despite the emergence of innovative technologies, such as organoids, comprehensive evaluation of MSK-related diseases still relies on animal models, particularly for assessing systemic functional recovery, a capability beyond current *in vitro* systems. This review systematically explores the complementary roles of MSK system organoids and animal models, highlighting the potential for organoid technology to reduce reliance on animal experimentation. It also critically evaluates the current limitations of MSK organoids and outlines future directions for improving organoid construction and functionality. Overall, the review provides an overview of recent advances and applications in both MSK system organoids and animal models of the MSK system, aiming to offer valuable insights and references for researchers in this field.

2. Animal models of the MSK system

In MSK system biomedical research, skeletal animal models are indispensable tools for investigating disease pathogenesis, evaluating therapeutic interventions, and developing regenerative strategies. These experimental models replicate critical pathological features of human skeletal disorders, including OP, fracture nonunion, and bone neoplasms, through various construction methods, such as surgical intervention, pharmacological induction, genetic engineering, or age-related modeling. The establishment of reliable animal models provides an essential foundation for advancing the treatment of MSK diseases and helps to translate basic research results into clinical applications (Figure 1).

2.1. Bone-related animal models

2.1.1. OP animal models

OP is a systemic metabolic bone disorder and a major global public health concern, imposing substantial burdens on healthcare systems, particularly in aging populations.[27-29] Animal models of OP are essential for studying bone metabolism disorders and evaluating therapeutic strategies.[30] The ovariectomy (OVX) model remains the gold standard for postmenopausal OP research, demonstrating characteristic trabecular thinning and reduced bone mineral density that mirror clinical manifestations.[30-33] Glucocorticoid-induced OP, established through chronic administration of prednisolone or similar agents, replicate iatrogenic OP and are valuable for assessing anabolic treatment.[30,34] Disuse OP models employ tail suspension or surgical immobilization to study mechanical unloading effects, enabling investigation of mechanotransduction pathways and physical rehabilitation interventions.[35,36] Age-related OP models utilize senescent rodents (typically 18–24 months old) to investigate low bone turnover and progressive bone loss.[37-39] Genetic modification OP models involve manipulation of key regulatory genes (e.g., receptor activator of nuclear factor-κB ligand [RANKL], osteoclastogenesis inhibitory factor [OPG], and sclerostin) and elucidate specific genetic contributions to bone homeostasis and disease progression.[40,41] These models collectively advance understanding of OP pathogenesis and therapeutic development.

2.1.2. Fracture animal models

Animal fracture models serve as essential tools for investigating bone regeneration mechanisms, evaluating therapeutic interventions, and examining disease-related influences on fracture healing.[42] Current modeling approaches primarily focus on distinct clinical fracture etiologies, including traumatic fractures, osteoporotic fractures, infectious fractures, and non-union conditions.[43]

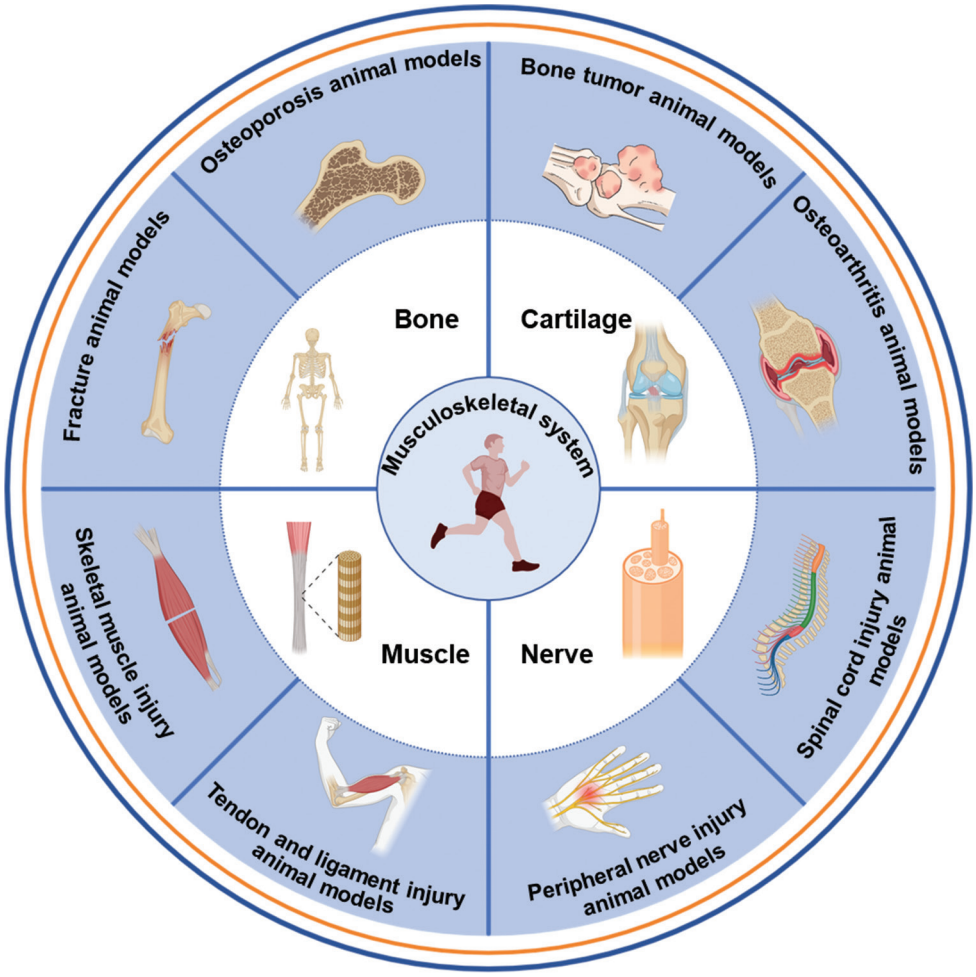

Figure 1. The types of musculoskeletal system animal models for various diseases

Most clinical fractures are caused by trauma, such as traffic accidents and falls, with long bone diaphyseal fractures accounting for a significant proportion. The animal models for traumatic fractures are categorized into closed and open fracture models. Closed fractures are commonly induced in long bones through controlled three-point bending or impact techniques, effectively modeling high-energy traumatic injuries, which are particularly valuable for studying normal fracture healing processes and evaluating callus formation-promoting therapies.[44-46] Open fracture is established through surgical osteotomy according to experimental protocols, frequently accompanied by internal fixation or the implantation of bone repair biomaterials, allowing for the assessment of osteogenic performance in clinically relevant settings.[47-51]

Osteoporotic fracture models are established through initial induction of OP, followed by controlled fracture creation.[43] Senescent animal models have been used for the study of geriatric OP fractures.[52] The most common site of OP fracture is the epiphysis. The distal femur and proximal tibia are generally used as the study sites in rat models. Alt *et al.*[53] and Nozaka *et al.*[54] established osteoporotic fractures in rats after OVX, which reveal osteoporotic bone status and fracture healing in osteoporotic fracture defects. These models accurately replicate postmenopausal or senile fragility fractures and are indispensable for investigating the effects of anti-osteoporotic agents on fracture healing.[55,56]

The fracture-related infection (FRI) is a serious complication. Helbig *et al.*[57] developed a new sequential animal model for FRI. In the study, the rats underwent transverse osteotomy of the femur with a 5 mm defect, followed by inoculation with *Staphylococcus aureus*. Infectious fracture models incorporate bacterial inoculation at the fracture site to study osteomyelitis pathogenesis and evaluate antibiotic-loaded biomaterials.[58]

2.1.3. Bone tumor animal models

Bone is a common site of metastasis for many prevalent tumors, contributing significantly to cancer-related mortality worldwide.[59] Several strategies have been developed to date, yet most demonstrate limited or no efficacy in patients. Therefore, there is an urgent need to better understand the mechanism

underlying bone tumors. Bone tumor animal models serve as indispensable tools for investigating tumorigenic mechanisms, screening potential therapeutics, and evaluating treatment efficacy.[60] These models can be broadly categorized into three principal types based on their methodological approaches: spontaneous models, transplantation models, and genetically engineered models, each offering distinct advantages for specific research applications.

The spontaneous model employs local administration of Aflatoxin B1 or radioactive substances to recapitulate environmentally induced carcinogenesis, and most commonly involves dogs, which is similar to humans at the histopathological and genetic level.[61-63] At present, the spontaneous osteosarcoma (OS) model is rarely used because of the prolonged period needed and the heterogeneity of the tumor, transplantation models represent the most widely utilized methodology, which involve implanting cancer cells or tumoral tissue fragments directly into mice or rats, which can recapitulate key aspects of primary bone cancer.[64] Patient-derived xenograft models maintain the histopathological and molecular characteristics of primary tumors, making them particularly suitable for personalized therapy development.[60] Genetically modified animal models serve as valuable tools for elucidating the molecular pathways involved in bone sarcoma pathogenesis and enable preclinical evaluation of novel therapeutic approaches. In the context of OS, conditional activation of oncogenes or deletion of several suppressor genes known to be implicated in tumor initiation and progression is a common method applied to construct transgenic mouse models.[64]

2.2. Cartilage-related animal models

2.2.1. OA animal models

OA, a chronic condition characterized by pain and significant discomfort, is one of the most prevalent forms of arthritis. Contemporary modeling approaches target distinct etiological factors, including mechanical injury, metabolic dysregulation, aging processes, and genetic predisposition. Mechanically induced OA models represent the most widely utilized approach for studying abnormal stress-induced joint degeneration, including anterior cruciate ligament transection and meniscectomy. The models replicate post-traumatic secondary OA pathology and provide insights into it.[12,65,66] Metabolic OA models employ high-fat diets to induce obesity and metabolic dysfunction, facilitating investigation of metabolic syndrome-associated OA.[67-69] These models are particularly relevant for elucidating the obesity-OA relationship in contemporary populations with increasing metabolic disorders. Chemically induced models (e.g., sodium monoiodoacetate or collagenase injections) produce rapid chondrocyte death and matrix degradation.[70,71] However, the acute pathological processes differ substantially from the gradual progression of human OA.

2.2.2. Rheumatoid arthritis (RA) animal models

RA is a chronic systemic autoimmune disorder with an incompletely understood pathogenesis. Various methods, including immune dysregulation, genetic predisposition, and environmental triggers, have been applied to induce RA in animals, which have been proven to be effective approaches for RA research. Induced arthritis models remain the most widely utilized models. The collagen-induced arthritis model faithfully reproduces characteristic synovial hyperplasia and bone erosion observed in human RA.[72] Spontaneous genetic models have emerged as powerful tools for studying RA pathogenesis.[73] Interleukin (IL)-1 receptor antagonist knockout mice develop spontaneous arthritis resembling human disease.[74] Tumor necrosis factor-α transgenic mice exhibit chronic progressive polyarthritis, while SKG mice (carrying a zeta-chain-associated protein kinase 70 mutation) demonstrate β-glucan-sensitive autoimmune arthritis.[75,76]

2.3. Skeletal muscle injury animal models

Skeletal muscle injuries occur from traumatic incidents, including car accidents, surgical resections, and injuries sustained on the battlefield, leading to functional loss of the injured muscle. Muscle injury experimental models are primarily selected based on distinct pathogenic factors and research objectives. Mechanical injury models, including volumetric muscle loss, contusion, and stretch models, simulate trauma in rodents, providing insights into muscle repair.[77,78] However, standardization of injury severity remains challenging. Chemical injury models, such as cardiotoxin or barium chloride injections, enable controlled muscle fiber damage but carry systemic risks.[79,80] Genetic engineering models, including muscle-specific gene knockout constructs (e.g., MyoD$^{-/-}$) and muscular dystrophy models, serve as indispensable tools for elucidating genetic myopathies and gene-specific functions.[81] Future research should better mimic human muscle pathology and implement standardized assessment protocols to improve clinical translatability, advancing preclinical MSK research.

2.4. Tendon and ligament injury animal models

Animal models of tendon and ligament injury are essential for investigating connective tissue repair mechanisms. Acute trauma models, including complete/partial transection, enable precise investigation of early healing response, recapitulating clinical pathology with inflammatory infiltration and collagen disorganization.[82] Chronic degeneration models utilize mechanical overloading or enzymatic induction to simulate tendinopathy, including glycosaminoglycan (GAG) accumulation and neovascularization.[83] Surgical repair

models further allow direct assessment of reconstruction techniques and biomaterial integration.[84-86] Despite their utility, these models face limitations, including interspecies variability, difficulty in replicating human biomechanical environments, and ethical concerns.

2.5. Neurological dysfunction-related animal models

2.5.1. SCI animal models

Spinal cord contusion-induced limb motor dysfunction represents severe orthopedic diseases with a high morbidity and disability rate. Various animal models of SCI effectively assess post-injury MSK alterations.[87] Traumatic injury models predominate, with controlled contusion devices replicating clinical spinal cord contusions.[88-90] Complete/partial transection models enable axonal regeneration studies,[91,92] while distraction injuries model vehicular trauma.[93] These reliably reproduce hallmarks of traumatic SCI, including hemorrhage, edema, and neuroinflammation. Ischemic injury models, induced through vascular occlusion or photochemical techniques, exhibit distinct pathophysiology.[94-97] Compression models simulate disc herniation or hematoma effects, particularly valuable for myelopathy research. Chemical models using targeted neurotoxins permit focused investigation of apoptosis and demyelination mechanisms.[98-101]

2.5.2. Peripheral nerve injury models

Peripheral nerve injury animal models are crucial for investigating nerve damage-induced MSK system dysfunction.[102] Crush injury models, including standardized forceps compression and chronic constriction, effectively simulate clinical nerve entrapment syndromes and chronic neuropathies while preserving epineurial integrity, facilitating the study of Wallerian degeneration and axonal regeneration processes.[103-105] Transection models are classified by injury completeness, with complete transection enabling nerve graft evaluation and partial transection permitting study of spontaneous regeneration.[106-108] Chemical models utilized neurotoxic agents for selective fiber-type damage or ethanol for focal demyelination, though they require a carefully optimized dose due to potential systemic toxicity.[109-111]

3. Advances in MSK organoids

With the rapid advancement of organoid technology and growing ethical and scientific imperatives to reduce reliance on animal models, MSK organoids have emerged as a transformative tool in regenerative medicine. These 3D, multicellular constructs recapitulate key structural and functional features of native bone, cartilage, and muscle tissues, offering unprecedented opportunities to study developmental biology, disease mechanisms, and therapeutic interventions. MSK organoids not only serve as an alternative system for simulating complex tissue functions but also provide an ideal platform for gaining more intuitive and in-depth insights into disease pathogenesis and treatment (Table 1). Therefore, a comprehensive understanding of recent advances in MSK organoid research is crucial for their future development, refinement, and translational application.

The development of MSK organoid technology has undergone a critical evolution from single-tissue modeling to multi-system integration. Research on MSK organoids can be traced back to as early as 1990, when Zimmermann *et al.*[112] developed a cartilage organoid culture to study endochondral mineralization. Subsequently, Sass *et al.*[113] applied limb bud mesenchymal cell organoids to drug screening for evaluating the teratogenic potency of retinoids. In the early 2000s, Vandenburgh *et al.*[114] utilized primitive embryonic avian or neonatal rodent myoblasts to create uniformly batch-producible muscle organoids, advancing the technology toward practical applications. As single-structure organoid techniques matured, research focus shifted to multi-structure integrated organoids. Muraglia *et al.*[115] formed chondro-osseous organoids through bone marrow stromal cells, while Mizuno *et al.*[116] developed spherical organoids with depth-specific architecture, longitudinal depth zones in articular cartilage. By 2020, Hall *et al.*[117] proposed the "callus organoid" concept to predict long bone healing mechanisms, followed by Akiva *et al.*[118] in 2021, constructing a 3D self-organizing co-culture of osteoblasts and osteocytes for early-stage woven bone formation, establishing the most complete 3D living *in vitro* model system. Dai *et al.*[119] further engineered *in vivo* osteo-organoids using bone morphogenetic protein (BMP)-2-loaded scaffolds, pioneering novel osteo-organoid-derived cell therapeutic strategies. In pathological modeling, Hu *et al.*[120] established bone metastasis organoids from lung adenocarcinoma to validate denosumab efficacy. Recently, Yin *et al.*[121] generated self-organized human neuromusculoskeletal organoids (hNMSOs), achieving cross-tissue functional regulation through neuromuscular junctions (NMJs) and marking an unprecedented level of technological sophistication. The rapid advancement of organoid technology has made it increasingly feasible for organoids to replace animal experiments (Figure 2).

3.1. Bone organoids

Bone organoids refer to 3D, miniaturized, and simplified bone tissues generated *in vitro* using stem cells or progenitor cells, aiming to mimic the structure, function, and intercellular communication of natural bone tissue.[122] They consist of various cell types, including bone mesenchymal stem cells (BMSCs), osteoblasts, osteoclasts, and mature osteocytes, and undergo a mineralization process to form a rigid, bone-like matrix.[123] Current research in bone

Table 1. Strengths, weaknesses, and unique applications of MSK organoids

Animal models	MSK organoids		
	Advantage	**Weaknesses**	**Unique application**
Bone-related animal models	• High human relevance • Scalable production • Reduced ethical concerns	• Structural simplicity • Insufficient mechanical strength	• The study of systemic interactions (gut-bone axis/nerve-bone axis) • Investigation on biomechanical loading effects (fracture healing under weight-bearing)
Cartilage-related animal models	• Controllable metabolism (hypoxia) • Controlled microenvironment	• Lack of systemic interactions • Limited mechanical loading	• Study of the mechanical differences among the various layers of cartilage • Investigation of immune-mediated cartilage destruction (RA model)
Skeletal muscle injury animal models	• Study of a single muscle fiber • Genetic/disease modeling	• Limited maturity/functional strength • Lack of a full injury microenvironment	• Study of neuromuscular diseases (e.g., ALS) • Model for chronic muscle degeneration & fibrosis (e.g. DMD)
Tendon and ligament injury animal models	• Human-specific physiology • Controlled mechanobiology studies	• Lack of a systemic healing environment • Limited mechanical strength • No native bone-tendon junction modeling	• Study of whole-joint biomechanics (e.g., ACL reconstruction) • Investigation of chronic degeneration (e.g., supraspinatus tendinopathy)

Abbreviations: ACL: Anterior cruciate ligament; ALS: Amyotrophic lateral sclerosis; DMD: Duchenne muscular dystrophy; RA: Rheumatoid arthritis; MSK: Musculoskeletal.

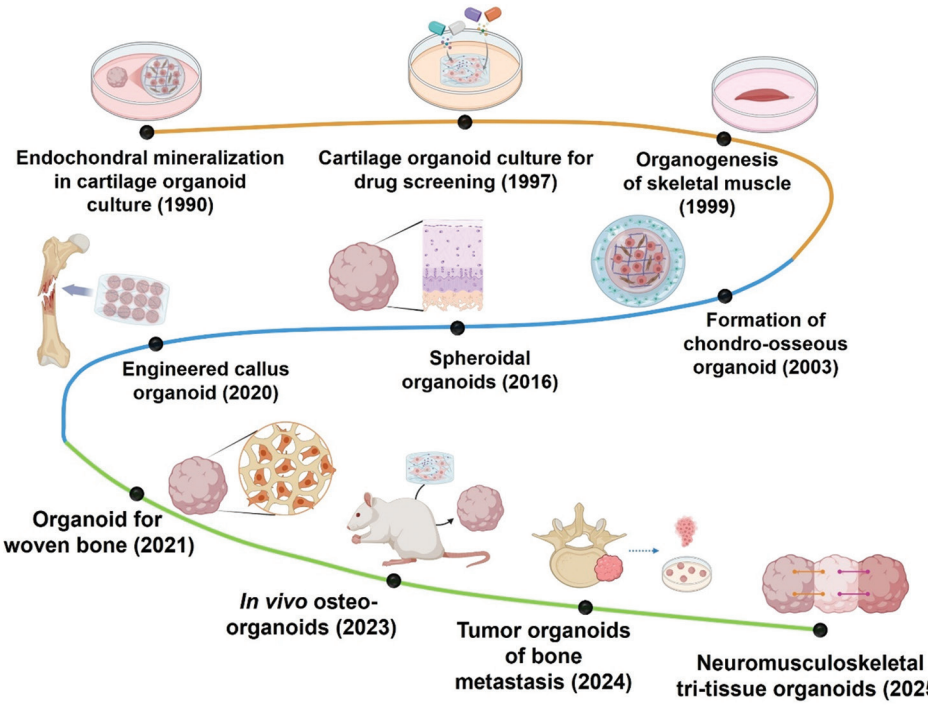

Figure 2. A summary of key landmark studies and breakthroughs leading to the establishment of various organoid technologies

organoid development has primarily focused on achieving high-fidelity replication of native bone tissue architecture and biomechanical function, as well as advancing innovative methodologies for organoid construction and maturation. These efforts aim to bridge existing gaps between *in vitro* models and *in vivo* bone physiology.

The fundamental challenge in bone organoid engineering lies in recapitulating the native cellular heterogeneity of bone tissue. Osteoblasts, serving as the principal effector cells in bone formation and regeneration, constitute the essential cellular component for developing functional bone organoids with osteogenic potential.[124] Current

methodologies predominantly employ stem cell-derived osteoblasts,[125] with BMSCs representing the most widely utilized progenitor cell source due to their well-characterized osteogenic differentiation capacity. In addition, attempts have also been made to construct bone organoids from pluripotent stem cells (PSCs), including induced PSCs (iPSCs), periosteum-derived cells (PDCs), and embryonic stem cells (ESCs).[126,127] These alternative cell sources offer distinct advantages in terms of scalability and patient-specific applications. The critical role of osteoclasts in bone remodeling processes has prompted innovative co-culture approaches.[128] Iordachescu *et al.*[129] developed an advanced model system utilizing decellularized bovine femoral cancellous bone scaffolds to support the 3D co-culture of osteoblasts and osteoclasts, thereby better mimicking the dynamic equilibrium of bone formation and resorption. Osteocytes, representing approximately 90–95% of all bone cells, serve as the primary mechanosensory cells responsible for maintaining bone homeostasis and orchestrating remodeling processes in response to mechanical stimuli.[130] The growth and maintenance of osteocytes require long-term mechanical loading, which presents significant technical challenges for utilizing organoids. To address this challenge, Zhang *et al.*[131] fabricated a compression bioreactor to deliver long-term mechanical loading to osteocyte bone organoid (Figure 3A-G). This technological advancement represents a crucial step toward recreating the native osteocyte microenvironment in 3D culture systems. Despite these developments, the application of osteocytes in bone organoid construction remains at an early stage of investigation. Several critical aspects, including mechanical loading parameters, long-term maintenance of osteocyte networks, and standardized characterization protocols, still need further exploration.

Beyond cellular composition, the extracellular matrix (ECM) represents a fundamental component in bone organoid engineering, serving both structural and regulatory functions. The ECM not only provides essential support for cell adhesion, growth, proliferation, and differentiation but also plays a crucial role in the spatiotemporal control of organs. The ECM-derived materials have emerged as particularly effective substrates for accelerating bone formation. The earliest biomaterial used in the design of bone organoids is demineralized bone matrix (DBM), which is advantageous for its wide availability and low immunogenicity. Park *et al.*[132] constructed a trabecular bone organoid to simulate local bone remodeling using a demineralized bone paper (DBP) made from biomaterials, which highlights the potential of ECM-based materials to recapitulate complex bone microenvironments *in vitro* (Figure 3H-J). In another study, Iordachescu *et al.*[133] utilized trabecular bone as scaffolds to co-culture osteoblasts and osteoclasts within the microporous structures, fabricating micron-scale trabecular bone organoids. This model successfully recapitulated microgravity-induced osteopenia, providing a valuable platform for studying bone loss under simulated space conditions. Alternative processing methods for DBM have further expanded its applications. For instance, Gai *et al.*[134] developed an innovative biomimetic matrix hydrogel by incorporating calcium phosphate oligomers into bone decellularized ECM, enabling continuous construction of bone organoids with vascularization and mineralization functions. At the same time, the Matrigel remains a widely utilized commercial ECM derived from Engelbreth-Holm-Swarm (EHS) mouse sarcoma.[135] However, the high cost, undefined chemical nature, and significant batch-to-batch variability of EHS lead to poor reproducibility and limited application in the construction of bone organoids. Therefore, lots of organic bioactive materials, including natural polymers such as silk fibroin, gelatin, chitosan, and hyaluronic acid, as well as synthetic polymers including polycaprolactone (PCL), polylactic acid, and polyethylene glycol (PEG), have been developed for the construction of bone organoids.[123]

3D bioprinting has emerged as a transformative technology for bone organoid engineering, which offers precise spatial control of cell-matrix deposition, enhanced structural complexity, and improved reproducibility. For example, methacrylated gelatin (GelMA) microspheres containing BMSCs were prepared using digital light processing 3D printing technology, and efficient bone regeneration-like callus tissue was cultivated through chondrogenic induction and osteogenic differentiation.[136] Another study created self-mineralizing large-sized bone organoids by combining GelMA, alginate methacryloyl, and hydroxyapatite (HA) in a bioprinting process, mimicking the complexity of the ECM and constructing bone-like organs with similar functions and mechanical properties to natural bone tissue.[137] Innovative *in vivo* approaches have also been developed, such as BMP-2-loaded scaffolds implanted in murine muscle pouches that generate functional bone organoids capable of treating liver injury.[119] Genome editing technology, such as clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein 9 (Cas9), also plays a critical role in the construction of bone tumor organoids.[138] Gerardo-Ramirez *et al.*[139] successfully established CD44-knockout OS organoids with validated genetic stability using CRISPR/Cas9 technology. Zhang *et al.*[140] developed OS organoid models using CRISPR/Cas9 technology and evaluated the combination therapy of protein kinase, DNA-activated, catalytic subunit inhibitor, 7-methyl-2-([7-methyltriazolo{1,5-a}pyridin-6-yl]amino)-9-(tetrahydro-2H-pyran-4-yl)-7,9-dihydro-8H-purin-8-one, with doxorubicin, pioneering novel experimental models

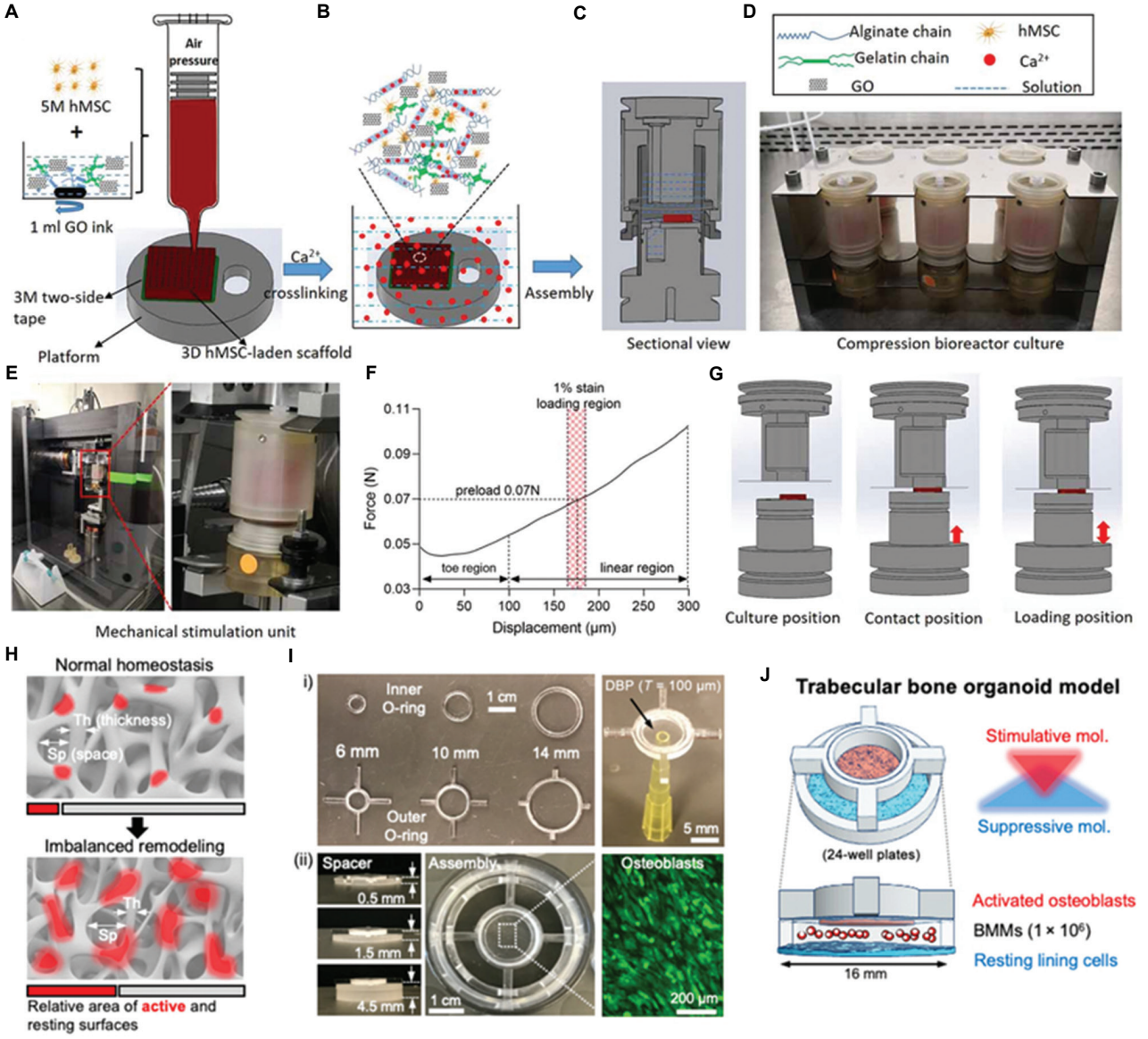

Figure 3. Construction of bone organoid. (A) 3D bioprinting processes; (B) Ca^{2+} crosslinking post-bioprinting; (C) section views to show the position of the 3D cell-laden construct in the compression bioreactor; (D) bioreactors containing 3D hMSCs-laden constructs were incubated in the in-house constructed rack; (E) the compression bioreactor was mounted into the mechanical stimulation unit like a cartridge; (F) a representative force and displacement curve of the mechanical test with a 3D bioprinted cell-laden constructs showing the preload and loading region; and (G) schematic illustration of the compression bioreactor showing the culture position, contact position and loading position during loading processes.[131] Reprinted with permission from Zhang *et al.*[131]; (H) excessive bone remodeling results in bone loss and changes in the trabecular bone morphology, which could compromise anatomical regulation of localized bone remodeling; (I) (i) DBP inserts prepared by securing 100-μm-thick DBP between two acrylic O-rings; (ii) suspended DBP inserts above DBP disks with ring-shaped spacers; osteoblasts cultured on a DBP insert; and (J) the trabecular bone organoid model consists of a DBP insert that has been activated by vitamin D3 and prostaglandin E2 suspended over a DBP disk containing bone lining cells.[132] Reprinted with permission from Park *et al.*[132] Abbreviations: 3D: Three-dimensional; DBP: Demineralized bone paper; hMSCs: Human mesenchymal stem cells.

and therapeutic strategies for precision oncology and bone tumor treatment.

3.2. Cartilage organoids

Cartilage tissue exhibits a unique stratified architecture composed of chondrocytes embedded within an ECM rich in collagen fibers and GAGs. The structure of cartilage can be divided into several different layers from the outside to the inside, each with distinct cellular and matrix characteristics. Cartilage obtains nutrients through the diffusion of water and nutrients in the matrix, with no direct vascular supply.[141] The lack of blood supply places chondrocytes in a relatively hypoxic environment.[142] This hypoxic environment not only results in poor self-repair

capabilities of cartilage after injury but also increases the difficulty of constructing cartilage organoids.

Different from bone organoids, cartilage organoids are composed solely of chondrocytes derived from multiple cellular sources, including isolated and cultured stem cells, namely, ESCs, iPSCs, mesenchymal stem cells (MSCs), PDCs, and other stem cell or pluripotent cell lines, as well as chondrocytes derived from autodigestion.[143-146] Abe *et al.*[147] demonstrated successful integration of iPSC-derived cartilage organoids in primate knee joint defect models, with subsequent remodeling into functional articular cartilage. Another study by Sun *et al.*,[148] who applied synovial mesenchymal stromal cells to generate 3D-cultured organoids for pre-clinical modeling and treatment of degenerative joint disease. In addition, Hall *et al.*[149] compared two types of cartilage organoids constructed from human PDCs (hPDCs) and iPSCs. The results revealed that the cartilage organoids derived from hPDCs were more similar to hypertrophic cartilage. In contrast, the cartilage organoids obtained from iPSCs exhibited ratios of acidic GAGs and aggrecan to total collagen that more closely resembled natural cartilage.

At present, there are mainly two types of organoid culture methods: Scaffold-based and scaffold-free. The construction of scaffold-free cartilage organoids primarily relies on the self-organization ability of cells. By placing cells in a suspension culture environment, cell aggregation is promoted to form 3D structures.[150] This method is simple and highly reproducible, allowing the rapid generation of organoids without the external scaffold materials. Yamashita *et al.*[151] demonstrated successful generation of hyaline cartilage organoids from PSCs using scaffold-free suspension culture. In addition, O'Connor *et al.*[145] developed a sequential differentiation protocol employing transforming growth factor (TGF)-β and BMP to model the cartilage-bone interface, thereby providing a well-established platform for studying the region between cartilage and bone. In addition to static cultures, centrifugal cultures without scaffolds have been used for the study of cartilage organoids. For instance, Irie *et al.*[152] utilized hollow fibers as cell culture devices, inducing chondrocytes to form cylindrical organoids through centrifugation. Steinwerth *et al.*[153] investigated scaffold-free 3D chondrogenic organoid formation in a rotating wall vessel bioreactor under simulated microgravity. These results demonstrated that chondrocytes were able to form dense 3D cartilage-like tissues without the addition of scaffolds. In contrast, scaffold-based culture methods can select scaffolds with corresponding properties according to the purpose of the cartilage organoids. For example, compared with elastic hydrogels, viscoelastic hydrogels can better support the growth and expansion fusion of cartilage organoids. The viscoelastic hydrogels prepared by Crispim *et al.*,[154] composed of alginate or decellularized ECM, provide a mechanical environment that allows cells to proliferate and differentiate into cartilage tissue, producing new hyaline cartilage with properties similar to natural cartilage. Inspired by the structure of cartilage tissue, researchers have successfully constructed highly biomimetic cartilage organoids by regulating the orientation of collagen fibers through plastic compression and introducing a gradient of chondroitin sulfate.[155] These organoids not only replicate the various heterogeneous features of natural cartilage but also achieve region-specific regeneration of cartilage tissue. In addition, the construction of hypoxic cartilage organoids provides an important model for studying the hypoxia adaptation mechanisms of chondrocytes. Yang *et al.*[156] have used a hyperdynamic hydrogel to construct cartilage organoids, which exhibit significant accumulation of lactate and histone lactylation in a hypoxic microenvironment, thereby promoting the proliferation and differentiation of chondrocytes.

3D bioprinting has emerged as a transformative technology in cartilage organoid research, enabling precise spatial control over cellular organization and ECM composition.[157] This advanced technique not only facilitates precise control over the layer-by-layer deposition of cells, matrix materials, and bioactive factors but also permits the construction of complex structures that closely recapitulate native cartilage morphology and biomechanical properties. Noteworthy applications include the work of de Melo *et al.*[158] and Xie *et al.*,[136] who employed suspension 3D printing technology to fabricate cartilage organoids through cell-laden microspheres. Recent advances in structural engineering have further enhanced organoid fidelity through the development of compression-based techniques that induce anisotropic collagen fiber alignment within hydrogel scaffolds.[155] Concurrently, gradients of varying concentrations of chondroitin sulfate have been established to mimic the composition of cartilage. The integration of CRISPR/Cas9 has expanded the experimental utility of these systems, as demonstrated by Wei *et al.*[159] through the creation of dual-fluorescence-labeled chondrogenic organoids. These cartilage organoid models offer a robust platform for investigating cartilage pathologies and advancing regenerative strategies.

3.3. Skeletal muscle organoids

The construction of skeletal muscle organoids first requires the precise reproduction of the cellular composition of skeletal muscle tissue, which is primarily composed of muscle cells. Current methodologies employ multiple cellular sources for organoid generation, including iPSCs, myoblasts, satellite cells, MSCs, and *in vitro*-derived satellite cells (idSCs).[160] iPSCs represent a particularly versatile option, as they can be directed through staged

differentiation protocols to generate either myogenic precursor cells or quiescent satellite-like cells, both of which subsequently mature into functional, contractile myotubes.[161] Primary myoblasts serve as another fundamental building block, possessing an intrinsic capacity to fuse and form multinucleated myotubes, the basic functional units of skeletal muscle.[162] Under 3D culture conditions, myoblasts can self-assemble into skeletal muscle organoids, simulating the structure and function of natural skeletal muscle. Moreover, myoblasts can dedifferentiate into cells similar to satellite cells under 3D culture conditions, known as idSCs, which can support multiple rounds of muscle regeneration, similar to natural satellite cells, after transplantation.[161] Satellite cells are the stem cells in skeletal muscle, responsible for muscle growth and regeneration.[163] However, it remains unclear how to effectively increase the number of satellite cells *in vitro* while maintaining their stem cell characteristics, especially their ability to repopulate the niche. In addition, both MSCs and idSCs can be induced toward myogenic lineages under appropriate conditions, further expanding the available cell sources for organoid construction.[164] This diverse cellular toolkit enables the generation of increasingly sophisticated skeletal muscle organoids with significant potential for applications. The construction of skeletal muscle organoids also requires suitable ECM materials. These primarily include Matrigel, collagen, and fibrin.[165-167] Synthetic hydrogel materials mainly consist of poly(lactic-co-glycolic acid) (PLGA), PEG, and polyacrylamide.[160] These ECM materials offer a diverse range of options for culturing skeletal muscle organoids, catering to various research and application needs.

The cultivation techniques for muscle organoids have seen significant advancements in recent years, particularly in the application of 3D culture systems and bioreactors.[168] These technologies provide conditions closer to the *in vivo* environment for constructing muscle organoids and promoting the alignment, fusion, and formation of muscle fiber-like structures. 3D culture systems, by simulating the mechanical tension within muscle tissue, offer an environment closer to that of the body for the construction of muscle organoids. For instance, Price *et al.*[164] developed a 3D organoid culture method to produce a large number of adult skeletal muscle satellite cells *in vitro*. This method not only increased the quantity of satellite cells but also maintained their stem cell characteristics, which is crucial for the growth and regeneration of skeletal muscle. Bioreactor technology has also played a significant role in the cultivation of muscle organoids. By introducing mechanical stretching stimuli in the bioreactor, researchers can simulate the physiological conditions of muscle tissue in the body, promoting the alignment and fusion of muscle fibers. Similarly, Chromiak *et al.*[168] applied an orbital shaker to culture muscle organoids, significantly improving the survival rate and differentiation efficiency of the organoids through sustained mechanical stimulation. To better study the function and mechanism of muscle organoids, researchers have also employed gene-editing and cell-labeling technologies. A recent study by Li *et al.*[169] developed skeletal muscle organoids using microdroplet-engineering technology. Derived directly from primary tissues without requiring primary cell culture, microdroplet-engineered skeletal muscle organoids significantly reduce the generation time of skeletal muscle organoids. In addition, while employing CRISPR/Cas9 technology to investigate the roles of specific genes in muscle development and diseases has become well-established, studies utilizing this technology to construct muscle organoids remain at a nascent stage.

3.4. Composite organoids/multi-tissue organoids

The MSK system does not exist in isolation; it is also under the control and regulation of the vascular and nervous systems. The vascular system supplies muscles and bones with oxygen and nutrients, ensuring their normal metabolism and functional maintenance.[170] The nervous system precisely controls muscle contraction and relaxation through nerve impulses, coordinates movement, and senses muscle tension and joint position to prevent injury.[171] The vascular, nervous, and MSK systems work together to ensure the body's mobility and overall health.

Extensive research has been conducted on the construction of vascularized bone organoids. Duan *et al.*[172] proposed a new strategy to produce pre-vascularized bone organoids with self-organizing vascularization and enhanced osteogenic properties on a large scale by combining MSCs, human umbilical vein endothelial cells (HUVECs), and osteogenic microparticles. Besides, Li *et al.*[173] successfully constructed vascularized bone organoids by leveraging the ability of dental pulp stem cells to differentiate into endothelial lineages in conjunction with BMSCs. This vascularized bone organoid not only increased mineralization deposition and reduced cell necrosis but also formed hollow structures, demonstrating good vascularization effects. In addition, Jusoh *et al.*[174] designed a microfluidic device composed of four parallel channels (vasculature, bone, medium, and lung fibroblasts), using fibrin and HA nanoparticles as the ECM to mimic the structure of real bone tissue. Although cartilage is devoid of vascularization, blood vessels play a critical role in constructing cartilage organoids. Chen *et al.*[175] leveraged the natural vascularization gradient within osteochondral tissues using single-BMSCs-derived cartilage organoids, achieving gradient heterogeneous osteochondral regeneration. Vascularization of skeletal muscle organoids can be achieved either through the

reassembly of undifferentiated endothelial cells (ECs) into capillaries or through organ-on-a-chip (OoC) technology, with the latter representing the prevailing approach in current research.[176,177] For instance, Wang *et al.*[178] cultured skeletal muscle organoids in an OoC system, monitoring their responses to both perfusion stimuli and electrical stimulation in real time.

The role of nerves must also be considered in the construction of MSK organoids. In the field of neurogenic muscle organoid research, co-culture and assembly techniques combine independently differentiated cell types, utilizing intercellular interactions and positioning to guide the formation of NMJs.[179] Moreover, by employing microfluidic chip technology, researchers can precisely control the co-culture conditions of nerve and muscle cells, further optimizing the construction of neurogenic muscle organoids (Figure 4A-E).[165,179] For example, researchers have successfully constructed functional NMJs by co-culturing motor neurons with skeletal muscle cells.[180] In another study, the first self-organized hNMSOs were constructed using human PSCs (hPSCs), providing a human *in vitro* model for studying the human neuromusculoskeletal axis and related diseases (Figure 4F-K).[121] Although extensive research has been conducted on the interaction between nerves and bones, the construction of neurogenic bone organoids remains an emerging field. Future research needs to further explore the mechanisms and strategies of neurogenic bone tissue engineering to achieve more efficient and physiologically relevant bone regeneration effects.

4. Attempts to replace *in vivo* experiments with organoids

Organoid technology represents a transformative advancement in biomedical research, offering significant improvements in precision, controllability, and ethical compliance compared to traditional animal models. By employing patient-derived cells or iPSCs to generate miniaturized tissue constructs, this approach recapitulates human organ functionality while overcoming the inherent limitations of interspecies variation that plague animal studies. The organoids enable unprecedented experimental control through the establishment of precise growth factor gradients through microfluidic systems, optimization of nutrient diffusion using 3D-printed biomimetic scaffolds with tunable porosity, and real-time monitoring of cellular dynamics through advanced live imaging techniques. These capabilities provide researchers with direct observational access to developmental and pathological processes that were previously only inferable through terminal animal experiments. Ethically, organoid technology significantly reduces the use of animals, aligning with the replacement, reduction, and refinement ("3R principles") and gaining policy support such as the Food and Drug Administration (FDA) Modernization Act 2.0, which permits bypassing animal testing in new drug development.[181]

Compared to traditional animal models, organoids offer significant advantages, including reduced culture timelines, enhanced capacity for high-throughput drug screening, and markedly lower animal experimentation costs. More importantly, these 3D *in vitro* models faithfully recapitulate human physiological and pathological processes by maintaining native tissue architecture and microenvironmental conditions, thereby providing a more clinically relevant platform. Beyond disease research and therapeutic development, organoids demonstrate remarkable versatility in tissue regeneration and regenerative medicine. This technology, through humanized modeling, precise microenvironment control, and high-throughput screening capabilities, bridges the gap between traditional animal experiments and clinical applications, propelling biomedical research from animal validation to human prediction.

4.1. The functions of MSK organoids

MSK organoids primarily model MSK development or study the pathogenesis of specific diseases by investigating intercellular interactions, cellular signaling pathways, and biomechanical responses. Compared to *in vivo* animal experiments, organoids demonstrate unique advantages. At the signaling pathway level, organoids can effectively isolate the interference from the variable systemic environments within animals (encompassing endocrine, immune, and metabolic factors). This capability enables the precise localization, simulation, and targeted intervention of key molecular signaling pathways (Wingless-related integration site [Wnt], nuclear factor-kappa B [NF-κB], TGF-β, etc.) that regulate developmental processes in tissues, such as muscle, bone, and joints, all under highly controllable conditions. For instance, neuromuscular organoids (NMOs) generated from amyotrophic lateral sclerosis (ALS) patient cells not only enable precise delineation of C9orf72 mutation-induced disruption of the Wnt/β-catenin pathway but also serve as platforms to validate the efficacy of R-like endoplasmic reticulum kinase inhibitor, GSK2606414, in ameliorating ALS phenotypes through restoration of Wnt downstream signaling.[182] Furthermore, the MSK OoC model enables investigation of hypoxia's impact on signaling pathways within the MSK system. Intermittent hypoxia (IH) downregulates muscle mitochondrial sirtuin 3 (SIRT3), activating the NF-κB pathway and promoting secretion of the myokine C-X-C motif chemokine ligand 5 (CXCL5), thereby suppressing osteogenic differentiation while enhancing osteoclast activity.[183] In addition, organoids derived from Duchenne muscular dystrophy (DMD) patients not only permit

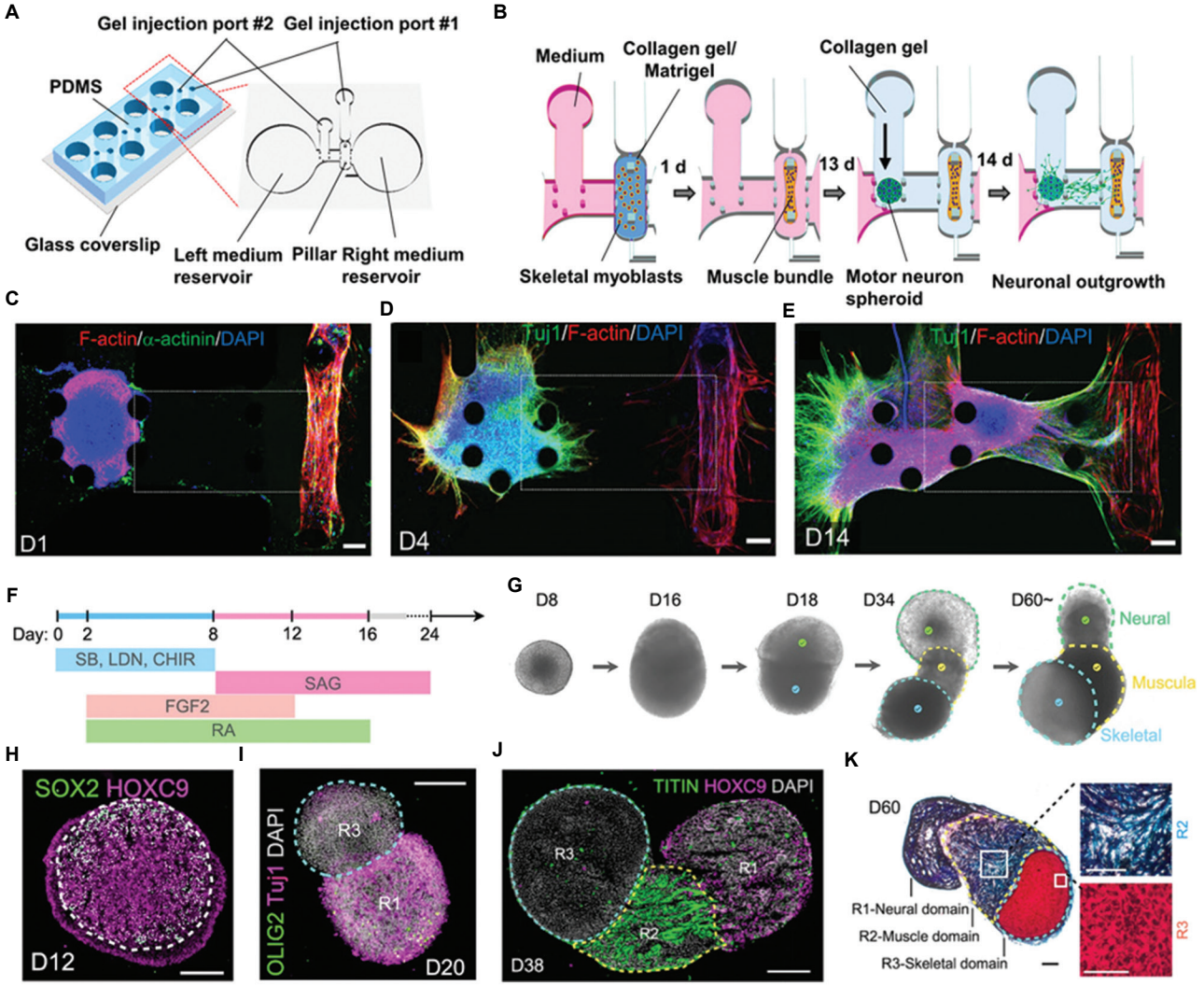

Figure 4. Construction of composite organoids/multi-tissue organoids. (A) The micro-fabricated motor unit-mimic device that forms four identical sites on a single chip, each composed of a muscle fiber bundle attaching pillar structures and culture MN spheroids; (B) A 3D muscle fiber bundle was fabricated in a microfluidic device; (C-E) Day 1, 4, and 14 of coculture, thick neural fibers were observed at day 14.[165] Some of MNs also migrated from their original position. Reprinted with permission from Osaki *et al.*[165]; (F-G) morphology of hNMSOs at different developmental stages; (H-J) morphology of hNMSOs at day 12, 20, and 38 of development; and (K) Safranin O and fast green staining of H9 hESCs-derived hNMSO.[121] Reprinted with permission from Yin *et al.*[121]

Abbreviations: 3D: Three-dimensional; hESCs: Human embryonic stem cells; hNMSOs: Human neuromusculoskeletal organoids; MN: Motor neuron.

investigation of TGF-β signaling in muscle fibrosis but also enable validation that CRISPR-mediated correction of TGF-β receptors restores myotube fusion capacity.[184]

For biomechanical research, organoids integrated with auxiliary systems, such as microfluidic chips and mechanical loading devices, not only streamline the complex mechanical environments inherent to *in vivo* animal experiments but also enable quantitative and qualitative analysis of biomechanics' impact on the MSK system. For example, Iordachescu *et al.*[133] engineered bone organoids by co-culturing osteoblasts and osteoclasts on trabecular bone scaffolds within microgravity bioreactors. These organoids faithfully recapitulate disuse OP under weightlessness, exhibiting a 45% reduction in mineralization under mechanical loading culture, conditions that are consistent with findings from rodent tail-suspension models. In another study, Melke *et al.*[185] employed spinner flask bioreactors to investigate the relationship between wall shear stress within the reactor and mineralization patterns in organoids during culture. In addition, Li *et al.*[186] developed a miniaturized joint-on-a-chip system to investigate the effects of fluid shear stress on 3D cartilage tissue. Their findings revealed that fluid flow-induced shear stress applied to the 3D constructs stimulated native-like chondrocyte alignment while upregulating lubricin expression by chondrocytes.

Research on intercellular interactions necessitates reliance on multicellular and multi-tissue coculture systems, even transcending the limitations of single-organoid models. For instance, Tong *et al.*[183] revealed cellular crosstalk between muscle and bone under IH using an MSK OoC platform. The results demonstrate that mitochondrial damage in muscle tissue triggers CXCL5 release, which suppresses expression of osteogenic markers (e.g., Runt-related transcription factor 2 [Runx2, Osterix) while enhancing osteoclast activity. Furthermore, Park *et al.*[132] engineered a trabecular bone organoid model using DBP. This trabecular bone organoid was employed to investigate intercellular crosstalk among osteocytes, osteoblasts, and osteoclasts during local bone remodeling regulation. For multi-organoids construction, Yin *et al.*[121] investigated the interactions among neuronal, skeletal, and muscular cells by generating hNMSOs from hPSCs. These hNMSOs demonstrated that: motor neurons can control skeletal muscle contraction through the NMJ; skeletal support fosters the development and maturation of human muscle; and pathological skeletal degeneration directly precipitates NMJ dysfunction.

4.2. Organoids for the developmental/physiological process

Organoids have significant advantages in the study of development and physiological processes. First, they can highly simulate the development of organs, providing a 3D structure that closely resembles the *in vivo* environment, allowing researchers to observe the dynamic processes of cell differentiation, tissue formation, and organ development *in vitro*.[187] Second, organoids can be used for personalized research using patient-derived cells, revealing individual developmental differences and disease-related mechanisms.[188] In addition, organoids are highly manipulable, enabling the study of the effects of specific genes or signaling pathways on development through various methods, such as gene editing and drug intervention.[189] This model not only serves as a powerful complement to animal models but also enhances research efficiency and precision, providing a robust tool for developmental studies.

Bone development involves two modes: endochondral ossification and intramembranous ossification.[190] Both processes work together to drive skeletal development and growth. A deep understanding of the mechanisms underlying bone development is of great significance for bone fracture repair, regeneration, and the occurrence and development of diseases. Endochondral ossification begins with the differentiation of mesenchymal cells into chondrocytes, forming a cartilage template. Subsequently, chondrocytes undergo hypertrophy and calcification, followed by the integration of blood vessels. Osteoblasts then replace the cartilage to form bone tissue, promoting skeletal growth. Inspired by endochondral ossification, Dong *et al.*[191] combined endochondral ossification with endogenous enzyme-induced mineralization to simulate the mineralization process in natural bone development, creating bone organoids based on the endochondral ossification model. In addition, Xie *et al.*[136] utilized 3D printing technology and hydrogel microspheres loaded with human BMSCs to construct osteo-callus organoids. They constructed osteo-callus organoids that recapitulate developmental processes, exhibiting cellular composition similar to that of developing endochondral ossification. In another study, Kesharwani *et al.*[192] utilized ESCs-derived organoids to model vascular dynamics on a microfluidic chip at the initial stage of endochondral ossification. These studies provide strong support for a deeper understanding of the endochondral ossification process and its mechanisms. Intramembranous ossification is a process where MSCs directly commit to osteoblasts and mineralize to form bone tissue, without transitioning through a cartilaginous intermediate stage. The majority of organoids are constructed by mimicking the developmental process of intramembranous ossification. For instance, Zhu *et al.*[193] developed a GelMA/DNA double-network hydrogel that significantly enhances osteogenic mineralization of BMSCs, while concurrently exhibiting anti-inflammatory properties and pro-angiogenic functionality. As for modeling physiological process, Park *et al.*[132] constructed trabecular bone organoids to investigate molecular mechanisms and cellular activities during localized bone remodeling.

The development of cartilage is a highly regulated biological event involving multiple aspects, including cell differentiation, matrix synthesis, and remodeling. During cartilage development, yes-associated protein (YAP), a key effector of the Hippo signaling pathway, directly influences the chondrogenic differentiation of MSCs through its subcellular localization and activity.[194] Zhu *et al.*[194] utilized MSCs in combination with Verteporfin to modulate the YAP signaling pathway, constructing hyaline cartilage organoids on decellularized cartilage matrix scaffolds to mimic the physiological and developmental processes of cartilage. As for modeling physiological processes, Liu *et al.*[144] constructed a condylar cartilage organoid to explore the primary cilia's functions. At present, there are relatively few studies using cartilage organoids to simulate the development of cartilage. A deeper understanding of this process is of great significance for investigating the pathogenesis of cartilage-related diseases and developing new therapeutic approaches.

Organoids also play an important role in simulating the development of skeletal muscle. For example, Shahriyari *et al.*[195] used hPSCs to construct a new skeletal muscle organoid model, successfully reproducing key stages of

human embryonic muscle development. Researchers have constructed new skeletal muscle organoid models using hPSCs and observed the construction of the models by monitoring gene expression, myocyte formation, and changes in contractile force.[165] Furthermore, Yin *et al.*[121] used hPSCs to construct the first self-organizing hNMSO, achieving spatial self-organization of nerves, muscles, and bones through a co-development strategy, revealing the key role of skeletal support in skeletal muscle development.

4.3. Organoids for the disease models

While animal models have played an important role in bone disease research, significant differences in pathological processes exist due to species differences, leading to many effective treatments in animal experiments failing to achieve the same results in clinical applications. MSK organoids, built using patients' cells, can highly simulate the cellular composition, tissue structure, and physiological functions of the human MSK system, providing a platform closer to the real human environment for disease research. This high degree of human simulation enables researchers to precisely observe the disease development process *in vitro* and investigate the interactions between cells and changes in signaling pathways, offering strong support for the analysis of disease etiology and pathogenesis.

MSK organoids have wide applications in simulating diseases, including trauma, inflammation, neoplasms, and hereditary disorders. First, MSK organoids can simulate the complex pathological process of fracture healing, including inflammatory response, new bone formation, and bone remodeling. Research by Price *et al.*[164] successfully converted mouse muscle precursor cells into idSCs using an organoid culture system. These idSCs exhibit robust self-renewal capacity and myogenic potential *in vitro*. In injury models, idSCs effectively fuse into myofibers, replenish the satellite cell niche, and support muscle regeneration through matching freshly isolated satellite cells. Compared to *in vivo* trauma models, organoids, relatively isolated microenvironments, enable precise control over injury magnitude and frequency, while circumventing stress responses and mortality risks associated with repeated animal injuries. In addition, when investigating cellular composition and behavioral changes in the MSK system post-trauma, *in vivo* models often involve multiple cell types with mutual interference. Hence, researchers engineered osteo-callus organoids incorporating hydrogel microspheres encapsulated with BMSCs, which effectively model the cellular composition and dynamics of endochondral ossification following long bone injury.[136]

Second, MSK organoids play an important role in simulating inflammatory diseases such as OA and RA. For instance, researchers have successfully induced OA-like inflammatory responses and cartilage degeneration in cartilage organoids by introducing pro-inflammatory cytokines, such as IL-1β.[196] Organoids not only simulate the pathological processes of OA, such as chondrocyte death, inflammatory responses, and degradation of the ECM but also allow for dynamic monitoring and quantitative analysis of the inflammatory responses and ECM degradation during the inflammatory process. Nevertheless, research and evaluation methods in OA animal models are predominantly confined to imaging, gait analysis, and histological staining, which inherently limit quantitative analysis and impede real-time monitoring of disease progression. In addition, Occhetta *et al.* developed a "cartilage-on-a-chip" model that simulates the mechanical factors in OA pathogenesis by applying compressive forces, thereby inducing a shift in cartilage from homeostasis to catabolism and hypertrophy.[197] Compared to the complex biomechanical loading *in vivo*, cartilage organoids engineered through "cartilage-on-a-chip" not only deliver hyperphysiological compression to cartilage but also enable systematic investigation into mechanical alterations throughout OA pathogenesis. When investigating the relationship between tissues/organs and biomechanics, OoC platforms and bioreactors exhibit exceptional capabilities. For example, Iordachescu *et al.*[133] utilized a microgravity bioreactor to simulate reduced mechanical stimulation, constructing an osteoporotic organoid model *in vitro*. This model enables the study of OP and bone remodeling processes. Organoids can also be utilized to simulate the pathological processes of RA and investigate the interactions between synovial tissue and immune cells.[198] For example, co-culturing cartilage organoids with synovial cells can mimic the inflammatory responses observed in the joints of RA patients.[199] For immune-mediated inflammatory diseases, specialized animal models or complex modeling approaches are typically required, whereas organoids offer simpler, more stable construction and significantly higher success rates than *in vivo* models.

Moreover, MSK organoids can be utilized to model both tumors and hereditary diseases. However, modeling animal tumors poses several challenges: (i) significant biological disparities exist between animal and human tumors; (ii) constructing primary tumor models is technically demanding; (iii) tumor models exhibit poor stability and reproducibility; and (iv) stringent animal ethics regulations and oversight impose limitations. MSK organoids, with advantages including: (i) high-fidelity recapitulation of human tumor microenvironments; (ii) enhanced model robustness and reproducibility; (iii) capability for real-time monitoring and long-term culture; and (iv) absence of ethical constraints, are increasingly favored for establishing animal tumor models, superseding animal models as the preferred approach for tumor modeling. The raw materials

for constructing tumor organoids can be categorized as tissue-derived or cell-derived. For tissue-derived organoids, patient tumor tissues undergo initial processing, mechanical dissociation, and enzymatic digestion, yielding tissue fragments that serve as tissue-derived organoid seeds. For example, Nie *et al.*[200] constructed OS organoids using 20 biopsy tissues and 12 surgical specimens from OS patients, with success rates exceeding 90%. Notably, these tissue-derived OS organoids formed within 2 weeks and demonstrated sustained proliferation for several months while maintaining phenotypic stability. Further, Suzuki *et al.*[201] developed tumor-derived organoids from human malignant giant-cell tumors (GCTB) tissues and genetically confirmed that the developed organoid lines represented malignantly transformed GCTB. Cell-derived organoids are developed from single cell types or established cell lines. Common cell sources include stem cells, tumor cell lines, or specialized cells. Forsythe *et al.* developed patient-specific sarcoma organoids using patient-derived tumor cells, and these organoids provide a vital platform for personalized oncology and rare tumor research.[202] Compared to animal models for tumors with unclear pathological mechanisms or rare tumors, tumor organoid cultivation offers a standardized and controllable environment, thus ensuring reproducibility and robustness in subsequent therapeutic testing.

The construction of animal models for genetic diseases represents a significant challenge in MSK disease modeling. While the rapid advancement of gene-editing technologies has substantially reduced modeling complexity, persisting issues include off-target effects, low knockout efficiency, uncertainty in integration sites, and animal ethics restrictions. MSK organoid models not only circumvent ethical issues associated with animal experimentation but also offer advantages, including scalable production and high construction rates. These genetically diseased organoid models exhibit high-fidelity recapitulation of pathological phenotypes, complete retention of disease-associated mutational profiles, and robust capabilities for multi-gene editing. For example, Gao *et al.*[182] successfully constructed NMOs from patient-derived iPSCs as an *in vitro* disease model for ALS. These NMOs recapitulate disease-relevant pathological features, including impaired skeletal muscle contraction, NMJ degeneration, and aberrant protein aggregation. In another study, the 3D skeletal muscle model constructed from DMD patient-derived iPSCs accurately recapitulates the pathological features of DMD.[203] This model integrates fully human, iPSC-derived, complex, multilineage muscle constructs containing key isogenic cellular constituents of skeletal muscle.

4.4. Organoids for the drug screening

Although animal models remain the primary method for drug screening, they exhibit significant limitations. Animal models' predictive validity for human drug responses is insufficient (with a preclinical translation failure rate exceeding 90%), and even drugs passing animal testing must undergo four costly and protracted clinical trial phases.[204,205] Clinical research trials consist of four phases: Phase I: Establishment of tolerability and safety in healthy volunteers; Phase II: Evaluation of therapeutic effect and adverse reactions in patients; Phase III: Establish the effectiveness and safety of the drug compared with placebo or current standard treatment; and Phase IV: Determination of benefits and risks after authorization. It may take between 10 and 15 years to bring a new drug to clinical use.[206]

MSK organoids offer significant advantages in drug screening as an alternative to animal experiments. They closely mimic human tissue structure and function, providing more accurate insights into drug mechanisms and efficacy while reducing errors caused by interspecies differences in animal models. In adition, organoid experiments are characterized by shorter durations and lower costs, enabling high-throughput screening and enhancing research efficiency. They also circumvent ethical concerns associated with animal testing, providing a more efficient platform to enhance predictive accuracy of drug development and shortening drug development cycles. For example, Occhetta *et al.*[197] encapsulated cartilage cells in PEG hydrogel and subjected to high compressive loads to simulate the pathological process of OA, with various anti-inflammatory and anti-catabolic drugs added for testing. O'Connor *et al.*[145] developed osteochondral organoids with a cartilage core and a calcified outer ring by inducing iPSC microclusters with TGF-β and BMP-2 to simulate endochondral ossification. This model can be used to screen potential OA-modifying drugs, as OA affects not only cartilage but also subchondral bone. As for high-throughput drug screening, Wei *et al.*[159] developed a human cartilage organoid-based system for high-throughput drug screening. From a library of over 2,000 FDA-approved drugs, they identified the α2-adrenergic receptor inhibitor phentolamine as a compound capable of simultaneously promoting chondrogenesis and suppressing hypertrophy. Meanwhile, the drug was further demonstrated to promote hyaline cartilage regeneration in mice and minipigs. In the evaluation of pharmacodynamic efficacy, researchers utilized osteochondral organoids to simulate the pathological processes of OA by inducing inflammatory responses through the addition of pro-inflammatory cytokines, such as IL-1β.[196] They tested the therapeutic effects of the adenosine A2A receptor agonist 2-(4-[2-Carboxyethyl]phenethylamino)-5'-N-ethylcarboxamidoadenosine on OA. The drug significantly upregulated the expression of forkhead box O (FOXO) 1 and FOXO3 in the cell nucleus, proteins that are typically suppressed or abnormally expressed in OA.

4.5. Organoids for tissue engineering

With the rapid advancement of tissue engineering technologies, the synthesis and fabrication of biomaterials have experienced exponential growth. The ultimate goal of tissue engineering is to facilitate the *in vivo* regeneration and clinical translation of biomaterials while ensuring their biocompatibility and repair capabilities. At present, the biocompatibility, regenerative ability, and clinical translation potential of biomaterials are predominantly evaluated through animal models, with groundbreaking advances achieved particularly in the field of MSK systems. As for evaluation of biocompatibility and repair ability, researchers conducted an evaluation of cranial bone repair using hydrogels mineralized with distinct metal ions in a rat model.[207] *In vivo* experiments demonstrated significantly enhanced osteogenic efficacy. In addition, Sharma *et al.*[208] developed a poly(ethylene glycol) diacrylate-based bioadhesive to repair focal cartilage defects in a caprine model. This soft hydrogel adhesive enhanced the efficacy of microfracture treatment, promoting cartilage regeneration. In the context of clinical translation, biomaterials require further evaluation in large animal models (porcine, ovine, and non-human primates) before human trials. For example, researchers evaluated the osteogenic ability of porous ceramic scaffolds in a 48 mm ovine tibia defect model. Results demonstrated that the scaffold was gradually replaced by new bone over a year and was fully absorbed within 2 years.[209]

Due to individual variations in animal model establishment, biases in construction methods, as well as the prolonged experimental periods and high costs of large animals, animal models are increasingly becoming constraints on the development of tissue engineering and clinical translation of biomaterials. MSK organoids offer advantages such as low cost, high reproducibility, broad applicability, and scalability, and have already become complementary experimental models to animals in tissue engineering. MSK organoids can be utilized not only to evaluate the biocompatibility of biomaterials but also to analyze their ability to facilitate osteogenic differentiation and bone tissue formation. For instance, Mikael *et al.*[210] employed bone organoids to investigate the significance of patient-specific biomaterials in promoting bone regeneration. Similarly, in cartilage repair, organoids provide a dynamic system to assess the mechanical and biological properties of biomaterials. The study by Vainieri *et al.*[211] demonstrated that biomaterials should not only support the growth of chondrocytes but also respond appropriately to mechanical stimuli, which is crucial for the successful integration and function of repaired cartilage tissue. The application of MSK organoids in biomaterial clinical translation remains at an early-stage phase. Establishing standardized evaluation criteria and methodologies tailored for biomaterial translation is imperative, as this will accelerate the exponential advancement of tissue engineering and propel the rapid clinical deployment of biomaterials.

5. The challenges of replacing animal experiments with organoids

5.1. Insufficient system complexity

Due to the high complexity of tissues and organs, their intricate structural relationships and crosstalk effects have not yet been effectively recapitulated *in vitro*. Despite significant structural and functional advances in MSK organoids in recent years, most existing organoids are limited to simple simulations of a single tissue type, lacking significant system complexity.[212,213] For example, skeletal muscle organoids usually form only myotubular structures, but lack key elements, such as vascularization, innervation, and tendon connections, which significantly limit the in-depth study of muscle contraction function and mechanical properties.[123,214] The physiological functions of bone, cartilage, and skeletal muscle tissues are far beyond the independent action of a single cell and rely on the sophisticated synergy of vascularization, nerves, mechanical feedback, and hormonal signals.[215-217] Current single-tissue organoid models of the MSK system lack cross-system functional integration, making it challenging to realistically reproduce the complex physiological and pathological processes of the MSK system *in vivo*. Therefore, the construction of organoids *in vitro* that can comprehensively recapitulate the MSK system still faces significant challenges.

5.1.1. Absence of vascular network

The integration of a perfusable vascular network into MSK organoids is a key breakthrough in achieving their functional maturation and reaching adequate tissue size. The lack of vasculature in traditional organoid cultures results in insufficient nutrients and oxygen for the internal cells, as well as accumulation of metabolic byproducts, thereby limiting the growth size and functional maturation of the organoids.[218,219] Especially in MSK organoids, the high energy demand and metabolic activity make the vascularization of organoids even more important.[220,221]

The introduction of blood vessels addresses the physical limits of nutrient diffusion. According to the "diffusion limit" theory, the thickness of non-vascularized tissues usually does not exceed 200 μm; otherwise, the core area will be necrotic due to hypoxia and nutrient deficiency.[222] By engineering a perfusable vascular network, it is possible to mimic blood circulation *in vivo* and support the survival of millimeter-sized or even centimeter-sized organoids. Second, vascular networks can promote the

functional maturation of MSK organoids. For example, ECs in skeletal muscle not only provide blood supply but also regulate the alignment and contractile function of muscle fibers by secreting growth factors, such as vascular endothelial growth factor (VEGF) and insulin-like growth factor (IGF)-1.[223,224] In addition, in the skeletal system, osteogenesis is associated with a specific capillary EC subtype, termed type H.[218,225] This subtype shows high expression of the markers CD31 and endomucin, which are present in the epiphysis and endosteum of postnatal long bones. In addition to inducing vascular growth, type H ECs also act on osteogenic progenitor cells through molecular signaling.[226] Therefore, the introduction and integration of a functional vascular network into MSK organoid cultures could address the challenge of organoid undernutrition and promote functional maturation of organoids.

Recently, researchers have made some attempts, and Wang *et al.*[227] 3D printed a stable vascular network structure (Figure 5A). The vascular network can achieve blood flow perfusion through surgical anastomosis of the circulatory system (Figure 5B), innovatively providing a unique vascularized tissue suitable for implantable applications. Liu *et al.* developed an *in vitro* vascular model in which ECs germination and subsequent formation of a perfusable lumen were enabled by chemokine guidance and degradation of the matrix hydrogel (Figure 5C and D).[228] It was found that the adhesive and degradable properties of the hydrogel enabled ECs to invade collectively and generate perfusable tubes, providing a new understanding of cell-matrix material interactions and regulating angiogenesis. Tsigkou *et al.*[229] seeded collagen-fibronectin gel containing HUVECs and human MSCs (hMSCs) on the scaffold. Surprisingly, hMSCs assumed the role of perivascular cells and acted as effective stabilizers of the engineered vessels, allowing the HUVECs to form tubular structures 7 days after scaffold implantation *in vivo*. Although some human-derived HUVECs remained after 5 months, the vascular system of most grafts had been remodeled by host rat cells. This study confirms the ability of HUVECs to function as organoid vascular building cells and to form connections with blood vessels *in vivo* to accomplish nutrient transport. These studies offer a new strategy for inducing angiogenesis *in vitro*, promoting a significant step forward for vascularized organoids, which can effectively overcome the challenge of oxygen and nutrient diffusion, as well as expand the size and scale of organoid culture.

5.1.2. Incomplete innervation

In addition to providing electrical stimulation signals in the MSK system, innervation is a key regulatory component in the development and repair of the MSK system. Functional NMJ cannot mature without motor neurons.[230] The axons of motor neurons can recognize, grow directionally, and connect precisely to specific regions of the muscle fiber to form synaptic structures. This process triggers muscle fibers to form mature terminal zones at the junction, characterized by a high density of acetylcholine (ACh) receptor clusters.[231] Complex molecular signaling exchanges between axons and muscle fibers drive junction formation and end zone maturation. Not only that but innervation also profoundly

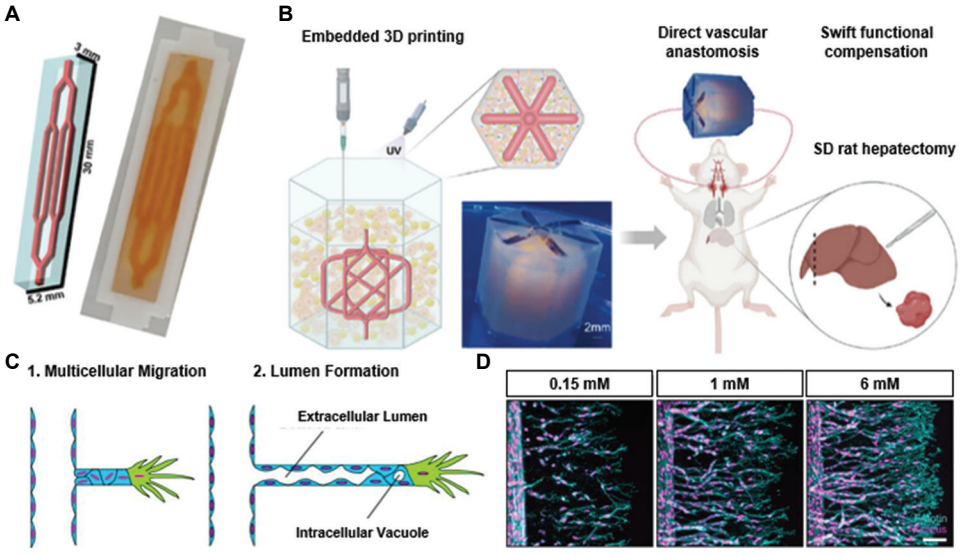

Figure 5. Attempt to construct vascular network. (A) Scheme and optical images of multichannel engineered blood vessels printed in hydrogel hybrids; (B) scheme of 3D printed engineered blood vessels implanted into liver tissue of Sprague-Dawley rats with liver failure.[227] Reprinted with permission from Wang *et al.*[227]; (C) vessel lumen formation involves two consecutive steps: HUVECs migrate into DexMA first, followed by lumen formation; and (D) intervention of HUVECs in DexMA hydrogels with different concentrations of adhesion peptides, the number of HUVECs germinated increased with increasing concentrations of adhesion peptides.[228] Reprinted with permission from Liu *et al.*[228]
Abbreviations: 3D: Three-dimensional; Dexma: Dextran methacrylate.

influences the construction of muscle tissue by directing the orientation of muscle fibers, delineating muscle bundle boundaries, and even shaping the overall morphology of the muscle to avoid the formation of disorganized cell clusters.[232] At the same time, the loss of innervation is the most important cause of muscle atrophy (both wasting atrophy and neurogenic atrophy). This highlights the dual critical role of nerves on muscles. On the one hand, nerves provide essential trophic factors that maintain muscle mass and metabolic activity. On the other hand, the electrical activity of nerves is a central stimulus that maintains the balance between protein synthesis and degradation and inhibits apoptotic signaling within the muscle. At the core of many diseases of the MSK system lies, a disruption of the NMJ or a lesion of the nerves/muscles themselves, for example, ALS, spinal muscular atrophy, and myasthenia gravis. Simple muscle organoids lacking the nervous system cannot accurately mimic key pathological processes, such as secondary muscle damage, due to neurogenesis. Martins *et al.* induced hPSCs to generate spinal cord neurons and skeletal muscle cells in 3D culture, which were able to self-organize and form NMOs containing functional NMJ.[233] This organoid model provides a powerful tool for exploring the developmental process of functional neuromuscular networks and the underlying communication mechanisms. Notably, in this type of organoid, sustained innervation is equally necessary to maintain the long-term survival of muscle fibers and prevent their degeneration, further confirming the indispensability of nerves in muscle homeostasis.

In recent years, it has been reported that the nervous system not only regulates muscle activity but also directly affects bone development and metabolism through a complex signaling network. Among them, sympathetic nerves mainly act through the release of norepinephrine and neuropeptide Y.[234-236] These neurotransmitters have been shown to modulate osteoblast activity and osteoclast-mediated bone resorption. In contrast, parasympathetic nerves act through ACh and vasoactive intestinal peptide.[237-239] Significant cholinergic innervation is observed during skeletal growth and development and during fracture healing. These neurotransmitters not only promote the proliferation of osteoblasts but also induce apoptosis of osteoclasts, thus maintaining the dynamic balance between bone formation and bone resorption. The role of sensory nerves in bone regulation is also not negligible. Calcitonin gene-related peptide, as the main transmitter of sensory nerves, can significantly increase the expression level of osteogenic differentiation markers in BMSCs, suggesting that it may be involved in the process of bone repair through the promotion of differentiation of BMSCs to osteoblasts.[240,241] Together, these neuromodulatory mechanisms constitute a complex network of neural-bone interactions that maintain bone homeostasis by precisely regulating osteoblast and osteoclast activity.

In conclusion, innervation in the MSK system not only provides electrical stimulation signals but is also deeply involved in the physiological functions of muscle and bone. The incorporation of the nervous system can further enrich the pathology model of MSK organoids. However, researchers are currently unable to extract primary functional neurons from patients, which pose a significant challenge to the study of NMOs. To address this issue, researchers have turned to patient-derived iPSCs as an alternative. By inducing iPSCs through different processes, researchers can differentiate them into muscle cells and neurons, successfully forming NMOs.[233] These organoids effectively mimic muscle weakness and NMJ defects, serving as models for neuromuscular diseases and platforms for screening therapeutic drugs. They provide a valuable platform for studying disease mechanisms and potential therapeutic interventions.

5.1.3. Deficiency of the immune system

Whereas traditional anatomy views the MSK system as a mechanical support structure and categorizes the immune system as a defense network, contemporary research over the past two decades confirms their profound integration through osteoimmunology.[242] In the muscle microenvironment, the development and repair of muscle cells are mainly regulated by T cells and macrophages. T cells express amphiregulin, epidermal growth factor receptor, and suppression of tumorigenicity 2, which promote myotube maturation and contractile function.[243] Macrophages, on the other hand, secrete factors such as IGF-1 and TGF-β through the M2 type to reduce fibrosis and drive functional myofiber formation.[244] In the skeletal microenvironment, the bone marrow serves as the core hematopoietic site, providing a symbiotic ecological niche for immune cells (T/B cells, macrophages, and granulocytes) and skeletal cells (osteoblasts, osteoclasts, and osteocytes). Among them, the RANKL/receptor activator of nuclear factor-κB (RANK)/OPG pathway is a key hub for bone immunoregulation.[245] RANKL was initially discovered in activated T cells and regulates T-cell function. Subsequent studies have shown that osteoblasts also express high levels of RANKL, and that RANKL-RANK binding directly drives osteoclast differentiation, activation, and survival, leading to bone resorption.

In addition to T cells, cytokines secreted by other immune cells profoundly affect the balance of bone formation and resorption by modulating this pathway. Neutrophils infiltrate during the acute inflammatory phase, releasing reactive oxygen species and proteases that directly damage bone tissue and indirectly accelerate bone resorption through pro-inflammatory factors.[242]

Peroxidase, secreted by eosinophils, inhibits osteoclast overactivation and plays a protective homeostatic role in inflammation. Macrophages, as osteoclast precursors and microenvironmental coordinators, dominate bone homeostasis, injury repair, and pathological destruction by secreting signaling molecules and intercellular contacts.[246]

From this perspective, the immune system plays an indispensable role in the development and normal function of MSK organoids. The absence of the immune system may lead to an imbalance in the osteoblast-osteoclast coupling due to the lack of T-cell-derived RANKL.[245] It may also result in the sustained release of local inflammatory signals from injured tissues due to the absence of neutrophils and macrophages, leading to delayed regeneration. The absence of an immune microenvironment significantly hinders progress in MSK organoid research. To establish an effective immune microenvironment in MSK organoids, co-culture is a mature and feasible method.[247] Researchers can simulate the immune microenvironment in the MSK system by combining cells, drugs, or gene editing technologies. In addition, the development of microfluidic chip technology enables researchers to precisely regulate cell interactions and the delivery of soluble factors in co-culture systems. These methods provide powerful tools for studying the dynamic interactions between immune cells and MSK organoids, accurately simulating key pathological processes such as RA, OP, and muscle atrophy.

5.1.4. Absence of hormonal regulation

The endocrine system realizes the precise regulation of the MSK system at multiple levels through hormonal messengers, and its mechanism of action is both extensive and complex. During the development of the MSK system, the growth hormone/IGF axis and the hypothalamus-pituitary-thyroid axis play a critical regulatory role.[248] Activation of these endocrine axes is capable of finely regulating bone and muscle growth processes. However, hormone release is not simply constant, but shows a highly dynamic pattern that includes pulsatile secretion, circadian fluctuations, and stress-responsive release. This complex pattern of secretion enables the endocrine system to be precisely regulated according to the needs of the organism, but, at the same time, poses a great challenge for *in vitro* studies. Although significant progress has been made in organoid culture technology, it is still unable to realistically simulate the dynamic hormone fluctuation environment in the human body, which is a limitation that may lead to serious bias in drug screening and organ function studies. In the case of juvenile thyrotoxicosis, for example, the disease is caused by abnormally high levels of thyroid hormone release. Excessive thyroid hormone accelerates the process of bone development, leading to premature fusion of the growth plates, which ultimately results in the premature termination of the patient's height growth and the development of short stature.[249] This pathological process fully reflects the importance of precise regulation of hormone levels, which is achieved through the positive and negative feedback regulation of the endocrine system. Feedback regulatory mechanisms ensure that hormone levels are maintained within a narrow range of physiological requirements and that they respond precisely and dynamically to adaptive changes in the external and internal environment. The absence of this mechanism is particularly evident in the field of organoid research. For example, corticosteroids are unable to mimic clinically important phenomena, such as adrenal suppression and disturbances in endogenous cortisol secretion resulting from long-term drug use in organoid models that lack negative feedback regulation. This lack may lead to grossly underestimating the risk of drug-induced rhabdomyolysis or ignoring the adverse effects, such as muscle weakness, that may occur after drug withdrawal.[250,251] Of greater concern is the fact that organoids are cultured in isolation so that they can neither sense and respond to changes in the whole-body state nor trigger the complete endocrine cascade. This endocrine dysfunction impairs hormonal regulation of organoids, a process often critical in clinical applications, thereby compromising the functionality of MSK organoid models. These limitations suggest that the development of organoid technology should focus on constructing culture systems that closely resemble the *in vivo* microenvironment and integrating dynamic hormone regulatory networks to more accurately mimic the complex physiological and pathological processes in the human body.

5.1.5. Difficult to reproduce mechanical stimuli

As the core system responsible for human limb movement, the development, maintenance, and functional realization of the MSK system are always closely linked to mechanical stimulus signals.[252] In a complex biomechanical environment, the bone is continuously subjected to a variety of stresses, such as compression, bending, and torsion. Moreover, its density and microstructure are dynamically remodeled in response to changes in mechanical loading. Muscle fibers are subjected to active contraction and passive pulling forces, adjusting their thickness in response to changes in strength training. Joints are subjected to repeated cyclic compressive and shear stresses to maintain interface lubrication and limb mobility. These phenomena profoundly reveal the high dependence and active adaptation of the MSK system to mechanical stimuli. From the cellular and molecular level, mechanical signaling is a key regulator that is indispensable for maintaining the maturation phenotype of cells, such as osteoblasts, chondrocytes, and tendon cells. In the absence of appropriate mechanical stimulation,

the molecular mechanisms of intracellular mechano-signaling are significantly disrupted.[253,254] For example, YAP/transcriptional coactivator with postsynaptic density protein 95/Drosophila disc large tumor suppressor/zonula occludens-1-binding motif transcriptional co-activators is unable to efficiently translocate into the nucleus, and the Wnt/β-catenin signaling pathway activity is abnormal, which leads to the expression profiles of key downstream genes regulating bone formation and the dominant factors of myogenic differentiation to deviate from physiological status. This dysregulation at the molecular level will trigger a profound shift in cell fate. For example, the mineralization capacity of osteoblasts and the anabolic activity of chondrocytes will decrease. The ultimate manifestation is cellular dedifferentiation or reduced function, which completely fails to reflect their true state of activation in the mechanical microenvironment *in vivo*.

However, current organoid culture for MSK systems is mostly limited to static environments or primary devices that can only provide simple unidirectional cyclic strain, making it difficult to effectively reproduce the complex and variable mechanical environment *in vivo*. This serious lack of mechanical simulation has led to significant deviations from the *in vivo* situation in terms of structural integrity, functional maturity, and physiological responsiveness of organoids. Several studies have provided empirical evidence for the critical role of mechanical stimulation in organoid culture. Schädli *et al.*[255] inoculated hMSCs in PLGA scaffolds and subjected the scaffolds to cyclic compression through a bioreactor. It was found that hMSCs cultured under dynamic conditions of cyclic compression secreted more collagen, while the scaffold mineral density increased significantly. These findings establish the necessity of cyclic mechanical simulation in inducing the maturation of functional bone organoids. In the study of the muscle mechanical response mechanism, Bieling *et al.*[256] resolved the force feedback regulation mechanism of the actin network. It was reported that when the self-assembled actin fiber network encounters external mechanical resistance, it results in a significant increase in the density and structural rigidity of filaments within the network. This strengthening effect, directly driven by mechanical stimuli, is essentially a microscopic pre-adaptation of the muscle in response to mechanical loading. This discovery elucidates at the subcellular level how mechanical stress regulates cytoskeletal dynamics and ultimately influences the maturation of muscle structure and function at the tissue level. By accurately modeling the complex mechanical environment *in vivo* and reintroducing the core element of force into the organoids of the MSK system, can we promote the organoids of the MSK system to truly become organoids capable of moving?

5.2. Non-uniform construction standards

The successful transition of MSK organoids from the laboratory research stage to the field of wide application must first overcome the key challenge of standardization. The current lack of standardization in the preparation of organoids has become a major bottleneck constraining their development. This lack of standardization is reflected at multiple levels. In terms of experimental reproducibility, it is often difficult to corroborate the results of different research teams or even different batches of experiments by the same team. Regarding data comparability, various research methods make it difficult to compare and analyze the data horizontally. In terms of large-scale production, the lack of standardization poses a significant challenge to stable production. Together, these factors seriously limit the potential of organoid technology for translational applications.

Achieving organoid standardization is an extremely complex, systematic project, as it covers the complete process chain from cell acquisition to final data analysis, each of which contains variability factors that may lead to biased results. The primary source of variation is the heterogeneity of cell sources. Stem cells from different donor sources will exhibit inherent differences in genetic and epigenetic characteristics due to differences in age, gender, genetic background, and health status of individual donors.[257,258] These differences will directly affect the differentiation potential of stem cells, their proliferative properties, and the phenotypic characteristics of the final organoid formed. To unify the heterogeneity of tumor cells, Ji *et al.*[259] collected 65 types of liver cancer tissues from different patients and constructed a liver cancer organoid biobank (LICOB). Employing this LICOB for multiple omics analyses and high-throughput drug screening can comprehensively collect histological and molecular characteristics of various types of liver cancer, avoiding the limitations of a single cell type.

Secondly, Matrigel and fetal bovine serum, the critical components of the organoid culture, have a significant "black box" effect problem. These two substances, as indispensable culture matrices for maintaining the normal proliferation and functional status of stem cells, contain a variety of proteins and cytokines that are essential for cell behavior.[260,261] They cannot be completely replaced by synthetic means. However, the marked batch-to-batch variability of these naturally occurring substances, as well as the risk of immunogenicity, can significantly interfere with cell behavioral patterns and the stability of organoid developmental processes. The core of solving this problem is to decipher the secrets of Matrigel and fetal bovine serum active ingredients, and formulate an economically effective synthetic substitute. Sekine *et al.*[262] developed a completely synthetic, chemically well-defined animal-free culture

medium. This medium can effectively reduce the risk of batch variation, ensure the reproducibility of the culture system, and successfully realize the process of culturing iPSCs into liver organoids.

The culture protocol of organoids also suffers from great inter-laboratory variation, with significant differences in the induction protocols (type of growth factors, concentration, time of addition, etc.) used by different researchers during cell culture. This technical operation, which is highly dependent on the operator's personal experience, leads to poor experimental reproducibility. This ultimately results in inconsistent organoid differentiation results, creating obvious inter-batch differences. The key to addressing batch-to-batch variability in organoid culture processes is the use of high-throughput technology. High-throughput technology encompasses automated sample processing, microfluidics, OoC systems, and high-content imaging techniques. By leveraging these high-throughput technologies, researchers can efficiently and rapidly complete the culture and analysis steps in organoid research, ensuring consistency and reproducibility across experiments. Schuster *et al.*[263] developed a standardized, miniaturized, and automated high-throughput microfluidic platform. This platform enables the cultivation of organoids or 3D cell cultures in a reliable environment with minimal reagent consumption and reproducible conditions, while simultaneously monitoring and screening cell phenotypes. This allows researchers to obtain a large amount of reliable experimental data at a lower cost. Li *et al.*[264] also developed a high-throughput polymethyl methacrylate microfluidic chip that can be used to study the growth of tumor spheroids under different combinations of factor concentrations and to perform combined drug screening on tumor spheroids. These studies have demonstrated the importance of high-throughput technology in organoid research, effectively addressing the shortcomings of traditional research methods.

In addition to the lack of uniform standards in the culture process, the characterization system of organoids also faces the dilemma of insufficient standardization. At present, there is a lack of clear and quantifiable evaluation standards for the key parameters of organoids, such as the degree of mineralization of bone organoids, the spatial distribution characteristics of proteoglycans in cartilage organoids, and the arrangement pattern of myotubes in muscle organoids, all of which lack a unified evaluation system. Meanwhile, the expression profiles of key functional genes and proteins in MSK organoids at the molecular level have not yet been established as standardized databases. These standardization deficiencies have hindered the practical application of organoids.

Overall, there is a lack of a standardized system for key aspects, such as cell acquisition, culture, and functional evaluation. At present, no strict and universally binding operational and quality control standards have been established globally to standardize the organoid culture process. The absence of such a standardized system will inevitably introduce additional variables into organoid-related research, thereby affecting the reliability and comparability of research results. At present, only a limited number of guidelines for organoid quality control have been published. ISSCR has released the "Standards for Human Stem Cell Use in Research," standardizing processes such as the sourcing of hPSCs/hASCs, ethical approval, and cell identity verification. The European Committee for Standardization and the European Committee for Electrotechnical Standardization, among other organizations, have jointly published the "Roadmap for Organ-on-Chip Standardization," comprehensively deploying OoC standardization efforts and accelerating the standardization process for OoC. The Organoid Standards Initiative released the "Organoid Manufacturing and Application Guidelines" in 2024, systematically outlining the complete process from organoid preparation to application, to establish global unified standards for organoid culture, validation, and application. Although various institutions have introduced standards and specifications applicable to their operations, a consensus on organoid standards remains unavailable internationally.

5.3. Challenges facing clinical translation

Although technological breakthroughs in MSK organoids are emerging in cutting-edge fields, their translation into preclinical and clinical applications is accompanied by serious and complex obstacles.[265] The first challenge arises from the research starting point: cell source and informed consent. Organoid culture is highly dependent on biological samples from human donors. During the process of collecting samples, many volunteers who donate samples often find it difficult to understand the cutting-edge concept of organoids and the complex application scenarios that their cells may undergo in the future. This dilemma is exacerbated by the prevalence of broad consent.[266] Donors, especially those seeking treatment for MSK injuries or illnesses, may be unaware that their samples will ultimately be used to construct organoid models, which may in turn be involved in drug screening chains, biomaterials development, and even grafts. It is even more challenging to anticipate the enormous commercial value that their samples may generate. This information asymmetry and ambiguity in informed consent constitute a significant contradiction in the current development of organoids, which seriously undermine the rights and interests of donors. A traceable and updatable electronic consent system should be established for new scenarios, such as organoids, drug development, and commercial transformation, where samples may be used in the future,

allowing donors to withdraw or update authorization at any time. At the same time, a new strong regulatory framework is needed to ensure the autonomy of donors. At present, countries are promoting special legislation for organoid research and clarifying the bottom-line rules for informed consent, data use, and commercial transformation.

Second, although organoids perform well *in vitro*, their functional integrity and maturity still differ significantly from human organs. The survival, integration, and functional recovery of organoids after transplantation into the body lack systematic verification. To overcome this challenge, the simplest approach is to first validate organoids in animal models. Verifying the ability of organoids to integrate with host tissues and assessing their feasibility as potential transplants is crucial. However, the implantation of human-derived organoids into animal models raises deep cross-species ethical concerns.[267,268] When human tissues are highly developed in animals and may even partially participate in or influence host MSK functions, traditional species boundaries are substantially impacted. This not only challenges existing biological perceptions and socio-ethical concepts but may also raise profound questions about animal welfare and potentially uncontrollable biological risks. Therefore, the development of detailed and rigorous ethical review guidelines specific to organoid chimera research has become an urgent scientific and ethical task. Transplanting organoids back into the human body to repair damaged tissue is arguably the ultimate goal of research, but it also faces high-risk ethical considerations unique to human trials. Although preclinical studies provide fundamental data, the unknowns of human transplantation remain enormous. Issues such as immune rejection and long-term safety cannot be guaranteed.[269] Therefore, the ethical review system must play a crucial role in centering on the strict application of the principle of risk minimization.[268] This requires an extremely rigorous assessment of the trial design, including the selection of the most appropriate group of subjects, the setting of clear and monitorable safety endpoints, the formulation of detailed contingency plans, and the establishment of a long-term follow-up mechanism. This ensures that the life safety and health rights of the subjects are prioritized to the highest degree.

Finally, the rapid development of organoid technology has generated a huge commercial wave, which cannot be separated from issues of cost and benefit. Although the combination of iPSCs and gene editing technology can achieve individualized modeling of organoids, the high cost and long cycle of the technology mean that MSK organoids can currently only be developed on a small scale in laboratories and are unlikely to be widely adopted throughout society in the short term. In addition, cell samples from patients undergo a complex research and development process, which may ultimately result in diagnostic tools, therapeutic products, or biomaterials of significant value, potentially generating enormous profits.[270] Ensuring a balance between the interests of clinical donors providing biological samples, research institutions investing intelligence and resources, and investors taking risks to provide funding is a key issue in the equitable distribution of the enormous benefits of these commercial transformations.[271] There is an urgent need to explore innovative governance models and protocol frameworks that meet basic ethical licensing requirements. A dynamic and sustainable balance needs to be found between adequately incentivizing scientific and technological innovation and ensuring that the technological dividend reaches society at large.

6. Construction techniques of next-generation organoids

6.1. Organoid-on-a-chip

Organoid-on-a-chip, a multi-channel 3D microfluidic platform recapitulating organ-level activities, biomechanical properties, and physiological responses, will bring a revolutionary breakthrough in the cultivation and application of MSK organoids.[272] Its key strength lies in enabling continuous perfusion and dynamic medium renewal through integrated microchannel networks, thereby overcoming the inherent limitations of conventional static cultures.[273,274] This biomimetic fluid environment is crucial for metabolically demanding sports tissues, especially skeletal MSK organoids that consume large amounts of oxygen and glucose and produce lactic acid. For instance, Kesharwani *et al.*[192] used organoids derived from human ESCs to model vascular dynamics at the initial stage of endochondral ossification on a microfluidic chip. This model not only elucidates novel aspects of human endochondral ossification but also demonstrates broad application prospects for modeling bone disease and drug screening. Another study conducted by Whelan *et al.*[275] established a microphysiological model of vascular invasion during bone development and regeneration to recapitulate endochondral ossification. This process was achieved by integrating ECs with organoids representing distinct stages of endochondral bone development within a microfluidic chip.

More importantly, OoC empowers researchers with unprecedented spatiotemporal precision manipulation of the experimental microenvironment. By integrating a sophisticated flow control system, growth factors or drug molecules can be precisely applied to organoids at preset spatial and temporal points.[276,277] This capability enables *in vitro* recapitulation of complex dynamic signaling cascades in MSK organoids, thereby modeling *in vivo* developmental, reparative, or pathological processes.

For example, in bone organoid construction, osteogenic differentiation and mineralization of MSCs can be precisely regulated by adjusting the concentration of delivered BMPs.

Organoid-on-a-chip also provides a unique platform for in-depth analysis of metabolic characteristics and drug response in MSK organoids. Its inherent microscale nature facilitates the integration of real-time pH, oxygen concentration, and metabolite sensors.[278] This enables monitoring of the organoid microenvironment and secreted components, providing direct data for assessing energy metabolism or cellular differentiation processes. For example, Scheinpflug *et al.*[279] developed a microphysiological system capable of studying human skeletal biology under simultaneous control of oxygen tension and mechanical loading. Moreover, Tong *et al.*[182] developed an MSK OoC to investigate the mechanisms of muscle-bone communication under IH. They revealed that muscle mitochondrial protein SIRT3 modulates bone metabolism through regulating the myokine CXCL5. This study reports a novel microphysiological model for muscle-bone axis research. The powerful interconnectivity of OoC also makes it an ideal framework for building multi-OoC systems.[280] Functional crosstalk between the MSK organoids and other key organoids through microfluidic channels can establish highly biomimetic circulation. Jin *et al.*[281] used a high-throughput microfluidic array platform to integrate other types of organoids with 3D micro-hepatic tissue to establish a multi-organ model that simulates drug absorption and metabolism (Figure 6A-D). This closed-loop design not only realistically reproduces the dynamic material exchange and signaling between organs but also constructs a complex *in vitro* microenvironment for the study of systemic physiopathology and prediction of pharmacokinetics. This greatly improves the physiological relevance and accuracy of drug safety evaluation and efficacy prediction.

6.2. Assembloids

The construction of MSK organoids is a complex project involving the assembly of multiple cell and tissue types. To break through the bottleneck of current single-tissue-confined organoids for biomimetics and to move towards the system-level simulation that realistically reproduces the overall physiological functions of the MSK system, the core strategy lies in the adoption of the "Assembloids" technology.[284] From molecular signaling, intercellular communication to inter-tissue fusion, assembloids can rebuild the intrinsic multi-level interactions network of the MSK system and ultimately realize the system-level physiological response of neural command to muscle contraction, driving the skeletal structure to produce coordinated movement.[122] However, realizing this revolutionary breakthrough is by no means an easy task. To overcome the bottlenecks encountered in the construction of MSK organoids using assembloids, a synergistic approach combining co-culture and bioprinting can be adopted. Co-culture is not merely a simple mixture of cells. It involves spatio-temporal programming of multi-lineage stem cells at the molecular and cellular level, combined with multi-modal bioprinting to synchronously construct mechanical adaptation, vascularization, and neural innervation at the macro scale. This approach can systematically recreate the complex physiological process where neural signals drive muscle contraction, which, in turn, coordinates skeletal movement, thereby overcoming the functional limitations of current organoid consortia. This provides a new paradigm for modeling MSK diseases, drug testing, and regenerative repair.

It is encouraging to note that there have been encouraging pioneering explorations on the path of multi-tissue integration to build composite organoids to support MSK organoids. Pirosa *et al.* innovatively utilized PCL/HA/GelMA composite scaffolds as the structural foundation, loaded with hMSCs and HUVECs. A vascularized osteochondral organoid was successfully constructed with the dual-chamber microphysiological system bioreactor.[285] The key breakthrough of this model is that it integrates blood vessels, bone, and cartilage, which are physiologically closely related and functionally interdependent tissue units, to achieve functional crosstalk. Histological and gene expression analyses demonstrate that hMSCs exhibited clear osteogenic and chondrogenic differentiation features in different spatial regions of the scaffold, suggesting that spatial regulation of the microenvironment induced heterogeneous differentiation of cell fates. More importantly, the capillary-like network formed by the self-assembly of HUVECs not only provided channels for nutrient and oxygen delivery but also its secreted paracrine factors were also demonstrated to significantly promote osteogenic and chondrogenic differentiation of hMSCs, which vividly recapitulated the central regulatory role of the vasculature *in vivo* on skeletal development and homeostasis. This demonstrates that it is feasible to functionally integrate multiple heterogeneous tissues *in vitro* and achieve promotive interactions between them. This is a promising and fundamental step toward the construction of more complex assembloids.

6.3. Artificial intelligence (AI)

AI is profoundly changing the current research method, and through data-driven intelligent optimization, analysis, and control, it can significantly improve the efficiency of organoid research in MSK systems. In the optimization of organoid construction and culture, AI can break through the traditional empirical trial-and-error method. Through machine learning algorithms,

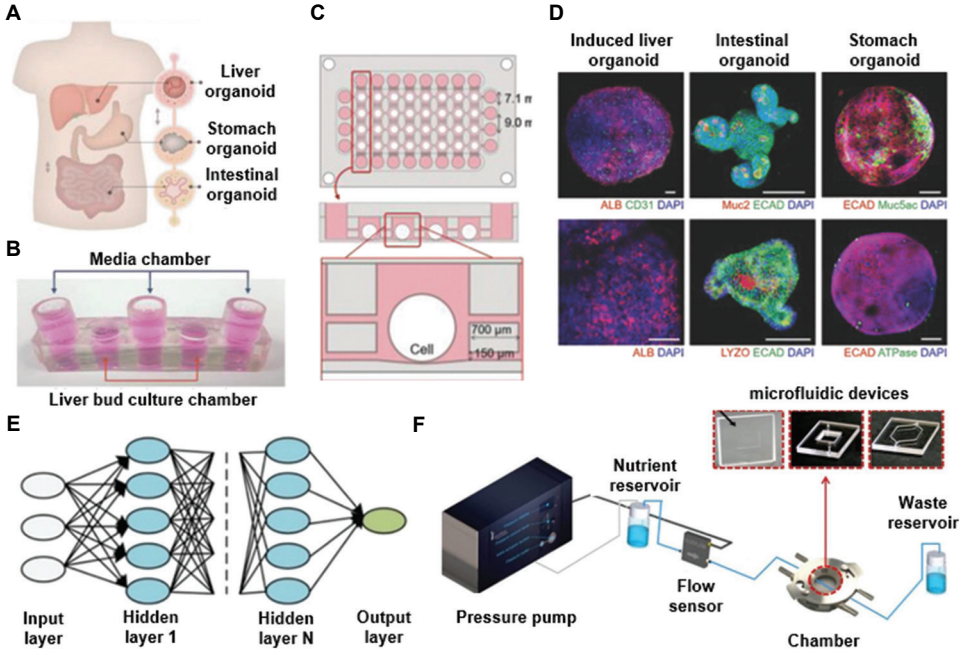

Figure 6. Construction of next-generation organoids. (A) Schematic illustration of the multiorgan model of liver, small intestine, and stomach; (B) pictures depicting the front view of the microfluidic device; (C) schematic diagram of front view of the microfluidic device in a microplate array format; (D) immunostaining analysis of specific markers (albumin for liver, Mucin 2 and lysozyme for intestine, and Muc5ac and Hydrogen/Potassium ATPase for stomach) (scale bars = 200 μm).[281] Reprinted with permission from Jin *et al.*[281]; and (E) conventional model construction in deep learning.[282] Reprinted with permission from Zare Harofte *et al.*[282]; (F) Illustration of the microfluidic system with precisely controlled flow.[283] Reprinted with permission from Babaliari *et al.*[283]

especially deep learning of the complex nonlinear relationship between massive culture parameters (such as growth factor combinations and concentrations, mechanical stimulation modes, and ECM components) and organoid culture results (survival rate, differentiation efficiency, and functional indexes), AI can provide the optimal culture program (Figure 6E).[282,286,287] For bone organoids, machine learning algorithms can dynamically regulate the mechanical loading parameters, delivery time of BMP-2, VEGF, and signal pathways to precisely balance osteogenic differentiation and angiogenesis. This addresses the persistent challenges of core necrosis and inadequate vascularization commonly encountered in static organoid cultures.[288] For skeletal muscle organoids, AI optimizes the synergy between electrical stimulation patterns and cytokines to maximize myofiber growth and functional maturation. At the level of phenotypic analysis and functional assessment, deep learning-based computer vision technologies can accomplish high-throughput, non-invasive, quantitative resolution of the structure, and composition of organoids. Traditional methods that rely on histologic sections and manual measurements are inefficient and subjective. In contrast, AI-driven image analysis tools can automatically process 3D organoid images acquired by bright-field, fluorescence, or confocal microscopy.[289] AI can also be integrated with automation, microfluidic techniques, and biosensors to build a perception-decision-execution system, which can promote the standardization, scale-up, and personalization of MSK organoids research. For example, an intelligent bioreactor with integrated real-time sensors (pH, O^2, metabolite, and mechanical sensors) can be constructed (Figure 6F).[283] The collected microenvironmental data can be input into the AI model, which dynamically adjusts the perfusion flow rate, nutrient supply, drug dosage, or mechanical stimulation parameters after machine learning, to maintain the optimal physiological or pathological state.[290] Soon, the deep integration of AI and MSK organoids can significantly enhance the degree of biomimetics, standardization, and scaling of organoids. This will provide an unprecedentedly powerful engine for precision regenerative medicine, innovative drug development, and deciphering the mechanism of MSK system diseases.

6.4. CRISPR and associated protein 9

Gene editing technology enables precise DNA modification of target genes. Among these, CRISPR-Cas9 has become the most mainstream and cutting-edge tool due to its simple design, low cost, and ability to edit multiple genes simultaneously.[291] Introducing this technology into organoid research is expected to accelerate the optimization of organoid models and significantly improve the efficiency of drug screening and disease mechanism research. Taking OS as an example, Xu *et al.*[292] successfully induced

a cisplatin-resistant model using OS organoids, finding that cisplatin treatment significantly upregulates excision repair cross-complementation 6 (ERCC6) expression, and that this expression level is correlated with patients' clinical pathological characteristics. Subsequently, the team used CRISPR-Cas9 to knock down ERCC6, significantly restored the organoid's sensitivity to cisplatin and promoted apoptosis, thereby rapidly validating the gene's potential as a therapeutic target. CRISPR-Cas9 can also be applied to the treatment of genetic diseases. Saito *et al.*[293] extracted cells from patients with craniosynostosis (cleidocranial dysplasia [CCD]) and induced them into iPSCs. CCD is a dominant genetic skeletal disorder, and researchers corrected the mutation in CCD-derived iPSCs using CRISPR/Cas9 technology. The edited iPSCs demonstrated the ability to stimulate bone regeneration in rat cranial defects, thereby demonstrating the potential therapeutic applications of CRISPR/Cas9-reprogrammed iPSCs. Similar strategies can also be extended to the construction of MSK organoids. For example, CRISPR-Cas9 technology can be used to overexpress RUNX2 to enhance stem cell osteogenic differentiation and promote bone matrix mineralization. Alternatively, DNA methylation modifications can be employed for epigenetic regulation to precisely coordinate osteogenesis. Although the application of CRISPR-Cas9 in skeletal muscle organoids is now in an early stage of development with limited research, its immense potential in bone regeneration, disease modeling, and clinical translation is undeniable, and it will undoubtedly play a pivotal role in the future.

7. Future perspectives

Organoids have emerged as powerful *in vitro* models that complement, rather than replace, animal studies by addressing key limitations while preserving the unique advantages of whole-organism systems. Unlike traditional cell cultures, organoids recapitulate human tissue architecture and function, making them particularly valuable for studying species-specific disease mechanisms and drug responses. However, organoids currently lack the systemic complexity of animal models, including functional immune, vascular, and neural networks, as well as organism-level behaviors and pharmacokinetics. This makes them unsuitable for studying multi-organ interactions, systemic drug effects, or complex phenotypes such as pain, cognition, or motor function, areas where rodent and other animal models remain indispensable.

Organoids excel at high-throughput early-stage screening of drug candidates or genetic therapies, significantly reducing the number of animals needed for subsequent validation. For example, liver organoids can first identify hepatotoxic compounds *in vitro*, minimizing unnecessary animal testing, while cardiac organoids can predict arrhythmia risks before proceeding to *in vivo* electrophysiology studies. Conversely, findings from animal models can guide the optimization of organoid systems, such as incorporating mechanical cues from load-bearing joints into cartilage organoids or immune cells into tumor organoids, to better mimic *in vivo* microenvironments. This iterative synergy enhances research efficiency and predictive validity.

Despite their advantages, organoids face challenges in scalability, standardization, and functional maturation that limit their standalone utility. Vascularization, innervation, and longevity remain significant hurdles, particularly for modeling chronic diseases or systemic effects. Thus, while organoids are transforming preclinical research by offering human-reducible reductionist models, they are not yet positioned to replace animal studies entirely. Instead, their greatest value lies in their integration with *in vivo* systems, refining hypotheses, reducing animal use through the 3Rs, and accelerating translational research. Future advancements in multi-organoid systems, OoC technologies, and computational integration with animal data will further solidify this complementary partnership, offering a more holistic and human-relevant approach to biomedical discovery.

8. Conclusion

Over the past decade, tissue engineering has experienced a remarkable surge in the development of MSK organoids. These organoids have emerged as a powerful alternative to traditional 2D cultures and animal models, offering a more physiologically relevant representation of native tissue architecture, cellular composition, and function. By combining the accessibility of *in vitro* systems with the complexity of *in vivo*-like environments, MSK organoids have become invaluable tools in disease modeling, drug screening, and the exploration of regenerative repair strategies. Their adoption has not only accelerated the advances in regenerative medicine but also deepened our understanding of the pathogenesis of MSK disorders. Despite these advancements, the intricate nature of the MSK system means that organoid technologies remain in an early stage of development. Looking ahead, the continued evolution of biomaterials and biotechnological innovations is expected to substantially broaden the applications of MSK organoids in tissue engineering.

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Conflict of interest

Bin Li is an Editorial Board Member of this journal, but was not in any way involved in the editorial and peer-review process conducted for this paper, directly or indirectly. Separately, other authors declared 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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Availability of data

Data will be made available on request.

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