In-Vitro Models for Osteoarthritis Drug Development: Past, Present, and Future Directions

BSc. Dorota Piechowiak

Student number: 1717367

Supervised by:

MSc. Núria Ginés Rodríguez

Examiner:

Prof. Dr. Ir. Jos Malda

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Abstract

Osteoarthritis (OA) poses a global health challenge, impacting millions of patients with debilitating symptoms. Despite available symptom-alleviating treatments, a lack of long-term treatments persists. No Disease-Modifying OA Drugs (DMOADs) have demonstrated efficacy in OA patients, partly due to an incomplete understanding of OA pathogenesis and heterogeneity, hindering the identification of a universal therapeutic target. This review emphasizes the need for predictive preclinical *in vitro* models to expedite pharmaceutical agent development and enhance drug response prediction in humans.

This review outlines recent advancements in *in vitro* models for OA modeling and drug development, encompassing 2D and 3D cell cultures, explant models, scaffold-free and scaffold-based models, and microphysiological systems, including tissue- and organ-on-a-chip and joint-systems. Emphasis is placed on models featuring multi-joint tissue cultures facilitating crosstalk, mimicking OA inflammation, applying mechanical stimulation, and incorporating immune cells.

Microphysiological systems, such as Organ-on-a-Chip (OoC) and Joint-on-a-Chip (JoC), emerge as promising tools for drug development, accurately recapitulating organ-level physiology and pathophysiology. This enhances predictive accuracy for drug safety and efficacy and positions them as potential platforms for personalized medicine. This article concludes by outlining challenges and opportunities for future advancements in *in vitro* disease modeling. It contributes to the ongoing dialogue on improving preclinical models for a more effective and targeted approach to OA drug development.

Keywords: disease-modifying osteoarthritis drugs; osteoarthritis modelling; microphysiological system; osteoarthritis; organ-on-a-chip.

Layman Summary

Osteoarthritis (OA) is a widespread disease affecting millions of people and causing severe symptoms. While treatments relieve symptoms, finding a lasting solution has proven challenging. Scientists are struggling with developing drugs that can modify the OA's due to an incomplete understanding of its development and variations.

This review outlines using advanced lab models to speed up the development of new medicines and better predict how they will work in humans. These lab models, such as 2D and 3D cell cultures and cultures involving structural support for the cells, aim to mimic aspects of OA for improved drug creation. These models focus on recreating a more realistic tissue environment by including multiple types of tissues, mechanical movement, and elements of mimicking inflammation like immune cells.

Innovative systems, such as Organ-on-a-Chip (OoC), act like miniature human organs. These tiny, specialized devices show promise in accurately mimicking the functions of organs and improving the ability to predict how drugs will perform in the human body. This not only can make drugs safer and more effective but also open doors for tailoring treatments to the individual patient needs. This review explores challenges and future possibilities in lab-based disease modeling, discussing ongoing efforts to create better and more specific treatments for OA.

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Introduction

Osteoarthritis (OA) is a debilitating and chronic joint disorder with multifactorial origins, imposing substantial social and healthcare costs worldwide. The occurrence of this disease is expected to increase as population life expectancy is ever increasing as well as the onset of metabolic diseases as obesity and hypertension (1,2). Approximately 250 million people worldwide are currently living with OA. Around 85% of documented OA cases manifest on the knee. However other joints, such as the hip, hands, feet, and ankle can also be affected by the condition (3,4).

Pathogenesis of Osteoarthritis

The onset of OA manifests in two distinct forms: primary and secondary. The primary form develops in previously healthy joints without a discernible triggering cause. It is often intricately intertwined with the aging process, significantly impacting adults aged 45 and above (4,5). The wear and tear on joints can lead to cartilage damage, and due to the low regenerative capacity of cartilage, this damage can initiate aberrant repair mechanisms (6). On the other hand, secondary OA stems from underlying predisposing factors. The onset might appear following a secondary trigger. Such triggers can derive from enhanced mechanical stress (attributed to factors such as obesity, traumatic injuries, or joint infections), endocrine and metabolic disorders, inflammatory joint diseases like rheumatoid arthritis, and genetic predispositions such as congenital abnormalities (5).

In contrast to primary OA, which predominantly affects an aging population, post-traumatic osteoarthritis (PTOA) has a greater impact on a younger and more active demographic. This susceptibility in the younger age group is attributed to the increased risk of sports injuries, often acquired through participation in activities with a heightened risk of joint injury (7). PTOA develops in response to joint trauma, which involves articular fractures, damage to the articular cartilage, meniscus tears, and subchondral bone injuries. Alternatively, PTOA may arise from joint instabilities and impaired biomechanics caused by ligament damage or patellar dislocation (8,9).

OA, whether in primary or secondary form, is a complex and progressive disease affecting various joint structures, involving a combination of conditions that result in structural alterations and loss of articular cartilage. Over time, the progression of the disease leads to severe pain, joint stiffness, and reduced mobility. This has a profound impact on the patients' quality of life, affecting them physically and mentally (10). As patients become less active, they are more susceptible to associated morbidities such as decreased productivity, weight gain and heightened social isolation (5). Managing OA has deep socioeconomical and quality of life impacts with short-term and long-term financial and personal consequences (11). These factors collectively contribute to psychological stress and may result in an elevated risk of developing depression and other mental health disorders (4).

Growing evidence indicates that OA is a multifaceted condition that affects various components of the joint (Figure 1.) (12). Central to these components is Articular Cartilage (AC), a specialized connective tissue covering joint surfaces. AC covers the epiphysis of long bones and protects them from direct bone-bone contact. To achieve such a protective function, unique extracellular matrix (ECM) composition and anisotropic structure. This includes zone-dependent collagen fiber orientation and osmotic pressure resulting from glycosaminoglycans (GAGs) interacting with retained synovial fluid (SF) (13). SF is maintained by synovium, which is a specialized connective tissue lining diarthrodial joints and producing components, like lubricin and hyaluronic acid. This fluid protects joint integrity, lubricates cartilage, and supports chondrocyte nutrition (14). Consequently, AC effectively transmits loads while enabling friction-free joint movement (15). Beneath the cartilage lies the subchondral bone (SB), together forming the osteochondral unit crucial for bearing and distributing joint loads. SB not only provides structural support but also plays a role in supplying nutrients to the adjacent cartilage. Changes in the SB microenvironment can therefore impact cartilage metabolism (12). OA manifestations within the osteochondral unit encompass a spectrum of changes, including AC

degradation and thinning, synovial inflammation, joint space narrowing, SB thickening, and SB remodeling by osteophyte formation, subchondral sclerosis, and cyst development. Additional features involve ligament degeneration, joint capsule hypertrophy, and alterations in periarticular muscles as well as nerve and vasculature outgrowth at later stages (10).

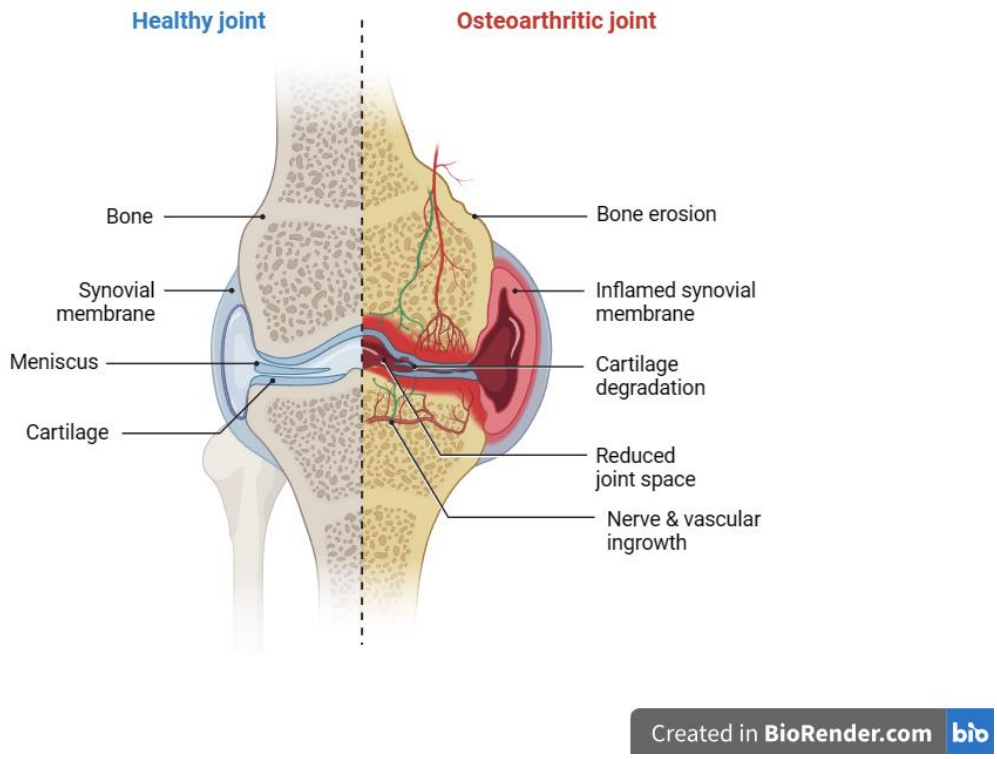

Figure 1. Schematic figure comparing the state of the knee joint in the healthy state and OA. Figure created with BioRender.com.

Despite ongoing efforts to establish delivery targets for Disease-Modifying Osteoarthritis Drugs (DMOAD), the underlying cellular and molecular mechanisms driving OA onset and development remain only partially understood (4). It is acknowledged that OA arises from the intricate interplay of biochemical, cellular, and mechanical processes (5).

The pathogenesis of OA involves inflammation and disruptions in both innate and adaptive immune systems, setting off a broad inflammatory reaction (16). In OA, activated Toll-like receptors (TLRs), macrophages, T cells, and B cells drive the release of pro-inflammatory cytokines, including Tumor Necrosis Factor-α (TNF-α), interleukin-1β (IL-1β), and IL-6 and chemokines into the synovial membrane and fluid (17). Subsequently, these inflammatory cytokines migrate into the cartilage. Chondrocytes, the primary cells in AC, maintain catabolic and anabolic homeostasis within a healthy joint (18). Various stimuli as inflammation, excessive mechanical loading, and hypoxia, promote catabolic activity of articular chondrocytes, amplifying the release of degradative enzymes such as MMPs (MMP-1, MMP-3, MMP-13 and ADAMTS-5) and aggrecanases (10,19,20).

This results in cartilage ECM degradation, primarily marked by the loss of aggrecan and collagen type II, as well as fibrillation and erosion of the cartilage surface, matrix calcification, chondrocyte senescence, and apoptosis (10,21,22). The release of cartilage breakdown products into synovial fluid amplifies inflammation, prompting synovial cells to produce pro-inflammatory cytokines. This leads to excess catabolic enzyme production, causing further cartilage degeneration (14). In the advanced stages of OA, pores develop at the chondral junction, permitting neuronal fibers to grow into the

subchondral bone and contact the calcified cartilage zone. This event triggers pain sensation, prevalently experienced by OA patients. Chondrocytes play an active role in this process, generating nerve growth factor (NGF), vascular endothelial growth factor (VEGF), and platelet-derived growth factor (PDGF) (23,24). Elevated levels of these growth factors coupled with other synovial mediators stimulate the growth of new nerves and blood vessels (17).

Previous studies have utilized these cytokines, either individually or synergistically, to stimulate conditions resembling OA in *in vitro* models. In this context, *in vitro* models provide practicality in dissecting this degenerative disease into distinct pathways, enabling a precise understanding of downstream signaling. From a drug development perspective, untangling molecular cross-talk and targeting specific pathways not only facilitates tracking of complex interactions but also enhances the assessment of their efficacy (22,25).

Current Treatment Landscape and Challenges in DMOADs Development

Currently, there is no curative treatment for OA. Therefore, there is an urgent demand for precise and effective therapies (1). Conventional treatments for OA are limited, revolving around symptom management, with a main emphasis on alleviating pain; or enhancing the structural integrity of affected joint tissues (26). Despite this emphasis, these approaches have fallen short of effectively halting the progression of OA and providing lasting symptom relief (27).

Current OA drug targets include AC, SB, inflammatory processes, and pain processes. Guidelines recommend a fundamental set of non-pharmacological measures, including education, dietary changes, and weight loss, preceding drug treatments. This combination has shown potential for enhancing functional status and reducing inflammatory markers (26,28). Commonly used drugs for OA, and primary pain management, include, non-steroidal anti-inflammatory drugs (NSAIDs) and intra-articular corticosteroid injections, though they pose challenges due to moderate efficacy and potential long-term side effects and toxicity (26,27). Alternatives include hyaluronic acid (HA) products, as well as emerging/evolving therapies like growth factors (derived from platelet-rich plasma), biologics targeting IL-1 or TNFα and stem cell preparations. It is crucial to approach these emerging treatments with caution due to their non-standardized nature and limited clinical evidence (29). No DMOADs have received approval for widespread clinical use by regulatory bodies in the United States or Europe, despite ongoing clinical trials (4). Consequently, patients are faced with limited treatment options.

Surgical and regenerative treatments, such as Autologous Chondrocyte Implantation (ACI) and Matrix-Induced Autologous Chondrocyte Implantation (MACI), aim to repair damaged cartilage. Although they demonstrate proven efficacy and safety, extended follow-ups beyond 5 years raise concerns, particularly regarding postoperative hypertrophy and deterioration of the repaired cartilage. Additionally, these methods are only suitable for treating focal AC defects (30). In late-stage OA, total joint replacement becomes the imperative solution for long-term pain relief. However, this approach is not without drawbacks, since it entails a significant surgical procedure with extended recovery time and requires revision surgery every 20 years (27). The failure rate is higher in younger, more active individuals (29,31).

The challenges associated with developing effective DMOADs are rooted in the intricate nature of OA. OA manifests heterogeneously across multiple tissues, differing between joint types and among patients (32). Developing new therapies for osteoarthritic degeneration requires a profound understanding of disease mechanisms and progression at the time of intervention. Which include the consideration of paracrine interactions among different joint cells (19). Navigating the transition from preclinical models to clinical trials is paramount to bring therapies closer to patients. Therefore, to bridge the gap between *in vitro* preclinical and clinical studies, it is necessary to create comprehensive *in vitro* tissue models. These models should have the capability to faithfully recapitulate the interactions and complexities of *in vivo* tissue environments within the knee joint (4,33).

In vitro Models for OA Studies

In vitro models can be classified into 2D cell culture (monolayer or co-culture), 3D culture systems (either scaffold-free or scaffold-based), explant-based culture and microfluidic models. At present, there has been a growing interest in cutting-edge techniques like 3D biofabrication and human-specific *in vitro* organ mimetic and microphysiological systems such as organoids and organ-on-a-chip (OoC) (34). The development of versatile *in vitro* OA models serves a dual purpose: deepening our understanding of the OA pathology and exploring new treatment options with a focus on translatability to a clinical setting (35). Ensuring adaptability over time and relevance to OA clinical stages is essential for ultimately improving OA patient outcomes.

2D Cell Culture Models

The use of two-dimensional (2D) culture methods marked the initial approach to studying chondrocytes, in the context of osteoarthritis (OA), over 50 years ago (4). Typically, monolayer models involve culturing of either primary cells or cell lines on a flat surface of a culture flask. In this configuration, the single-layer cells are exposed to uniform distribution of the surrounding media, which contains key nutrients and growth factors for growth and proliferation (1). The advantages of monolayer models for therapeutic testing lie in their convenience, reproducibility, cost-effectiveness, and ability to provide rigorous control over experimental conditions (4,36).

Primary cells closely mimic the *in vivo* phenotype but are costly to obtain, possess limited proliferative capacity, and face challenges in subculturing due to potential de-differentiation. Conversely, cell lines provide a more abundant cell source, high proliferative capacities, and suitability for long-term studies. However, they may display altered expression of key markers and reduced ECM secretion in comparison to primary cells, potentially compromising translatability to the native state (1).

Over the years, the evolution of monolayer chondrocyte cultures has played a pivotal role in advancing our understanding of chondrocyte behavior. These 2D *in vitro* models have provided valuable insights into various aspects, such as differentiation, cytokine effects, and responses to growth factors. Specifically, they offered early insights into the impact of inflammatory cytokines, as IL-1β, on cartilage, enabling the exploration of downstream inflammatory pathways (37,38). Furthermore, monolayer models serve as a common platform for testing and screening chondroprotective compounds and potential new therapeutics, aiming to mitigate and counteract factors contributing to articular cartilage degradation. Evaluations of biocompatibility, cytotoxicity, and efficacy, particularly in cases of local administration where the compounds interact with designated cellular sites or functions (39). Recent studies exploring emerging trends such as the use of nanoparticles in drug delivery, contribute to the ongoing evolution and application of these monolayer models (40,41).

In a recent study, a drug-loaded nanocarrier treatment strategy was tested, utilizing liposomes for the delivery of the anti-inflammatory drug D-glucosamine sulphate (GAS) (40). The study aimed to assess sustained release and the impact on TNF-α treated primary mouse chondrocytes. Due to the bioactivity limitations of oral administration for GAS, liposomes were investigated to enhance therapeutic efficiency of the drug for potential intra-articular injection. GAS released from DSPC–GAS liposomes promoted primary mouse chondrocyte viability and demonstrated anti-inflammatory and chondroprotective effects. It mitigated TNF-α-induced degeneration by modulating key factors: inhibiting IL-1β and IL-6, reducing TAC1 and MMP1 related to pain and cartilage degeneration, and increasing Agg and Col2α mRNA expression in inflammation-induced chondrocytes, indicating chondroprotective potential. In a related study DIA-loaded poly(d,l-lactide-co-glycolide) nanoparticles (DIA/PLGA NPs) were synthesized to achieve prolonged drug release (41). *In vitro* assessment using rat

synoviocytes under inflammatory IL-1- and lipopolysaccharide (LPS)-stimulated conditions, revealed dose-dependent reductions in mRNA levels of pro-inflammatory cytokines and enzymes, including IL-1, IL-6, TNF-α, MMP-3, MMP-13, COX-2, and ADAMTS-5 in synoviocytes. Furthermore, the results were corroborated in *in vivo* rat models, showing comparable efficacy in inhibiting inflammation and protecting against cartilage degradation in OA.

Researchers have also explored co-culture models involving other joint tissues. The modification in 2D culture method and use of Transwell plate models enables the study of cell-cell communication through the secretion of soluble factors. In this system, cells are seeded in the lower chamber of multi-well plates, an insert is placed in the well that contains a permeable membrane where further cells can be seeded. This setup allows cell communication without direct contact (1).

This method was employed to investigate the impact of Adipose-Derived Mesenchymal Stem Cells (AD-MSCs) on inflammatory factors in co-culture of OA chondrocytes and synoviocytes (42). Key inflammatory factors, such as IL-1β, IL-6, IL-8 and TNF-α, exhibited down-modulation at both the mRNA and protein levels. Notably, these anti-inflammatory AD-MSC effects were suggested to depend on the existing status of OA inflammation in the model. In a similar study, cell viability was assessed in a co-culture system involving AD-MSCs and primary chondrocytes, where TNF-α stimulation, mimicking OA conditions, induced chondrocyte apoptosis (43). Chondrocytes co-cultured with AD-MSCs demonstrated consistently higher viability compared to those cultured in isolation, both with and without TNF-α. The paracrine effect of AD-MSCs further reduced MMP-13 expression, emphasizing their chondroprotective impact (44).

It's important to recognize that 2D cell culture models have notable disadvantages, relating to their lack of complexity representing *in vivo* conditions. The planar environment, characterized by the absence of 3D scaffolding, nutrient flow, gradients, and the inability to replicate native-like mechanical forces, result in altered cell morphology, polarization, de-differentiation, and decreased cell connections. This absence of cellular niche impact mechanotransduction and cell signaling, hindering an accurate simulation of physiological conditions (1,45). To create a more reliable model of OA, it becomes essential to consider the various articular joint tissues as a unified biological unit. Therefore, 3D models emerged as a viable alternative.

3D Cell Culture Models

3D models come in various forms such as explants, scaffold-free, and scaffold-based systems, each has unique strengths and weaknesses. Explant models, also known as *ex vivo* cell cultures, involve the extraction of tissue fragments directly from living organisms (36). Maintaining cells within their native three-dimensional ECM environment, these tissue fragments largely preserve the structural and biomechanical properties of native tissues (46).

Explant tissue models

Explants of human AC or whole osteochondral plugs have proven to be valuable models in OA research. These models provide an opportunity to explore the effects of compressive loading on cartilage, shedding light on its influence on disease progression (47). Moreover, explant models offer a more comprehensive assessment of the osteochondral tissue responses, investigating relationships between various tissues in the osteochondral plug, including AC and underlying subchondral bone (SCB) tissue (48).

Geurts et al., demonstrated the establishment of cartilage degradation explant tissue model by cultivating tissue explants with collagenase II. The model proved to be a valuable *ex vivo* tool for replicating biological processes associated with OA within a controlled laboratory environment (49).

This *ex vivo* model was used for protein profiling to investigate diverse protein interactions underlying cartilage degradation. Specific proteins, including MMP9 and IFNϒ, emerged as potential biomarkers, distinguishing between healthy and pathological states. The model's utility extends to drug development, where it can aid in identifying potential treatment candidates through systematic *in vitro* analyses. Simultaneous measurement of multiple proteins enables a comprehensive characterization of molecular processes related to cartilage degradation. Consequently, similar models could play a crucial role in drug development by assisting in uncovering regulatory feedback mechanisms, validation of disease-modifying drug targets, and ultimately accelerating drug development (48).

Despite their advantages, explant models have certain limitations. One of the prominent drawbacks is their relatively short lifespan, rendering them less suitable for long-term studies (4). Cells located at the surgical edge of the explants are prone to cell death during or shortly after harvesting. Over time, cells derived from explants may undergo de-differentiation and morphology changes (50). Furthermore, the availability of tissue from a single biological source is limited, which can introduce variability in experimental outcomes, rendering explant models less suitable for high-throughput experimentation (36).

Scaffold-free Systems

Scaffold free systems facilitate 3D cell culture without the need for artificial scaffolds. They mimic self-assembly and differentiation processes observed in native tissue development, and promote essential interactions between cells and their ECM (33,51). The most common techniques are spheroid formation, micromass, pellet, hanging drop cultures, and organoids (34).

Pellet culture involves aggregating chondrocytes into spherical pellets, formed by centrifugation or in hanging drop culture by gravity-induced suspension (34). Pellets are valuable for studying cell-cell and cell-matrix interactions. As well as early condensation of progenitor stem cells, and the direct impact of therapeutic molecules on cells in suspension for potential OA treatments (34,36). It promotes chondrogenesis and a more organized 3D structure, though not as complex as native cartilage (34). Employing a pellet cell culture, a senescence-relevant model simulating OA-like cartilage was developed using human Bone Marrow-derived Mesenchymal Stromal Cells (BM-hMSCs) up to passage 10 (P10-MSCs). This model replicated key senescent traits observed in OA patients, encompassing telomere shortening, expression of senescence biomarkers, and the senescence-associated secretory phenotype (SASP) (52). Comparative analyses with cartilage derived BM-hMSCs highlighted the emergence of OA-like characteristics in the BM-hMSC-derived cartilage, eliminating the need for additional OA-inducing agents. The utility and clinical relevance of this model in drug development were assessed through testing of DMOADs and senolytics. The selected compounds, with a particular focus on their proposed role as a new type of DMOAD were evaluated for their efficacy. They demonstrated both positive effects in removing senescent cells and potential detrimental impacts on cartilage resembling OA development (53).

In micromass culture, cells are densely seeded in 3D arrangements within culture medium droplets. This technique maintains the chondrogenic phenotype and encourages cartilage-specific ECM production (4). This method is therefore well-suited for in-depth studies of single-cell behavior. A recent implementation of an *in vitro* model based on micromass culture has contributed to the development of a drug delivery system utilizing rapamycin encapsulated in poly(lactic-co-glycolic acid) (PLGA) microparticles (54). The system demonstrated prolonged drug release over weeks, effectively preserving *in vitro* cartilage and its key functions. It prevented senescence under oxidative and genomic stress conditions. This type of model allows testing of immunomodulatory drugs, overcoming challenges associated with high dosages and systemic toxicity. Drawing parallels with clinically used

drug carriers like PLGA micro/nanoparticles, as seen in triamcinolone acetonide formulations for OA patients, underscores the clinical translation of this platform (55). Overall, this approach holds promise for the exploration of clinically translatable materials for enhanced OA treatment strategies.

Scaffold-free models may encounter challenges such as the presence of a necrotic core within the spheroids due to restricted nutrient and oxygen diffusion, which affects cell viability (34,36). While these conditions lead to chondrogenesis, a hypoxic environment may concurrently hinder osteogenesis (56). To create an anatomically defined osteochondral model, it is essential that the model adequately accommodates various cell types present within an articular joint.

Scaffold-based Systems

Scaffold-based 3D cell culture involves fabrication of complex matrixes that emulate the intricate 3D structure of osteochondral tissue. This requires a careful selection of biomaterials, cellular, and acellular components, integrating interconnected pores, permeability, and modifiable surface chemistry. Collectively these components create biomimetic environments, mirroring the anatomical and physiological complexities of native tissues (57,58). Scaffolds play a crucial role, providing structural support for ECM organization. Additionally, they actively facilitate critical cellular processes – adhesion, proliferation, protein secretion, ECM composition remodeling, and maintenance of a specific tissue phenotype (59). In the context of cartilage production, a scaffold should be characterized by biocompatibility, biodegradability, mechanical stability, non-immunogenicity, and permeability. This multifaceted profile facilitates the efficient transport of growth factors, nutrients, and cytokines, essential for cartilage development (17,60).

Cartilage exhibits complex zonal biomechanical properties influenced by depth-dependent ECM composition and anisotropy (61). Cartilage is organized into distinct functional zones based on their proximity to the articular surface: the superficial zone, transitional zone, and deep zone (62). In the superficial zone, chondrocytes and type II collagen fibers align parallel to the surface, dissipate shear forces during joint loading and secrete lubricin for lubrication and frictionless joint movement. The transitional zone, experiencing both compressive and shear forces, features randomly arranged type II collagen fibers to resist forces from multiple directions. In the deep zone, type II collagen fibers are thicker and arranged perpendicularly to the cartilage surface and eventually attach to the bone for resisting compressive loads and high proteoglycan concentrations aid in water retention (63).

Various biomaterials, categorized as either natural or synthetic, have been extensively studied for joint tissue applications. Hydrogel-based scaffolds dominate applications due to their unique properties of water absorption and swelling, closely mirroring the characteristics of the natural ECM. Beyond their inherent biocompatibility, they also excel in facilitating the formation of irregular shapes and porous structures (64,65). Hydrogels incorporating natural polymers—collagen, hyaluronic acid, alginate, chitosan, nitrocellulose, gelatin, silk fibroin (SF), and partially modified natural polymers like gelatin methacrylol (GelMA) and hyaluronic acid methacrylate (HAMA)—have been widely employed in the development of cartilage scaffolds (17,66,67). Additionally, natural polymers facilitate receptor-mediated cell adhesion, exemplified by interactions through the arginylglycylaspartic acid (RGD) peptide motif or CD44 receptors (67). Synthetic materials, such as Polyethylene glycol (PEG), polylactic acid (PLA), polycaprolactone (PCL), and poly(vinyl alcohol) (PVA), possess highly tunable properties and are frequently integrated with natural polymers to enhance the mechanical attributes of scaffolds (17,68). While these materials are inherently bio-inert and can't promote cell behavior on their own, bioactivity can be added to hydrogels through functionalization (60). To date, no individual hydrogel material has shown promise in engineering of bone, cartilage, and the osteochondral unit (66). Ongoing research focuses on developing composite material formulations, blending natural and artificial

materials to address individual material limitations, and overcoming challenges in creating functional tissue.

A recent study created a 3D tissue-engineered (TE) synovium model using fibroblast cell sheets embedded in Matrigel, replicating *in vivo* conditions more accurately than traditional 2D culture (69). These constructs, assembled in Transwell inserts, facilitated research on the role of synovium in OA progression. Introducing IL-1 and dexamethasone (DEX) provided insights into synovial responses to inflammation and anti-inflammatory interventions. IL-1 induced a proinflammatory environment, evidenced by increased NO production, hyperplasia, altered cellular composition, and decreased permeability compared to native synovial explants. IL-1 treatment led to an increase in MLS (macrophage-like synoviocytes) content and a decrease in FLS (fibroblast-like synoviocytes) content, indicating a shift towards a more inflammatory state. The model's versatility was demonstrated through inclusion of quantitative measurements of solute transport function and co-culturing various synoviocyte types, offering a promising tool for studying both healthy and diseased synovium, with applications in drug development.

To recreate calcified cartilage and subchondral bone components, a widely adopted approach involves the integration of bioceramics, such as hydroxyapatite, tricalcium phosphate (TCP), calcium phosphate cement (CPC), calcium silicate and glass-ceramics into hydrogel matrices. This approach aims to facilitate osteogenesis, osteogenic differentiation and enhance mineralization at the later stages of bone development, potentially improving mechanical properties and biological activity in the engineered osteochondral constructs (66,70,71).

Similarly, the developed hydrogel was designed to replicate the transitional gradient architecture of osteochondral tissue. It features an alginate/poly(vinyl alcohol) (PVA) semi–interpenetrating network hydrogel, with a polymer structure with alginate, is partially intertwined within the crosslinked network of PVA. (72). Alginate and PVA were strategically combined to complement each other's mechanical properties and enhance overall elasticity. By incorporating nanohydroxyapatite (HA) and chondroitin sulfate (CS) in distinct zones, the hydrogel not only acquired osteogenic and chondrogenic potential but also established a gradient interface crucial for preventing delamination. The resulting hydrogel, enriched with layer-specific bioactive molecules, supported co-culturing cells by influencing cell retention and interaction of osteoblasts and chondrocytes. Its spatial variation showed chemotactic potential, making it suitable for controlled co-culture applications in tissue engineering and serving as an *in vitro* model of chondral and subchondral zone interface.

3D bioprinting greatly advances scaffold production via precise control over the deposition of cells and materials in a 3D network in a highly reproducible and controlled manner. Predefined gradients are included to manipulate local tissue variations. This encompasses integrating chemical gradients with adhesion molecules, peptides, and growth factors; biological gradients with varied cell densities; and mechanical gradients achieved through optimized hydrogel–bioink composition, geometrical cues, or mechanical reinforcement via tailored microfiber orientation (73). Guiding cell and tissue functionality during biofabrication involves incorporating signals from microarchitecture, bioink constituent elements, and communication among diverse cell types within the 3D printed tissue construct (73,74).

The most commonly employed techniques to 3D print osteochondral models are extrusion-based techniques (75). Extrusion-based bioprinting continuously dispenses a bioink mixture in a layer-by-layer fashion through a needle, driven by mechanical or pneumatic pressure (76). This method is highly flexible, accommodating a broad range of materials, with subsequent crosslinking for solidification to prevent construct deformation (17). However, extrusion resolution and precision depend on bioink viscosity, and different viscosities suitable for cell support and printing may lead to either cell stress or

compromised printability (77). Additionally shear thinning properties aid in safeguarding the encapsulates cells, but finding materials with these properties while meeting other criteria like biocompatibility, printability, and mechanical properties poses challenges in bioink development (78). Achieving simultaneous deposition of multiple cells and biomaterials is essential for creating complex structures that mimic the microarchitecture and functionalities of native tissues *in vitro.*

In a recent advancement, a 3D bioprinted osteochondral construct was developed to replicate the early stages and inflammatory onset of OA in humans (22). Utilizing silk fibroin (SF)-based bioinks, the construct incorporated various silk types with polyvinylpyrrolidone (PVP) K90 for cartilage ink, and nano-hydroxyapatites (nHAp) for bone ink. The construct featured a distinctive architecture with interconnected macropores and micropores, ensuring high porosity that facilitated cellular interactions and biochemical signaling. Upon exposure to pro-inflammatory cytokines TNFα and IL-1β, the model effectively replicated early-stage OA characteristics. Anti-inflammatory drugs, cyclooxygenase-2 inhibitor (CXB) and robenacoxib (RHN), currently in clinical trials for knee OA, were then assessed in attenuating the pathological conditions. The inflamed osteochondral unit exhibited key pathological features, including ECM degradation and upregulation of inflammatory mediators. Encouragingly, both anti-inflammatory drugs inhibited inflammatory mediators, MMPs expression, and restored ECM production.

In a systematic approach to address material limitations and tailor a composite hydrogel scaffold for specific mechanical demands, researchers developed a new hydrogel formula using sodium alginate (SA), gelatin (GA), and HA (68). The hydrogel, formed by solely combining SA and GA, demonstrated high biocompatibility, yet it lacked sufficient mechanical strength. To address this, HA was incorporated to enhance mechanical properties. The SA-GA-HA composite hydrogel cartilage scaffold was prepared using layer-by-layer bioprinting with CaCl2 solution as a crosslinking agent, promoting the rapid formation of alginate gels. The material enabled the adherence and growth of cartilage cells and mitigated issues associated with standalone hydroxyapatite, which is known for its poor mechanical properties and difficulty in forming structures. Mechanical tests confirmed the fabricated scaffolds exhibited a high elastic modulus and tensile strength, resembling the mechanical properties found in native cartilage.

To improve mechanical properties, another approach was employed (66). Following a continuous, multinozzle printing method, researchers integrated different material composition and pore structure in distinct phases of the scaffold. The design of the scaffold incorporated three phases: a porous bone phase consisting of SA and mesoporous bioactive glasses (MBG), a middle dense phase comprising both SA and MBG, and a chondral phase composed of SA. The authors demonstrated that crosslinking SA post printing effectively united scaffold materials, ensuring integration across all phases. Mechanical testing revealed notable compressive and interface bonding strengths, mirroring the mechanical properties of native cartilage. This renders the osteochondral scaffold promising for further biological and clinical studies.

In another approach aimed at improving the mechanical properties of a bio-printed construct, a biphasic scaffold with a gradient in mechanical properties was designed. GelMA and SF, were strategically selected for their unique properties (79). GelMA provides biological functionality enriched with RGD sequences, while SF contributes inherent biocompatibility and biodegradability. SF was grafted with parathyroid hormone (PTH) to inhibit chondrocyte hypertrophy and covalent immobilization with methacrylic anhydride (MA) to enhance the mechanical strength of the bone phase. The resulting GelMA+SF-PTH and GelMA+ SF-MA bio-inks promoted hyaline cartilage extracellular matrix production and exhibited improved mechanical properties. This systematic approach underscores the tailored selection of bio-inks to enhance the overall efficacy of the scaffold.

A common practice, particularly for engineering hard tissues involves 3D printing technology of synthetic materials with posterior cell seeding. PCL, in particular, has gained prominence for its favorable physical properties (65). An *in vitro* osteochondral model mimicking the interface between the deep layer of AC and SB was recently developed (80). The AC phase incorporated gellan gum methacrylated (GGMA) and chondroitin sulfate/dopamine (CSDP) hydrogels, chosen for their favorable mechanical and biomimetic properties. Soft lithography was employed to print this phase, designed with native-like columns with chondrocytes. Simultaneously, the SB phase used PLA, functionalized with gelatin and nHA for enhanced bioactivity, replicating trabecular bone organization with high interconnected porosity via fused deposition modeling (FDM). Introduction of OA-like conditions with cytokines (IL-1β, IL-6, TNF-α) enabled demonstration of cell behavior in both phases. The engineered tissue reproduced AC and SB histological features, offering a valuable tool for OA study and drug development.

Emergence of Organ-on-a-chip technology

3D printed constructs often face limitations in dynamic functionality in mimicking physiological processes, which hinders the accurate representation of *in vivo* conditions (81). In contrast, organ-on-a-Chip (OoC) systems emerge as innovative micro-engineered devices. These systems integrate biomaterials, three-dimensional cell culture, bioreactors, and microfluidics to replicate native tissue microenvironments, stimulating organ-level structure, phenotype and functions (33,82). Leveraging microfluidics technologies, OoCs integrate and enhance the precision of dynamic features such as fluid exchange, and regulation of temporal and spatial flow. This facilitates organ perfusion, generation of concentration gradients, organ crosstalk, and application of fluid mechanical shear stress to cells (83). Microfluidic technology is crucial in modern biological sciences by enabling rapid and parallel collection and analysis of individual biological information. Moreover, the organ-on-a-chip platform offers reconfigurability, convenience, and near-full portability (81). The evolution of *in vitro* models for modelling OA and possible components of joint-on-chip devices are shown in Figure 2.

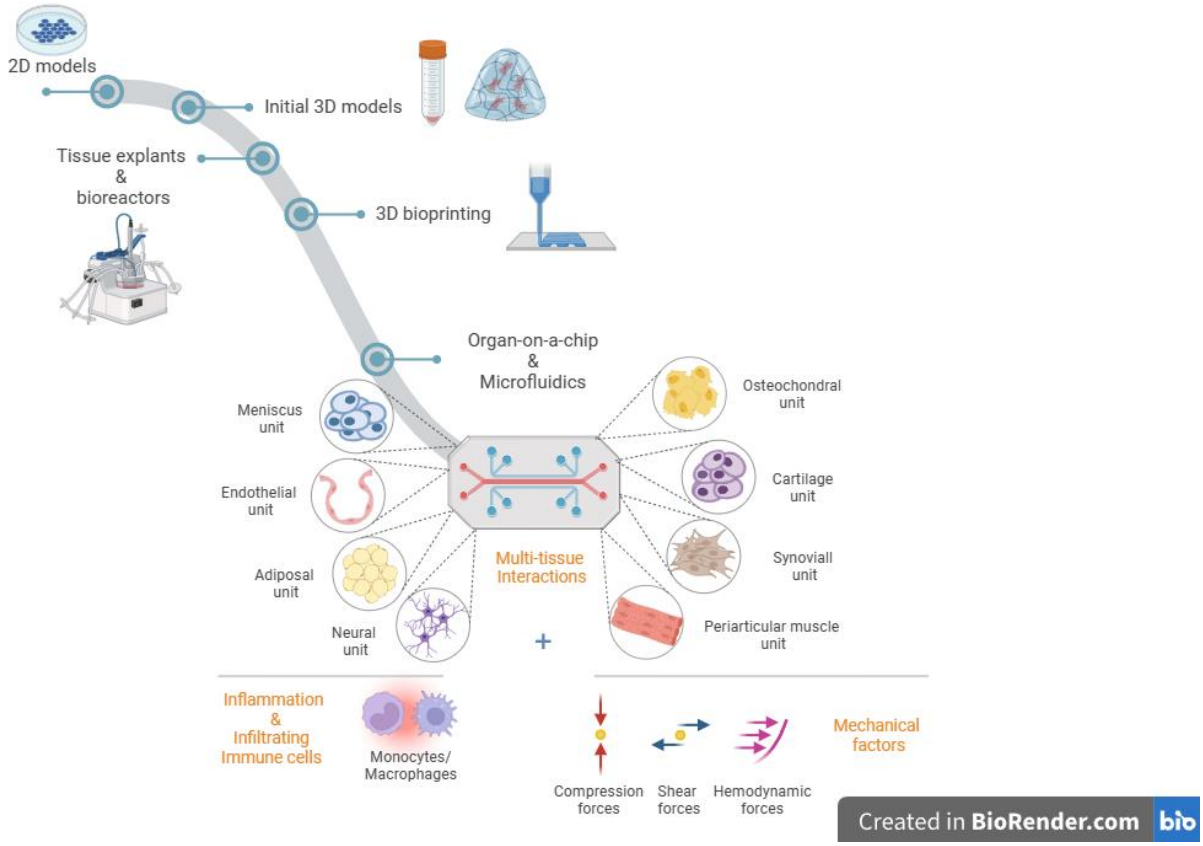

Figure 2. Evolution of *in vitro* models for cartilage and osteochondral tissue engineering, progressing from 2D cultures to 3D cultures, explants, bioreactors, and bioprinting. Bioprinting applications culminate in more complex microfluidic models, specifically organ-on-chip and joint-on-chip platforms. These platforms capture various microenvironmental aspects of OA, including mechanical stimulation, multi-tissue crosstalk, and inflammation through cytokine stimulation and infiltrating immune cells. The schematic joint-on-chip incorporates components included or yet to be included in existing joint-on-chip models (17,33). Figure created with BioRender.com.

Physiological compression in articular cartilage establishes a dynamic mechanical environment with variations in stress, strain, osmotic pressure, hydrostatic pressure accompanied by fluid flow (15). However, in OA due to pathological compression, repulsion of negatively charged aggrecan intensifies, triggering dysregulation of degradative enzymes. This, in consequence, contributes to breakdown of aggrecan molecules and loss of sulphated GAGs, compromising cartilage integrity. Load-based models have the potential to induce an OA-like response without requiring excessive concentrations of cytokine-based biochemical stimuli (84).

A Cartilage-on-a-chip model was developed by integrating a degradable poly (ethylene glycol) (PEG)-based hydrogel microenvironment with embedded human articular chondrocytes (hACs) (84). An actuation system, engineered for controlled hyperphysiological compression (HPC) at 30%, was used to apply mechanical stimulation. The chosen compression level demonstrated the ability to modulate inflammation while aligning with the range of cartilage deformations observed *in vivo*, thereby ensuring physiological relevance. The HPC triggered MMP-13 production and inflammation, resulting in increased expression of the pro-inflammatory cytokine IL8. Additionally, hypertrophic traits were acquired, as evidenced by heightened expression of COL10A1 and IHH genes (85). Concurrently, expression of BMP and Wnt signaling antagonists (GREM1, FRZB, and DKK1), associated with the onset of OA, was downregulated, indicating a gene profile correlated with OA (86). After the establishment of the model, proof of utility as a preclinical drug screening tool was provided. This was achieved by evaluating disease-modifying osteoarthritis (DMOA) candidates, using clinically approved or under-development anti-inflammatory and anti-catabolic drugs.

Synovial inflammation is a crucial aspect of OA, marked by changes in the synovium, such as tissue hypertrophy and abnormal macrophage infiltration from circulating monocytes that perpetuate inflammatory processes (14). A promising therapeutic strategy for OA involves identifying and targeting the excessive recruitment of monocytes through chemokine-signaling axes. A microfluidic chip model was designed to replicate monocyte extravasation into the OA joint synovium, comprising a synovial compartment with a perfusable endothelial channel and a cartilage compartment with a channel for synovial fluid (87). The model included pathological synovial fluid, synovial fibroblasts, chondrocytes, and monocytes isolated from OA patients. Endothelial cells underwent preconditioning with flow and TNF-α to simulate an inflammatory state. Subsequently, monocytes and synovial fluid were injected into dedicated channels to assess monocyte extraversion. Results presented evidence that OA synovial fluid induced monocyte extravasation, migration across the endothelial barrier and invasion into the extravascular matrix. The model effectively facilitated the screening of chemokine receptor antagonists, showcasing CVC's efficacy in antagonizing both CCR2 and CCR5, and significantly hindering monocyte extraversion by synovial fluid from OA patients. However, the therapeutic efficacy in OA and potential negative impacts on cartilage tissue from extravasated monocytes require further investigation.

In another study, an OoC cultured chondro-synovial dual organoid construct, utilizing self-organizing cells sourced from patients (88). The study aimed at creating spatially separated organoids that could communicate molecularly without direct contact, effectively mimicking the intricate human chondro-synovial niche. The research demonstrated that expanded synovial fibroblast cultures yielded organoids with higher precision and reproducibility compared to freshly isolated cells, due to a passaging effect that assimilated synovial cells *in vitro*. Examining serum content and growth factor concentrations, the study investigated their impact on organoid size and stability in the chondro-synovial model. Coculture experiments on the biochip revealed changes in chondrocyte morphology, characterized by an improved rounded phenotype. Furthermore, an analysis of cytokine secretion showed reduced VEGF levels in chondral organoids, indicating the influence of synovial interaction on molecule secretion. This model underscores the importance of reciprocal synovial-chondral tissue crosstalk in stimulating joint (patho)physiology and modeling arthritic diseases *in vitro*. It also holds implications for subsequent, more comprehensive analyses involving genomic and proteomic assessments, as well as drug testing studies (88,89).

OoC Applications

Drug failures in clinical settings are often linked to a lack of clinical efficacy, and safety-related issues, particularly non detection of adverse or side effects in preclinical phases. These safety concerns are primarily associated with liver and heart, stemming from the generation of harmful metabolites (90,91). Animal models, hindered by phylogenetic variations across species, often poorly predict human drug responses and lack precision for regulated mechanistic studies (83). OoC systems represent a promising alternative in therapeutic development, by replicating the environmental, functional, and interdependent features of tissues. This allows these models to demonstrate a superior predictive capability, facilitating the targeted design of new drugs (91,92). Thus, OoC, in addition to presently complementing animal and cell models, holds the potential to substitute these conventional models and, to some degree, reduce the necessity for human involvement in clinical studies (93). Integrated multiple organs on the single chip with increasingly more sophisticated representation of absorption, distribution, metabolism, excretion and toxicity (ADMET) process are being utilized to better understand drug interaction mechanisms in the human body, and thus showing great potential to better predict drug efficacy and safety (94). To validate OoC systems as a viable alternative,

addressing challenges in physical design, physiological representation, measurement systems, industrial-scale production and regulatory aspects is essential (91).

Bioreactor-Integrated OoC Model

A model named miniJoint was established using hBMSCs to create an osteochondral complex, synovial-like fibrous tissue, and adipose tissue analogs OoC. BM-hMSCs were encapsulated in photocrosslinkable GelMA after differentiation, and the resulting microtissues were integrated into a 3D printed bioreactor (95). The design facilitated real-time communication between tissues in real time, employing a shared medium that was perfused through the cartilage, synovial and adipose, and bone tissue, with direct physical interconnection between bone and cartilage. Additionally, to sustain the distinct tissue phenotypes, separate flows of osteogenic, fibrogenic, and adipogenic media were introduced, ensuring optimal conditions for each tissue type (96). Then the synovial tissues were treated with IL-1β to induce synovitis relevant OA model. The clinical relevance of the model was previously assessed by comparing outcomes of testing DMOADS in clinical trials with those in the *in vitro* disease model (97). In a recent study, the model's utility was tested by exploring new combinations of DMOADs. The investigation proposed the co-treatment with oligodeoxynucleotides (ODNs), an anti-inflammation agent and BMP-7. The results demonstrated that this combined ODN+BMP-7 treatment led to increased expression of chondrogenic genes, including aggrecan (ACAN) and collagen type II (COL2), and matrix production in cartilage. Simultaneously, there was a reduction in expression of pro-inflammatory cytokine genes and OA biomarkers in synovial-tissue (IL-1β; TNF-α; MMP-2, 3, and 13) of the miniJoint (95).

Advancements and Challenges in OoC Technologies

The *in vitro* OoC and JoC models mark significant progress in modelling the joint tissue microenvironment. Despite their potential, the broader acceptance and adoption of these technologies as mainstream research tools faces persistent challenges. These ongoing challenges include: engineering and technology development, faithful reproduction of the physiological joint environment, and acquisition of useful readouts—critical aspects to unlock the full potential of these models (33). By faithfully replicating inter- and intra-tissue communication, these models offer valuable insights into how individual tissues contribute to joint homeostasis and disease progression. Effective perfusion optimization is essential for connecting individual tissue units, with strategies such as using external capillary tubing linked to an external pump or integrating tissue-specific units into a microfluidic motherboard that includes all fluidic connections (98). These approaches offer flexibility in adjusting the JoC model configuration based on the specific objectives of the experiment. In cases where tissue units comprise two fluidic compartments, two independent perfusion systems are required—one to mimic the blood supply for tissues like the synovial membrane, ligaments, and subchondral bone, and the other to mimic the synovial fluid, ensuring recirculation flow among all joint tissues. Future developments in drug testing research will need to explore diverse avenues. Substances can be introduced either into the shared medium alone or into all mediums. This approach would respectively simulate "intraarticular administration" or "systemic administration," providing a comprehensive framework for investigating the efficacy and impact of drugs on the joint tissues (96).

Tailoring OA Models for Personalized Treatment

In response to the variability in disease phenotypes and drug responses, there is an increasing recognition of personalized medicine’s value in drug development. This approach prioritizes optimal drug regimen alignment with individual, genetically distinct patients, aiming to enhance efficacy and

minimize costs for new and existing drugs (99). Human-induced Pluripotent Stem Cells (hiPSCs) are gaining prominence in personalized OoC platforms due to their unlimited regenerative capacity and potency (100). Converting patients' somatic cells to hiPSCs allows the creation of customized healthy and disease models for personalized drug screening platforms tailored to patient's disease specific backgrounds. These platforms have the potential to closely recapitulate human physiology, surpassing the accuracy of animal models (101). Although iPSCs can be derived from easily accessible sources such as urine, skin, saliva, or blood cells, eliminating the need for multiple biopsies, they remain challenging to work with. The current availability of iPSC lines for genetic analyses, that require comparisons of a multitude of genetic backgrounds, is restricted. This challenge can be addressed by either combining existing iPSC lines stored in biobanks or by generating iPSCs from materials stored in biobanks accessible to researchers (100). To address differentiation efficiency and reproducibility issues, generating cell stocks from donors who are homozygous for human leukocyte antigens and represent a specific population is a more achievable goal, reducing associated costs in building personalized tissue models (94).

Future Data Acquisition Techniques

JoC development aims to create an accurate model to investigate arthritic diseases, encompassing a range of parameters and disease stages. Opportunities emerge by integrating advanced mechanical, biochemical, and optical sensing technologies, ideally paired with software analysis into OoC devices. Automated *in situ* data acquisition and visualization can offer significant benefits in translating JoC models to high-throughput applications, such as drug discovery (33).

Researchers can conduct a variety of assays on microsystems based on the chip's capacity and design. These assays range from traditional methods like ELISA, sequencing, and western blot to advanced techniques such as single-cell RNA sequencing, chromatography, and mass spectrometry (LC/GC-MS). Some multi-channel chip designs facilitate cell migration assays and the measurement of barrier function through transepithelial electrical resistance (TEER) assays. Despite the small volumes and sample sizes, advanced transcriptomic, proteomic, and metabolomic analyses can be achieved through on-chip lysis to release cellular contents, single-cell isolation, or the collection of supernatants from outlet channels (100). Online, real-time analysis can offer measurements of cell states and functionality, minimizing time delays. This is desirable as periodic measurements might overlook significant fluctuations in response. In OoC integrated electrodes can be tailored to measure electromechanical signals and integrated biochemical sensors can offer continuous measurement of various soluble analytes. The recirculating medium can be sampled for offline measurement of soluble biomarkers or analysis or circulating cells such as immune cells. Additionally, OoC can be connected to an optical set-up for live-cell imaging (102).

Ongoing developments on advancing high-content capabilities in existing microsystems are often demonstrated in proof-of-concept studies. For example, the next-generation OoC system integrated real-time sensing with high-throughput analysis, enabling simultaneous examination of multiple tissue cultures (98,100). This platform incorporated programmable flow control, electrical and oxygen sensors, and diverse cell types in a high-throughput layout of 96 individual OoC devices on a single plate. It seamlessly interfaced with industry-standard infrastructure and data collection tools, showcasing physiologically relevant flow patterns, real-time quantification of barrier function, optical access for direct measurement of tissue behavior, and compatibility with tools like HCS and RNA-seq. These features established it as a valuable tool for modeling various microscale tissues (liver, endothelial, intestinal, and kidney), thereby enhancing the predictive accuracy of *in vitro* drug screening (103). This exemplification illustrates the capacity of *in vitro* model technology to advance our understanding of complex physiological and pathological systems. Chondrocytes from intact and

damaged sites display unique gene expressions in over 1500 genes. Utilizing "omic" methods, like mentioned earlier mass spectrometry, could enhance future research, by refining patient classification and unveiling potential treatment outcomes that might go unnoticed by analyzing only “known” targets (4). Consequently, there is potential to expedite the clinical translation of novel therapies by enabling more comprehensive biological readouts through the integration of on-chip analysis and higher throughput screening (33).

Conclusions

In summary, various *in vitro* models have been developed for exploring the interactions among diverse joint tissues and advancing DMOADs development (17). The current state of the art emphasizes successful approaches involving cellular and acellular 3D bioprinting in constructing individual tissue components. Nevertheless, challenges persist in accurate recreation of physiological features, establishment of multi-tissue communication networks, achieving scalability, and ensuring reproducibility in bioprinted constructs (71). A highly diverse and adjustable joint-on-a-chip platform represents an active area of research with the aim to replicate the full spectrum of joint conditions, offering insights into the early onset of OA and longitudinal progression of OA in prolonged studies, which is a challenging aspect to observe in traditional clinical trials (33). Current systems primarily concentrate on mimicking the OA phenotype by means of mechanical stimuli or inflammatory mediators (17). As potential clinical diagnostic tools for OA, JoCs could assist in categorizing patients for personalized medicine interventions and enhancing therapeutic decision-making for optimizing patient outcomes. Additionally, microphysiological systems and animal models can complementarily predict drug toxicity and efficacy, reducing reliance on animal testing. Concurrently, drugs screened in animal studies could undergo further validation in *in vitro* systems, providing insights into patient subpopulations that may benefit from these drugs (4,104). As for now, no FDA-approved DMOADs exist, with multiple late-stage clinical trials showing inefficacy (105). This emphasizes the urgency for continued research in the coming years to advance fundamental insights into development of OA and discover new therapeutic approaches within complex multi-tissue interplay.

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