

3D *in vitro* approaches for modelling soft tissue joint diseases

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Abstract (240 words)

Diseases affecting the soft tissues of the joint represent a considerable global burden causing pain and disability, increasing the likelihood of developing metabolic co-morbidities. Current approaches to investigate the cellular basis of joint diseases including osteoarthritis, rheumatoid arthritis, tendinopathy and arthrofibrosis utilise the study of well-phenotyped human tissues, animal disease models and *in vitro* tissue culture models. Inherent challenges in preclinical drug discovery have driven the development of state-of-the-art *in vitro* human tissue models to rapidly advance therapeutic target discovery. Recently, such models have confirmed their clinical potential through successful recapitulation of the pathobiology of cancers, generating accurate predictions of patient response to therapeutics and providing a basis for equivalent musculoskeletal models. This review discusses the requirement for developing physiologically relevant 3D culture systems that could advance understanding of the cellular and molecular basis of diseases affecting the soft tissues of the joint. We discuss the specific practicalities and challenges associated with modelling the complex extracellular matrix of joint tissues including cartilage, synovium, tendon and ligament, highlighting the importance of considering the joint as a 'whole organ' to encompass crosstalk across tissues and between diverse cell types. Design of bespoke *in vitro* models for soft tissue joint disease has the potential to inform functional studies investigating the cellular and molecular mechanisms underpinning disease onset, progression, and drive towards resolution. Harnessing these models could inform precision therapeutic targeting and advance the field towards personalised medicine for patients with common musculoskeletal diseases.

Introduction

Musculoskeletal disorders involving joint disease are a considerable global burden featuring in the top ten causes for disability-adjusted life years¹. Whilst not immediately life threatening, joint diseases including osteoarthritis (OA), rheumatoid arthritis (RA), arthrofibrosis and tendinopathy are generally chronic conditions that cause prolonged pain and disability, significantly impacting patient life quality. Moreover, these conditions further increase the likelihood of developing comorbidities including obesity and diabetes². At present, incomplete understanding of the cellular and molecular basis underpinning joint disease has resulted in treatment strategies which are frequently non-curative. For example, surgical arthroplasty is performed in patients with end stage OA and demonstrably improves joint function, however, the underlying disease is not addressed. Consequently, further advancement in our

understanding of the cell types and interactions implicated in joint disease is required to develop robust and effective therapeutic approaches which address this unmet clinical need.

Recent research highlights the importance of considering the joint as a 'whole organ', whereby crosstalk between multiple tissue types including synovium, cartilage, and bone is central to pathology in OA and RA (Fig. 1). Recent investigations into the cell types comprising tissues of the joint have revealed insights into the functional biology of these tissues. For instance, we now understand the heterogeneity of fibroblasts in joint soft tissues (JSTs) such as synovium and tendon^{3,4} and that functionally distinct fibroblast subsets drive joint damage, inflammation and resolution^{3,5}. The microanatomical niches in which these cells occupy provide insights into cell behaviour during disease, including RA as a disease of the synovial sub-lining (where proximity of THY1^{pos} fibroblasts to NOTCH+ endothelial cells drives inflammation), in contrast to THY1^{neg} fibroblasts in the synovial lining mediating joint destruction^{3,6}.

Current approaches to advance understanding of the cellular basis of joint disease encompass the use of animal models, observational studies using human tissues, and *in vitro* studies utilising patient-derived cells or established cell lines. Advantages and disadvantages exist in each case, and frequently a multimodal approach is required for a step change to advance understanding of disease. Most commonly, observations from higher-throughput 2D cell monolayer research using cell lines or patient-derived cells informs experimental design in lower-throughput animal studies, which more faithfully recapitulate aspects of human physiology. Increasingly there has been a drive to incorporate more complex *in vitro* models into this pipeline, with models such as trans well co-cultures, spheroids, organoids, and on-a-chip platforms acting to down select candidate molecules or pathways from monolayer studies prior to animal work (Fig. 2). Here, animal models whilst being the most comprehensive model of joint disease, present a significant bottleneck in translational research due to cost, time, and ethical concerns. Moreover, a single animal model is not sufficient to reproduce both disease onset and progression, and animal models of disease must be induced or are uncharacteristically spontaneously resolving. For instance, the collagen-induced arthritis mouse model of RA exhibits clinical signs of arthritis 3-5 weeks after immunization against type II collagen, after which the condition is ultimately self-resolving unlike RA in humans. A multitude of animal models exist for OA, encompassing at least 18 different species, yet there remains no consensus toward a gold standard, with differences in anatomy such as cartilage thickness impeding translatability to humans. Finally, ethical concerns have resulted in a drive to limit the use of animals across all areas of research in accordance with the 3R's.

One approach to reduce, refine and replace the use of animal models of joint disease is to enhance the accuracy of *in vitro* studies such that they can narrow the experimental space for

animal research, or replace animal studies entirely in some cases. To date, efforts to advance 3D *in vitro* models (3DMs) have demonstrated real potential to provide functional mechanistic insights into the cellular basis of disease when compared to cell monolayers which are of more limited translational utility. Importantly, 3DMs typically include an extracellular matrix (ECM) component, a critical component of all joint tissues involved in disease, and a 3D space in which to spatiotemporally investigate crosstalk between cell types comprising the joint and to assess response to small molecules. In this review, we discuss recent developments in 3DMs which could recapitulate healthy and diseased JSTs including cartilage, synovium, tendon & ligament, advancing our understanding of soft tissue joint disease and informing new strategies to precisely target the cell types driving disease.

***In vitro* modelling of diseases affecting joint soft tissues**

Osteoarthritis

As the most common form of arthritis, OA presents a major disease burden with significant associated healthcare costs. OA is characterised by degeneration of osteochondral tissues of the joint which present as pain and stiffness that impair mobility and reduce functionality, with the knee being the joint most affected and accordingly the most studied in the context of disease models. Progressive loss of articular cartilage, subchondral bone thickening and osteophyte formation implicate the dysregulation of resident chondrocytes in the aetiology and progression of OA. However, synovial inflammation has been linked with more rapid cartilage breakdown, suggesting paracrine signalling between chondrocytes and synovial cells *via* synovial fluid can influence deterioration of the joint⁷. Current treatments for OA focus on symptom management and do not arrest or reverse the disease and joint arthroplasty remains the gold-standard in end-stage OA treatment. Consequently, there is a requirement to understand OA in the context of the whole joint to push forward interventional therapeutics which interrupt disease progression.

Chondrocytes maintain cartilage tissue homeostasis at the articular joint surface, expressing various ECM components and sulphated proteoglycans which incorporate foundationally into the tissue, promoting osmotic swelling and providing mechanical strength and joint lubrication. In disease, pathogenic chondrocytes are more catabolically active and upregulate ECM-degrading enzymes such as matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinases with thrombospondin motifs (ADAMTS). Chondrocyte behaviour has been modelled in scalable and inexpensive monolayer cultures, where stimulation by pro-inflammatory cytokines, which are upregulated in OA pathogenesis, drive chondrocytes

towards differentially activated states which mimic disease. For instance, IL-1 β is abundant in OA in both articular cartilage and synovium⁸, localising to chondrocytes⁹ which upregulate IL-1R1 and downregulate IL-1R α proportionally to increase OA severity¹⁰. *In vitro*, IL-1 β stimulation leads to MMP and ADAMTS upregulation in primary chondrocytes^{11,12} and chondrosarcoma cell lines¹³ in monolayer culture. Upregulation of other inflammatory cytokines such as TNF α , IL-6 and IL-8 are linked with IL-1 β stimulation of chondrocytes *in vitro* as well as downregulation of critical ECM-associated genes such as aggrecan and collagen II (Col II)^{14–16}. Beyond the chondrocyte, joint inflammation in OA promotes infiltration of immune cells including IL-1 β and TNF α -producing macrophages and T cell populations with high CD4+/CD8+ ratios¹⁷ which are linked with adverse clinical symptoms^{18,19}.

The numerous tissues and cell types implicated in OA present challenges to 2D culture systems which cannot recapitulate cell:cell and cell:matrix interactions active in native tissue and disease. 3DMs offer advanced control over matrix-related factors including biochemical cues, micro-architecture and mechanical stiffness as well as enabling incorporation of multiple cell types. Manipulating 3D culture environments is known to influence cell behaviour relative to 2D culture and can drive more physiologically relevant cell phenotypes. For instance, Col II, a key biomarker for healthy tissue-resident chondrocytes, can be stimulated or otherwise maintained in 3D scaffolds²⁰, whereas Col II expression degrades in monolayer culture for murine and human chondrocytes^{20,21}. Additionally, human stem cells have been shown to differentiate towards chondrogenic or osteogenic lineages when encapsulated in decellularized matrix (dECM)²², collagen²³, fibrin²⁴, alginate²⁵ or chitosan-based²⁶ hydrogels. As such, biomimetic 3D scaffolds could also be used to expand primary cells and address cell sourcing challenges of phenotypic drift and low cell numbers from tissue explants and digests.

Scaffold-free pellet culture, where cells are forcibly aggregated or otherwise gathered in low-adherent conditions, is the simplest 3DM of cartilage tissue, and has shown increased chondrogenic differentiation relative to monolayer cultures^{27,28}. However, high cell density in pellet culture drives necrosis of the core region due to limited nutrient diffusion, and the absence of an exogenous matrix component hinders the study of specific cell:ECM or stiffness-mediated interactions. Incorporating scaffolds into models of articular cartilage facilitates interaction between pathogenic chondrocytes and the surrounding matrix, which is largely comprised of collagen and aggrecan. For instance, Yeung *et al.* encapsulated primary human chondrocytes from OA patients in a collagen I gel, demonstrating that the OA phenotype is better maintained in a scaffold-containing culture model over the traditional pellet approach, as measured by expression of SOX9, RUNX2 and MMP13²⁹. Kaplan *et al.* cultured primary human chondrocytes for up to 21 days in a silk-protein scaffold in the presence of IL-

1 β and TNF- α , which upregulated MMP1, MMP3, MMP13 and ADAMTS4, while suppressing aggrecan formation³⁰. Galuzzi *et al.* produced models of articular cartilage comprised of either alginate, silk or dECM containing primary human chondrocytes. While culturing for up to 7 weeks, IL-1 β stimulation promoted aggrecanase activity which induced ECM degradation as measured by supernatant GAG concentration³¹.

More expansive models of OA should draw upon techniques from the field of regenerative medicine, where more relevant scaffold materials, architectures and mechanical forces have been introduced to biofabricate healthy tissue analogues. For instance, while collagen hydrogels commonly used in OA models facilitate cell:matrix interactions and associated signalling, these do not fully capture the architecture of collagenous tissues such as cartilage. By leveraging a biaxial compressive biofabrication technique, Zitnay *et al.* fabricated collagen scaffolds with tuneable collagen density, fibrillar alignment, anisotropic porosity, and mechanical strength in physiological ranges while supporting MSC culture³². Hydrogel gradients³³ or layered bioprinting³⁴ have also been used to recreate the zonal architecture of cartilage, driving differences in GAG and collagen content between zones. Mechanically actuated cartilage 3DMs draw on advances in actuated skin models³⁵, integrating physiological loading conditions which healthy chondrocytes require for proper functioning³⁶ into long-term tissue culture, demonstrating that mechanotransduction significantly effects expression of matrix remodelling genes such as ADAMTS1, as well as mechanical strength of the tissue construct³⁷.

Collectively, these 3DMs present promising approaches to recapitulating the OA microenvironment in a robust and scalable way, demonstrating key clinical hallmarks of OA such as dysregulated matrix synthesis, sensitivity to chemokine stimulation and corresponding expression of matrix-degrading enzymes. However, viable models for clinical application will need to be both more representative and scalable, incorporating more stratified tissue architecture, additional cell or tissue types and mechanical loading in conjunction with primary cells. At present, OA models lack a whole-joint approach which could reproduce biotribological factors underpinning joint mechanics and better predict patient outcomes such as mobility and pain. Here, new technologies and techniques such as 3D bioprinting and dynamically loaded bioreactors may serve to standardise and automate the biofabrication process to address scalability and reproducibility challenges.

Ultimately, more tractable models comprising primary human cells could better address the challenges facing OA research and complement animal models. For example, the pathogenic effects of IL-1 on chondrocytes in monolayer culture are well understood, however, animal models of OA are known to vary in response to IL-1 inhibition between species. Published

animal studies support the therapeutic role of IL-1 inhibition, such as surgically induced models of OA in rats³⁸ and dogs³⁹ showing chondroprotective effects of IL-1 inhibitors. Paradoxically, some mouse models suggest IL-1 β is not involved with synovial inflammation or cartilage destruction^{40,41}. Ultimately, robust clinical trials of the IL-1 inhibitors Anakinra⁴², lutikizumab⁴³ and AMG 108⁴⁴ have largely performed poorly against placebo. While further development of OA animal models and trial design will undoubtedly improve translational success rate, advanced culture models comprised of patient-derived cells may reduce discordance between monolayer models, animal models and the results of clinical trials.

Rheumatoid arthritis

RA is defined by synovitis and pannus formation which then causes degradation of both cartilage and bone; unlike OA, RA frequently involves multiple joints including digits, wrists, ankles, hips and elbows. No curative treatment exists for RA, with treatment strategy aiming to relieve pain and suppress disease activity. Clinically, current therapies benefit roughly half of patients, many of whom relapse upon cessation of treatment. Synovial expansion, inflammation and immune invasion is driven by production of inflammatory mediators such as IL-1 β , TNF- α , IL-6, IL-8, GM-CSF and MIF. Given the central role of synovial hyperplasia in RA pathology, the role of the synovial fibroblast has been studied extensively. Moreover, as an autoimmune disease, the immune component of RA is also of particular interest. Consequently, 3DMs of RA typically incorporate fibroblast-like synoviocytes (FLSs), endothelial cells and/or immune cells.

3DMs of RA using Matrigel-embedded FLS cells have been termed “micromass” cultures. Kiener *et al.* embedded primary human dermal fibroblasts and FLSs in micromass culture, with this work first establishing the tendency for primary FLSs to form distinct lining and sub-lining regions in 3D unlike dermal fibroblasts, mirroring physiology of the synovium⁴⁵. More recently, Wei *et al.* added an endothelial component to the micromass cultures, using HUVEC cells⁶. Here, HUVEC cells formed small vasculature-like structures in 3D co-culture with primary FLSs. With this positional cue, CD90 expression in FLSs was correlated to the vascular axis, with distant FLSs exhibiting the lowest CD90 expression. NOTCH3 signalling was associated with CD90 sub-lining differentiation and therefore identified as a possible target for inflammatory-associated fibroblasts. Critically, single-cell sequencing was integrally used to correlate and compare 3DM and native tissue FLS populations, for instance, by identifying CD90^{HIGH} FLS clusters uniquely present in co-culture micromasses and not in FLS monoculture micromasses⁶.

On-a-chip models represent another 3D culture approach in which samples may be cultured dynamically under specific flow regimes. Rothbauer *et al.* produced a synovium-on-a-chip model in which a FLS micromass culture could be rapidly optically measured non-invasively *in situ* using a light-scattering methodology, which could measure lining-layer expansion in response to TNF α stimulation⁴⁶. In a separate work, Rothbauer *et al.* generated an on-a-chip model of the synovial-chondro crosstalk using neighbouring 3D cultures separated by a fluid medium. Here, adjacent synovial and chondral 3DMs were separated by a fluidic channel to stimulate cellular crosstalk, demonstrating a stable co-culture in which cytokines could be measured over time⁴⁷. To enhance these fluidic approaches, advances in microfluidics could be used to produce models with greater throughput, which are highly standardised. For example, core-shell microfluidic techniques have been used in drug delivery and cell culture⁴⁸ and could pattern synovial lining and sub-lining layers in a microfluidic device, each layer comprised of different cell populations or biomaterials. Scalable, perfused microfluidic systems are already in use for other 3DMs could be used to maintain and stimulate RA 3DMs in an identical way, such as a 24-chamber perfused ANSI-standardised microplate for hepatic 3DM culture⁴⁹.

To date, 3DMs of RA containing primary FLS cells have demonstrated a clear utility for better understanding the role of FLS cells in synovial inflammation and expansion. Recent work incorporating single-cell technologies has highlighted the nuances in 3D culture compared to native tissue, whereby native cell types may be represented but significant cell clusters are absent or disproportionate. For instance, the CD90^{HIGH} cluster uniquely present in FLS/HUVEC micromasses identified by Wei *et al.* was not present in cultures containing the NOTCH inhibitor DAPT, supporting the hypothesis that endothelial cell NOTCH3 presentation is related to FLS CD90 expression. Consequently, granular technologies such as single cell RNA sequencing should be integrated with 3DMs moving forward to identify specific cell sub-populations implicated in disease.

Notably under-represented in RA 3DMs is an immune cell component, which is known to play an important role in disease. Synovial tissue macrophages (STMs) represent a large population in synovial tissue and are expanded in RA. Alivernini *et al.* have studied STMs with single cell sequencing, associating MerTK^{NEG}CD206^{NEG} clusters with proinflammatory cytokines, and MerTK^{POS}CD206^{POS} clusters with a repair phenotype found in tissue samples from patients with sustained RA remission⁵⁰. Moreover, MerTK^{NEG} STMs were found to stimulate cartilage-destructive mediators and pro-inflammatory cytokines in co-culture with primary FLS cells⁵⁰. Consequently, 3DMs foundationally incorporating macrophages or other immune cells is of clear importance for further developing RA 3DMs. Supporting this approach, advances in minimally invasive synovial tissue biopsies techniques will continue to aid

development of personalised RA 3DMs by increasing the availability of primary cells. Therapeutics for RA display a high failure rate in clinical trials, recently reported as 94% failure in phase I-II, with lack of efficacy the most common reason for withdrawal⁵¹. Here, personalised 3DMs could act to match patients to remission inducing treatments, whereby therapeutic molecules downregulate pathogenic interactions between the stromal compartment and immune cells that underly individual patient synovial pathology.

Tendinopathy

Tendons are connective tissues that routinely undergo high tensile loading given their role in transmitting forces from muscle to bone. However, repetitive, supraphysiological loading drives development of tendinopathies, a chronic pathological process that can predispose tendons to rupture⁵². Tendons are hypocellular and relatively avascular, leading to poor healing outcomes and resulting in biomechanically inferior tissue. Despite this pressing clinical burden, consensus biologics are currently unavailable for the treatment of tendinopathies, a consequence of inadequate understanding of the tendon microenvironment both at homeostasis and following insult⁵³.

Resident specialised tendon fibroblasts (tenocytes) represent the major cell population comprising tendons and are linearly aligned within the hierarchical collagen fibril structure of the tendon body. Tenocytes are highly mechanosensitive, requiring loading for proper function, shaping the way they respond to injury⁵⁴. *In vitro* 2D loading systems, whereby tenocytes are seeded on actuated silicone or similar membranes, have contributed significantly to the study of tendon mechanobiology. A uniaxial strain of around 4-8% has been shown to represent physiologically-relevant loading necessary to retain a tenogenic phenotype, while over- or under-loading may simulate injury conditions⁵⁵⁻⁵⁸. While these *in vitro* studies have set the stage for examining how tenocytes respond to various loading conditions, the models are often simplistic, lacking relevant extracellular matrix components. It is now understood that cell-matrix interaction is an essential consideration for tenocyte behaviour, with James *et al.* first demonstrating significant upregulation of the tenogenic marker scleraxis on electrospun scaffolds relative to 2D culture⁵⁹. Given the requirement for *in vitro* models to recapitulate the native tendon microenvironment, there is a significant need for application of tendon tissue engineering approaches to create applicable 3DMs featuring tenocytes embedded in a physiologically relevant matrix.

Biofabrication of 3D, cell-laden biomimetic scaffolds are the primary goal of tendon tissue engineering, and substantial progress has been made in optimising biomaterial performance and biofabrication techniques. One such approach, electrospinning, uses a high voltage field to create nanofibrous porous scaffolds out of liquid polymer, whereby fibre dimension and alignment can be tuned to simulate the microarchitecture and mechanical properties of native tendon ECM, in turn promoting cellular alignment, proliferation and gene expression⁶⁰. 3D bioprinting is another method used to form scaffolds with tuneable bioink and appropriate porous structure for cell infiltration⁶¹, moreover, such rationally designed bioinks can also be used as coating for electrospun fibres⁶⁰.

The choice of biomaterial is an important consideration, and ideally would mimic the microenvironment found in native tendon. Given that tendons are mainly composed of type I collagen, which is already widely used for biofabrication, researchers have commonly used purified collagen as a biomaterial for tendon tissue engineering. However, this greatly oversimplifies the tendon tissue microenvironment which may also contain up to 2% elastin and other proteoglycans, forming microdomains that alter the strain experienced by nearby tenocytes⁶². Decellularised matrix (dECM) has the advantage of more closely resembling the native tendon microenvironment in structural and biochemical properties, although challenges remain with regards to incorporation into a cell-laden scaffold. The enzymatic processes used for tissue decellularisation may degrade the remaining ECM composition, and converting dECM into usable bioinks for 3D printing has proven difficult due to poor gelation mechanics. Moreover, the process for bioink formation from dECM typically involves enzymatic digestion of a fine dECM powder, which results in a mechanically inferior material and homogenises the dECM bioink, removing any spatial matrix cues from the initial tendon sample⁶³. However, recent studies have demonstrated that the combination of dECM with other biocompatible materials, such as hyaluronic acid, may be used to improve mechanical performance⁶⁴. A recent study from Monteiro et. al introduces a novel method for supporting 3D dECM bioprinted scaffolds within a cellulose nanocrystal matrix, providing an innovative strategy towards faithful biofabrication of tendon 3D *in vitro* models⁶⁵.

While models of a tenogenic environment resembling mature tendon are improving^{66–68}, recreating the inflammatory tendon niche associated with tendinopathies remains challenging. A variety of approaches have been tested, including treatment of the tendon construct with inflammatory cytokines or co-culture with inflammatory leukocytes⁶⁹. These have mainly been performed in 2D culture systems, but growing evidence suggests that inflammatory cells, specifically macrophages, function differently in a 3D environment. A recent study has demonstrated that macrophages cultured on 3D networks secreted abundant IL-10, an anti-

inflammatory cytokine, whereas macrophages on 2D collagen surfaces secreted significantly more pro-inflammatory IL-12 and TNF α ⁷⁰. Addressing this, Stauber *et al.* have designed an elegant core-shell 3D hydrogel-explant model to examine how different tendon compartments interact with extrinsic immune cells during “stressed” conditions, studying the pathogenesis of tendinopathies in a validated culture system⁷¹. Another group have used a tendon-on-chip model to mimic endothelial-stromal cell interactions using cell-specific dECM, and this work has demonstrated endothelial cell migration towards the stromal compartment, resulting in enhanced tenogenic commitment and recreating the microenvironment of tendon development⁶⁵. Collectively, these studies underline the requirement for biomimetic 3D co-culture models to accurately reproduce cell:cell interactions.

Compartmental tendon models are of increasing interest due to the present view underpinning the importance of the interfascicular matrix (IFM) in tendon homeostasis. The IFM comprises a continuous compartment between fascicles (the fascicular matrix, or FM), housing the sparse vasculature of the tendon body, along with metabolically active mural and stromal cells⁷². These cells undergo large compressive and shear strains from fascicular sliding and play a role in homeostatic maintenance of the tendon as it undergoes loading⁷³. Evidence suggests that tendon pathology may derive from alterations in the structure and composition of the IFM^{72,74}, and recent work from Marr *et al.* identified a unique vascular niche residing within the IFM that expands following injury⁷⁵. Moreover, cellular crosstalk between distinct tendon compartments is increasingly thought to play a role in tendon pathologies. Here, interplay between the fascicular and IFM regions may play a role in inflammatory response and facilitating immune-stromal cell interactions^{76,77}.

To support advances in 3D scaffolds, new technologies have improved our understanding of the unique cellular composition of soft tissues. Sophisticated lineage tracing and single-cell RNA sequencing techniques have revealed unprecedented heterogeneity in resident tendon cell subpopulations, with potential implications for cell-cell communication and function^{4,78–80}. As different subpopulations differentiate into pro-fibrotic or pro-regenerative in nature, we may be able to take advantage of trajectory analysis to “push” cells towards a specific phenotype more consistent with regeneration. However, at present further consensus is required on the defining characteristics of tenocyte clusters from single cell analysis of primary tendon tissue.

Current work is ongoing in fine-tuning these approaches, the importance of adapting these 3DMs to reproducible, high-throughput methodology cannot be overstated. Opportunities also exist to model common comorbidities associated with JST disease (e.g., diabetic patients with tendinopathy), this could be accomplished by culturing 3DM’s in media containing high

glucose or advanced glycation end products. At present, the biofabrication of these constructs is pain-staking and difficult to widely reproduce, which limit potential for high throughput therapeutic drug screening. However, advances in next generation transcriptomics sequencing are driving the field forward by interrogating the cellular and molecular environment with ever more fidelity and will inform future work on more finely tuned, clinically translatable 3DMs.

Arthrofibrosis

Arthrofibrosis is the pathologic stiffening of JSTs due to an exaggerated inflammatory fibrotic response leading to the development of non-compliant scar tissue⁸¹. As a common complication following total knee arthroplasty, arthrofibrosis affecting the ligaments comprising the knee joint capsule is a significant cause of pain and disability with up to 25% of patients requiring additional surgery to restore adequate knee joint motion⁸². Interestingly, adhesive capsulitis (frozen shoulder) is also a manifestation of arthrofibrosis affecting the ligaments comprising the shoulder joint. In contrast to knee arthrofibrosis, frozen shoulder is uniquely self-limiting over time⁸³. The precise cellular and molecular cues that govern whether inflammatory fibrosis persists or resolves remain to be identified. Knowledge from how fibrosis successfully resolves in the shoulder joint capsule could inform the biological cues to push persistent fibrotic diseases like knee arthrofibrosis towards a resolving trajectory⁵. As an example of a condition without highly developed animal models, the development of 3DMs comprised of patient-derived cells isolated from resolving shoulder fibrosis could uniquely highlight critical cellular and molecular programmes that regulate the persistence or resolution of arthrofibrosis.

Conclusions and future directions

At present, therapeutically-applied 3DM of soft tissue joint disease lag behind advances in other fields where small masses of Matrigel-embedded patient cells, termed 'patient-derived organoids' (PDOs) have provided insight into disease mechanism and map on to clinical data. For example, gut PDOs can be stimulated to induce swelling, whereby differences in volumetric swelling correlate to disease severity for patients with cystic fibrosis, with some patients in this PDO study being approved for treatments based on their PDO data⁸⁴. Protocols developed for gut PDOs have been incorporated into multiple successful studies using colorectal cancer PDOs, including culture conditions and key media supplements such as R-Spondin and Noggin⁸⁵. Specifically in the cancer space, PDOs have been demonstrated to be predictive of patient response to therapy for a wide range of cancers. Critically, clinical non-responders have repeatedly been identified from cancer PDO studies, highlighting a clear first-

step in clinical utility; at the time of writing, 167 studies are listed on *ClinicalTrials.gov* containing the keyword “Organoid”.

To fully realise the potential of 3DMs for drug discovery and personalised medicine, standardisation must be introduced to drive scalability. PDO cultures have been adapted to 384-well formats⁸⁶ and advanced technologies such as bioprinting have also been used to rapidly scale 3DMs for drug discovery⁸⁷. Accordingly, it was recently published that a new bispecific anti-cancer antibody for KRAS^{mut} colorectal cancer patients entered clinical trials after large-scale functional screening using a PDO biobank (ClinicalTrials.gov identifier:NCT03526835)⁸⁸. This seminal work demonstrated the utility of advanced 3DMs when integrated into the drug-discovery pipeline, exhibiting an accelerated time from discovery to clinical trials of 5 years. PDO studies have also recently entered the personalised medicine space in an interventional capacity, although with mixed results. The SENSOR trial represents the first formal prospective interventional trial, whereby clinical decision making was guided on the basis of PDOs established from patient biopsy tissues⁸⁴.

In JST tissues, the presence of multiple tissue and cell types, complex tissue architectures and dynamic loading has been challenging for standardising and scaling 3DMs. For faithful recapitulation of native JSTs, it is likely that multiple biomaterials and cell types will be necessitated, for instance, softer FLS-laden synovial tissues and stiffer, collagenous, chondrocyte-laden cartilage are best replicated by separate biomaterials and cell sources. However, recent advances in biofabrication techniques, such as 3D bioprinting, electrospinning and on-a-chip technologies will serve to improve standardisation and thereby enable better scaling of JST 3DMs. Moreover, bespoke matrix components will improve the representation of relevant biological cues in 3DMs, such as dECM derived from healthy versus diseased tissues. Here, proteomic analysis of JSTs could help to rationally design biomimetic scaffolds with physiological ratios of ECM components. Ultimately, further incorporation of advanced biofabrication techniques will improve access to more physiological JST 3DMs.

Given the complex pathophysiology and tissue architecture of soft tissue joint diseases which incorporate multiple cell types, 3D *in vitro* approaches are a logical next step in advancing laboratory models of disease. Here, single-cell sequencing has been used to great effect, uncovering the diversity of tissue-resident stromal cell subtypes in health and disease in across a range of tissues⁸⁹. For instance, it is now clear that fibroblasts exist in a wide range of states, some of which contribute to disease by expressing inflammatory markers³. As single-cell technology becomes more accessible, its application has begun to expand to 3DMs. Bone marrow organoids⁹⁰, cancer PDOs⁹¹, synovial organoids⁶ and others have been sequenced and compared native tissue. Moving forward, patient specific 3DMs can be more finely

evaluated using this approach by elucidating which cellular subtypes are faithfully reproduced in the 3DM and also addressing questions behind patient variability in establishing successful organoid cultures or in response to therapeutics. Moreover, 3DMs may be established with enriched or depleted cellular subtypes of interest to evaluate their role in disease. Spatial sequencing may also be used in conjunction with organoids to map such subtypes to distinct regions in culture space, e.g., to hypoxic regions or in proximity to other cell types. However, despite this progress, challenges remain regarding patient variability, tissue heterogeneity and cell sourcing which limit scalability in 3DM fabrication and drive uncertainty when analysing 3DMs. To address this, advances in precision minimally invasive biopsies will enable greater selectivity in tissue sourcing, and primary cell expansion will need to be more finely evaluated to understand how expansion conditions are selective for specific cell types, or otherwise cause phenotypic drift resulting in cells which are no longer representative of *in vivo* phenotypes.

Ultimately, refinement of joint soft tissue models and incorporation with advanced sequencing methodologies is still nascent. However, it is likely such models will incorporate primary cells into bespoke 3D microenvironments which recapitulate physical and biochemical features of native tissue (Fig. 3). Critically, several key landmarks for 3DMs have now been met, including the first organoid-driven therapeutic to reach clinical trials⁸⁸ and the first organoid-based prospective interventional clinical trial⁹². Accordingly, given developments in 3DMs outside of soft tissue joint disease, it is clear that patient-derived 3D culture models will foundationally improve our understanding and treatment of a wide range of diseases, many of which have no established or representative animal model, such as frozen shoulder. Study of such organoids in conjunction with study of primary tissue samples will highlight differences between health and disease, particularly when sequenced at the single cell level. Building on this, future integration with *in silico* modelling such as disease network modelling may precisely identify molecular mechanisms underlying JST disease phenotypes and also elucidate drug action⁹³. Finally, as with the cancer PDO field, establishment of biobanks for soft tissue joint disease may become a powerful tool for fully exploiting JST 3DMs.

Search strategy & selection criteria

We searched PubMed, Web of Science and Google Scholar for publications spanning 3D models of OA, RA, and Tendinopathy from database inception to 20th June 2023, preferring recent and relevant developments. We also searched specialist journals encompassing musculoskeletal tissue engineering and regenerative medicine, selected through the expertise of the authors. Search terms included combinations of: "Osteoarthritis", "Rheumatoid Arthritis", "Tendinopathy", "Organoid", "3D", "3D Model", "3D in vitro model", "Tissue Engineering", "bioprinted", "electrospun", "microfluidic" and "joint on a chip". The search term "Organoid" was used when searching clinical trials via ClinicalTrials.gov."

Author contributions

Wrote the manuscript: PAJ, JAE, SGD

Reviewed, revised & edited the manuscript: PAJ, JAE, MC, CDB, MKS, SGD

Figures: JAE, PAJ, SGD

Declarations

The authors declared no conflicts of interest.

Figures

Figure 1

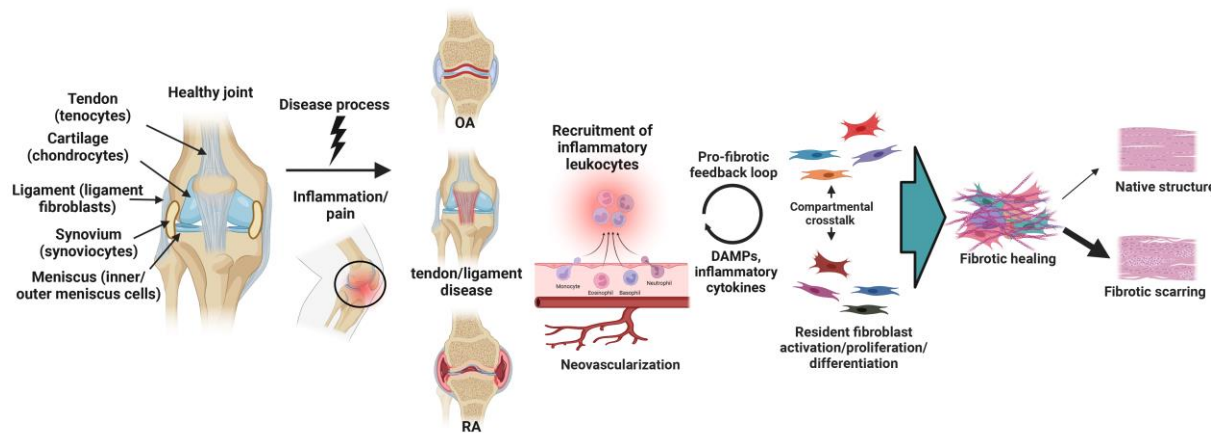


Figure 1: Overview of the soft tissues comprising the joint and the fibrotic disease process, whereby fibrotic tissue disrupts healthy tissue morphology in tendon, ligament, cartilage or synovium resulting in fibrotic scarring.

Figure 2.

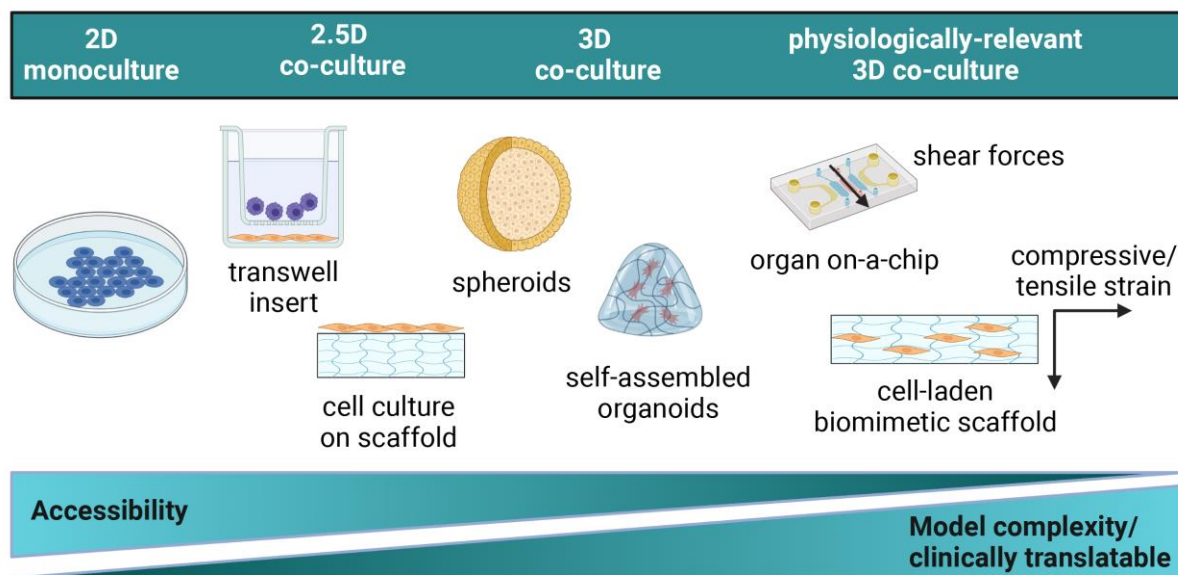


Figure 2: Overview of the current spectrum of *in vitro* models from 2D monoculture towards physiologically relevant, highly clinically translatable 3DM incorporating multiple cell types, an ECM component and tissue-appropriate biomechanical forces.

Figure 3.

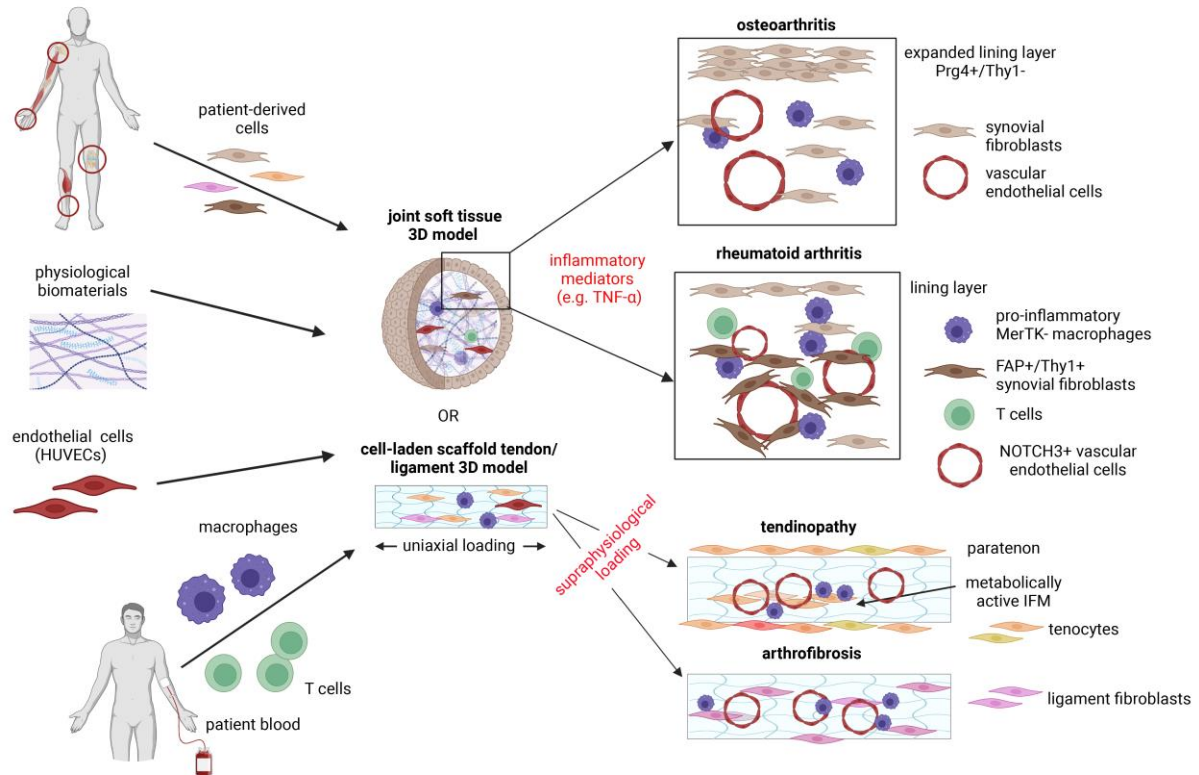


Figure 3: 3D model development for joint soft tissue disease. Combining patient derived cells with relevant matrix components will yield 3D cultures which recapitulate key tissue morphology such as an expanded synovial lining layer in OA or increased sub-lining vascularity in RA. Dynamic loading in tendon/ligament models will simulate the healthy tissue microenvironment and overloaded rupture or tear models.

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