Research Article

A Three-Dimensional Model to Study Human Synovial Pathology

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Abstract
Therapeutic agents that are used by patients with rheumatic and musculoskeletal diseases were originally developed and tested in animal models, and although retrospective studies show that these models have limited predictive value, their use has continued because of a lack of good in vitro alternatives. In this study, we developed a 3-dimensional synovial membrane model made of either human primary synovial cell suspensions or a mix of primary fibroblast-like synoviocytes and CD14+ mononuclear cells. We analyzed the composition of the mature micromasses by immunohistochemical staining and flow cytometry and showed that the outer surface forms a lining layer consisting of fibroblast-like and macrophage-like cells, reflecting the in vivo naïve synovial membrane. To model the affected synovial membrane in rheumatoid arthritis (RA), micromasses were exposed to the pro-inflammatory cytokine tumor necrosis factor alpha (TNF-α), which led to increased pro-inflammatory cytokine expression and production, and to hyperplasia of the membrane. To recreate the synovial membrane in osteoarthritis (OA), the micromasses were exposed to transforming growth factor beta (TGF-β), which led to fibrosis-like changes of the membrane, including increased alpha smooth muscle actin (α-SMA) and increased expression of fibrosis-related genes PLOD2 and COL1A1. Interestingly, the macrophages in the micromasses showed phenotypic plasticity, as prolonged TNF-α or TGF-β stimulation strongly reduced the occurrence of CD163+ M2-like macrophages. We show the plasticity of the micromasses as a synovial model for studying RA and OA pathology and propose that the synovial lining micromass system can be a good alternative for drug testing.

1 Introduction
Rheumatoid arthritis (RA) and osteoarthritis (OA) are the most common rheumatic diseases. The global incidence of RA is estimated to be 0.5%-1% (Gabriel and Michaud, 2009) and an estimated 12.1% of the adult population has clinical symptoms of OA (Lawrence et al., 2008). RA and OA are both viewed as multi-factorial diseases involving multiple risk factors (Alamanos and Drosos, 2005; Blagojevic et al., 2010) but their exact etiology is unknown. Both diseases cannot be cured at present, but disease-modifying drugs (DMARDs) are available for RA and non-steroidal anti-inflammatory drugs (NSAIDs) are commonly prescribed for OA.

During the abovementioned rheumatic diseases, many processes occur in the synovium. Already in the early phase of RA, hyperplasia of the synovial lining occurs and immune cells infiltrate the sublining (Timmer et al., 2007; Hitchens and El-Gabalawy, 2011). The cells in the synovium can produce several pro-inflammatory cytokines and matrix-degrading enzymes. At the interface of the synovium, cartilage and bone, pannus tissue is formed, which contributes to bone destruction. An important mediator of these processes is tumor necrosis factor alpha (TNF-α) (Vasanthi et al., 2007). Specific inhibitors of TNF-α are the most widely used biological therapeutics and can successfully inhibit the progress of RA in the majority of patients (Mewar and Wilson, 2011).

Synovial pathology is also described for OA. Although OA has traditionally been described as a wear-and-tear disease of the articular cartilage, synovitis is observed in up to 50% of the OA patients and may contribute to the disease process (Scanzello and Goldring, 2012). A second factor that contributes to the synovial thickening in OA is tissue fibrosis (Wenham and Conaghan, 2010). Synovial fibrosis is also associated with joint pain and stiffness (Remst et al., 2015). Although the pathogenesis of OA...
is poorly understood, the cytokine most critically associated with processes occurring in OA is transforming growth factor beta (TGF-β) (Fang et al., 2016). TGF-β is pivotal for the development and maintenance of the articular cartilage, but excessive signaling can have detrimental results. For example, adenoviral overexpression of TGF-β in mice resulted in osteophyte formation (Blaney Davidson et al., 2007). Moreover, TGF-β stimulates the differentiation of myofibroblasts and the expression of collagen type 1, which contribute to synovial fibrosis (Verrecchia and Mauniel, 2007; Remst et al., 2013).

Many experiments to study the initial stages of inflammation, hyperplasia, and fibrosis in the synovium have been performed in animal models of RA and OA. However, the predictive value of animal models for the screening of potential drugs in RA is variable and for OA, many therapies have been successful in treating experimental OA, but no treatment has been sufficiently effective in clinical trials in humans (Malfait and Little, 2015; McNamee et al., 2015). The translation of the animal findings to the human synovium is difficult because there is a lack of relevant human in vitro 3D models of synovial pathology, including hyperplasia and synovial fibrosis.

Translational arthritis research with human cells is often performed in monolayer culture with in vitro expanded synovial fibroblasts. Although monolayer culture with fibroblasts is easy to perform, the absence of extracellular matrix and other cell types results in alterations of cell functions and a rapid loss of phenotype (Zimmermann et al., 2001). One way to maintain the cell composition and matrix interactions of the cells is by using synovial explants instead of cultured monolayer cells. Although synovial explants better represent the original joint environment, important disease processes including hyperplasia and cartilage damage may have already taken place and can thus not be studied. Moreover, there is considerable variability between biopsies from a single joint, resulting from variations in lining thickness (Smith et al., 2003), sublining composition, and an unequal distribution of synoviocyte types in the synovial membrane (Shikichi et al., 1999; Iwanaga et al., 2000).

To circumvent these challenges and provide a relevant model for the synovial membrane in which cells can interact with extracellular matrix, Kiener and co-workers (2006) developed a 3-dimensional (3D) micromass model based on a mixture of fibroblast-like synoviocytes (FLS) and Matrigel® solution. When kept on ice, the Matrigel® was liquid and could be mixed with cells to obtain a homogenous solution. Single drops were pipetted into a culture well and became solid at 37°C. Within weeks, the FLS formed a lining layer on the Matrigel®-medium surface. A synovial lining was determined on 7 µm cryosections stained with hematoxylin and eosin (H&E) to confirm the synovial origin of the tissue (not shown).

2 Materials and methods

Patient material

Synovial biopsies from 5 RA patients were obtained during joint replacement surgery from the orthopedics department of the Sint Maartenskliniek, Nijmegen, The Netherlands. This material was considered surgery surplus material; therefore, its use did not need to be approved by an ethical committee. All patients adhered to the American College of Rheumatology (ACR) criteria and were end-stage RA. Patients gave written informed consent for the use of their material for research. The patient material was pseudonymized. Procedures were performed in accordance to the code of conduct for responsible use of human tissue in medical research1. The presence of a synovial lining was determined on 7 µm cryosections stained with hematoxylin and eosin (H&E) to confirm the synovial origin of the tissue (not shown).

Human CD14+ cells

Peripheral blood mononuclear cells (PBMCs) were obtained from 3 healthy donors by density gradient centrifugation using Ficoll-Paque PLUS (GE Healthcare, Chicago, IL, USA). CD14+ cells were isolated from the PBMC fraction using the MagniSort Human CD14 Positive Selection Kit (Invitrogen, Carlsbad, CA, USA) (purity > 90%) according to the manufacturer’s protocol.

Micromass production

Micromasses were produced from complete synovial cell suspensions from RA synovium as described previously (Broeren et al., 2016), or from a combination of primary RA fibroblast-like synoviocytes (FLS) after several passages and CD14+ monocytes. In short, for micromasses from complete cell suspensions,

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1 https://www.federa.org/code-goed-gebruik
the biopsies were digested using Liberase™ (Roche, Basel, Switzerland) for 1 h at 37°C and the cells were filtered through a 70 µm cell strainer (Corning, NY, USA). Red blood cells (RBCs) were lysed for 2 min at room temperature (RT) in 4 ml RBC lysis buffer (155 mM NH₄Cl, 12 mM KHCO₃, 0.1 mM EDTA, pH 7.3). The cells were mixed on ice with liquid Matrigel® and 25 µl droplets containing 5x10⁵ cells were pipetted on a poly-(2-hydroxyethyl methacrylate) (poly-HEMA) (Sigma-Aldrich, Zwijndrecht, The Netherlands) coated 24-well plate (Greiner Bio-one, Alphen a/d Rijn, The Netherlands). Alternatively, micromasses were produced from 2x10⁴ FLS combined with 10⁶ CD14⁺ mononuclear cells. FLS were obtained after biopsy digestion and RBC lysis by culturing the adhering cell fraction and passaging the cells at least once. After 30 min gelation at 37°C, 500 µl Roswell Park Memorial Institute (RPMI) medium (Gibco, Thermofisher Scientific, Waltham, MA, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS) (v/v) (Gibco, Thermofischer Scientific), 1 mM pyruvate (Thermofisher Scientific) and 1% penicillin/streptomycin (P/S) (v/v) (Westburg, Leusden, The Netherlands) was added. The micromasses were cultured for the time and conditions indicated in the results. In general, medium was replaced twice weekly and micromasses were cultured at 37°C and 5% CO₂.

Flow cytometry
Micromasses were kept on ice for 2 h and the cells were then washed to remove the liquified Matrigel®. Unspecific binding of antibodies to Fc receptors was blocked by incubation with hFc block (1:100 for 20 min at 4°C) (564220 BD Biosciences, San Jose, CA, USA). Cells were stained using mouse anti-human CD90-APC (1:20 for 30 min at 4°C), mouse anti-human CD68-PE (1:20 for 60 min at 4°C) and mouse anti-human CD14-APC (1:20 for 60 min at 4°C). The samples were measured on the CyAn ADP analyzer (Beckman Coulter, Woerden, The Netherlands) using an 488 nm laser in the FL7 and FL8 channels for Alexa Fluor 568/PE and APC, respectively. Gates were determined using fluorescence minus one (FMO). The gating strategy and unstained controls are shown in Figure S1².

Tab. 1: List of antibodies

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<th>Conjugate</th>
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APC, allophycocyanin; FC, flow cytometry; HRP, horseradish peroxidase; IHC, immunohistochemistry; N/A, not applicable; PE, phycoerythrin
* Similar concentration (not dilution) to primary antibody directed to antigen of interest.

² doi:10.14573/altex.1804161s
hyperplasia, Ki67, and fibrosis analysis, 3 sections were analyzed per micromass and per section up to 5 pictures were taken of the micromass lining after H&E-staining, Ki67 staining, or α-SMA staining, respectively. For CD163 analysis, pictures were taken of the complete micromass matrix. Images were analyzed using Leica Application Suite (LAS) software.

**Gene expression analysis**

The micromasses were dissolved in 500 µl Tri reagent (Sigma-Aldrich) and total RNA was isolated according to the manufacturer’s protocol. qPCR was performed as previously described (Vermeij et al., 2015). Primer sequences are listed in Table 2. Values are depicted as threshold cycle, corrected for GAPDH expression.

**Multiplex ELISA**

Cytokines and chemokines in supernatants were measured on a Bio-Plex 200 system using a magnetic bead-based multiplex immunoassay. Data analysis was performed with Bio-Plex manager software (both Bio-Rad).

**Statistical analysis**

The results are displayed as box plot (median) with whiskers: min to max. Statistical analysis was performed by Mann-Whitney U test using GraphPad Prism software v5.03. P-values below 0.05 were regarded as significant.

### 3 Results

#### 3.1 Analysis of micromass composition and formation

The composition of the cell suspension after digestion of a synovial biopsy was determined using cell surface markers. In the cell suspension prior to micromass formation, we observed cells positive for fibroblast marker CD90 (67.6%), monocyte/macrophage marker CD68 (23.1%), and monocyte marker CD14 (CD14<sub>low</sub> 22.9% and CD14<sub>high</sub> 4.58%). This cell composition was comparable in independent replicates. No

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These results show that the mixed cell population is maintained during micromass culture. The CD14\textsuperscript{high} fraction dropped to 3.38% and the CD14\textsuperscript{low} fraction was not detected on day 7. The distinct CD68\textsuperscript{+}/CD14\textsuperscript{low} cells had lost most CD14 expression after 7 days.

The kinetics of lining formation and cell migration in the synovial micromasses were assessed at different time points. Immediately after micromass formation, the cells had not regained their original morphology and all cells were round-shaped (Fig. 1B).

**Fig. 1: Flow cytometry and histological analysis of synovial cell suspension and micromasses**

(A) A RA synovial biopsy was digested using Liberase™. Cells were cultured for < 24 hours (Fig. 1A, day 0) or mixed with Matrigel\textsuperscript{®} and cultured for 7 days, after which the Matrigel\textsuperscript{®} was re-liquified on ice (Fig. 1A, day 7). The cell suspensions were subsequently stained for fibroblasts (CD90), macrophages (CD68), and monocytes (CD14). Similar profiles were obtained in at least 3 experiments with unique donors. FS, forward scatter; SS, side scatter. The gating strategy and unstained controls are shown in Fig. S1\textsuperscript{2}.

(B) Micromasses were fixed and embedded in paraffin at different time points (day 0, 4, 5, 7, 14, and 35). 7 µm sections were stained with H&E, anti-CD68, or antibodies against fibroblast marker 11-Fibrau. Pictures were taken at 200x magnification. Negative control IgG stainings are shown in Fig. S3\textsuperscript{2}.
Based on the observation that the synovial fibroblasts and macrophages were the two cell types that we could identify in the lining of micromasses composed of synovial cell suspensions and because these cells represent the two types of synovocytes found in the synovial lining, we switched in subsequent experiments to micromasses produced from cultured primary RA FLS and primary CD14+ cells purified from peripheral blood of healthy donors. This approach has previously been explored by Kiener et al. (2006). As expected, these micromasses produced a lining in 7 days (Fig. 2A), which contained both 11-Fibrau+ FLS and CD68+ macrophages (Fig. 2B,C).

### 3.2 Long-term exposure of synovial micromasses to inflammatory stimuli leads to inflammatory gene expression and cytokine production

We established the inflammatory response of micromasses to 3-week stimulation with 10 ng/ml recombinant human TNF-α (Abcam, Cambridge, UK) or TGF-β (Biolegend, San Diego, CA, USA), which was replenished twice per week. Control micromasses received culture medium without stimulation. (A) The gene expression of pro-inflammatory cytokines IL1B, IL6, IL8, and TNF was measured by qPCR. Gene expression values were corrected for GAPDH expression and are depicted as ΔCt. (B) Cytokine secretion was measured in the culture supernatant using a multiplex ELISA. n = 5/6 per group. Data points are combined from 2 independent experiments. Expression levels were compared to medium control by Mann-Whitney U test. *, p < 0.05; **, p < 0.01; ***, p < 0.001.

### Based on the observation that the synovial fibroblasts and macrophages were the two cell types that we could identify in the lining of micromasses composed of synovial cell suspensions and because these cells represent the two types of synovocytes found in the synovial lining, we switched in subsequent experiments to micromasses produced from cultured primary RA FLS and primary CD14+ cells purified from peripheral blood of healthy donors. This approach has previously been explored by Kiener et al. (2006). As expected, these micromasses produced a lining in 7 days (Fig. 2A), which contained both 11-Fibrau+ FLS and CD68+ macrophages (Fig. 2B,C).
with both TNF-α and TGF-β (Fig. 3B). IL-8 was only increased after stimulation with TNF-α, and TGF-β stimulation induced the release of TNF-α from the micromasses. These results show that the micromasses are sensitive to inflammatory triggers.

### 3.3 Long-term exposure of synovial micromasses to TNF-α leads to hyperplasia of the micromass lining and an altered macrophage phenotype

In addition to pro-inflammatory effects, we observed striking differences in the cells residing in the micromass matrix after long-term stimulation with TNF-α. When the micromasses were not treated with TNF-α, some CD68+ cells distributed throughout the complete micromass showed a hypertrophic phenotype (Fig. 4A). After stimulation with TNF-α, there was a strong reduction in cells displaying this phenotype and instead cells showed a spindle-like morphology. The large, round cells were positive-ly stained for CD163, a membrane marker associated with type 2 macrophages (Fig. 4B) (Kowal et al., 2011). These cells, which were distributed throughout the complete micromass, disappeared upon TNF-α or TGF-β stimulation (Fig. 4E).

### Fig. 4: Micromass lining hyperplasia and macrophage phenotype after long-term stimulation

Micromasses were generated from primary RA FLS and CD14+ PBMCs and stimulated for 3 weeks with 10 ng/ml recombinant human TNF-α or TGF-β, which was replenished twice per week. Control micromasses received culture medium without stimulation. Micromasses were stained for CD68 (A), CD163 (B) with H&E (C) and Ki67 (D). Black arrows indicate cells positive for the marker of interest. Grey arrows indicate a hyperplasic lining. (E) Quantification of CD163+ cells throughout the entire micromass. Values were corrected for the total surface area of the images analyzed and are depicted as fold change compared to the average of the unstimulated controls. (F) Quantification of H&E staining for lining hyperplasia. (G) Quantification of Ki67 staining for proliferation of the micromass lining. n = 5/6 per group and the average score of > 3 images per 3 sections of every micromass was calculated. Statistical analysis was performed by Mann-Whitney U test comparing to medium condition. *, p < 0.05; **, p < 0.01. Negative control IgG staining is shown in Fig. S3.
TNF-α, the lining thickness increased. The hyperplasia was assessed by quantifying the amount of hematoxylin staining and by the expression of the proliferation marker Ki67 in the lining in > 9 images per micromass. The cellularity and proliferation of the lining was significantly increased after TNF-α stimulation (Fig. 4D,F,G) whereas this was unaltered upon TGF-β stimulation.

### 3.4 TGF-β can induce fibrosis-like changes in the micromass lining

The effects of the 3-week stimulation on micromass fibrosis-associated processes were first assessed on the gene expression level. TGF-β signaling marker SERPINE1 was significantly induced by recombinant TGF-β, indicating active TGF-β signaling was induced in the micromasses (Fig. 5A). This resulted in an increased expression of PLOD2, Alpha Smooth Muscle Actin (ACTA2), and collagen type 1 (COL1A1), which are all associated with fibrosis (Remst et al., 2014). Histological staining of α-SMA showed that fibrotic-like changes had occurred in the micromass lining, which was significantly increased after stimulation with TGF-β (Fig. 5B,C). Moreover, TGF-β induced a stellate cell morphology of α-SMA+ cells, characteristic for myofibroblasts (Fig. 5B) (Bagalad et al., 2017).

### 4 Discussion

In this study, we further characterized the 3D synovial micromass model produced from primary human biopsies for application in both RA and OA research as an alternative to animal experiments. The model was first explored by Kiener and co-workers (2006) based on primary synovial fibroblast (FLS) culture. Those experiments were focused on cell-cell and cell-matrix interactions during the formation of the lining and the involvement of Cadherin-11 (Kiener et al., 2006; Lee et al., 2007). We adapted a procedure that was briefly explored in these studies to produce micromasses with both FLS and CD14+ PBMCs, which resulted in a lining that included 11-Fibrau fibroblasts and CD68+ macrophages (Kiener et al., 2010). These two cell types were the predominant lining cell types in the micromasses produced from the complete synovial cell suspension and correspond with the cell types observed in the intimal layer of the synovium, the type B synoviocyte (FLS) and type A synoviocyte (macrophage-like synoviocyte), respectively (Smith, 2011). The survival of the macrophage-like cells has previously been shown to be dependent on the presence of synovial fibroblasts (Kiener et al., 2010). The inclusion of these cells in the micromass model is important, because the macrophages produce large amounts of TNF-α and TGF-β and can activate latent TGF-β (Wahl et al., 1990; Kinne et al., 2000). Moreover, macrophages are involved in bone erosion, cartilage damage, and ectopic bone formation in RA and OA (Goldring and Gravallese, 2000; Blom et al., 2004; Bondeson et al., 2006).

For the micromasses that were generated from primary CD14+ PBMCs and primary FLS, FLS had first been cultured in vitro. It has been observed that directly after digestion of the synovium, the FLS have a disease phenotype, which includes the spontaneous production of pro-inflammatory cytokines (Firestein, 1996). However, after prolonged culture the disease imprint is gradually lost (Zimmermann et al., 2001; Hirth et al., 2002; Hardry et al., 2013), making the FLS more suitable for the micromass model in which part of the disease is mimicked by stimulation with TNF-α or TGF-β. The micromass model is therefore not dependent on the original disease phenotype of the fibroblasts. This fits with the observation that no spontaneous hyperplasia was observed without stimulation with exogenous TNF-α.

The survival of FLS and macrophage-like cells was observed both by immunohistochemistry and flow cytometry. Several results indicate changes in the macrophage population. During micromass culture, the number of CD68+ cells expressing CD14
decreased at day 7 compared to day 0. This can be the result of macrophage differentiation, which is associated with a decrease in CD14 expression (Ohradanova-Repic et al., 2016). During the 3-week micromass culture, we observed large CD68+ and CD163+ cells. These cells represent an M2-like anti-inflammatory macrophage phenotype (Vandooren et al., 2009; Kowal et al., 2011). They disappeared upon stimulation with TNF-α, which is known to polarize macrophages to a pro-inflammatory M1-like phenotype (Kennedy et al., 2011). We also observed significant down-regulation of CD163 by TGF-β, which has also been described previously (Pioli et al., 2004). Although additional markers are required for more accurate and reliable identification of specific cell types and cell subsets, the results clearly indicate plasticity of the macrophage-like cell population in the synovial micromass.

Interestingly, we found that when put on ice, the Matrigel® liquidized and could be washed away from the cells. The resulting cell suspension could be analyzed by flow cytometry without the requirement of enzymatic digestion, which further enhances the applicability of the micromass model.

We evaluated the long-term effects of TNF-α exposure on micromasses. In addition to the well-known role of TNF-α in RA, TNF-α produced by the osteoarthritic synovium is strongly associated with systemic low-grade inflammation and production of matrix-degrading enzymes by multiple cells in the joint (Bondeson et al., 2006; Ozler et al., 2016). The observation that systemic TNF-α levels are increased prior to the most pronounced cartilage damage supports the hypothesis that inflammation in OA is a cause rather than a symptom. We observed that the pro-inflammatory effects of TNF-α can be studied in the micromasses. In addition, synovial hyperplasia is observed in the synovium of both RA and OA patients (Bondeson et al., 2010). We could re-create this pathogenic process by stimulating the micromasses for 3 weeks with TNF-α, which is a major advantage compared to studies using synovial biopsies, in which hyperplasia has already occurred (Izquierdo et al., 2011).

Although both pro- and anti-inflammatory functions of TGF-β have been described for arthritic diseases, we observed a strong increase in expression of IL-6 and TNF-α after stimulation with TGF-β. IL-8 mRNA but not protein was significantly increased by TGF-β. The effects of TGF-β on IL-6 and IL-8 have been described before in PBMCs and synovial fibroblasts (Turner et al., 1990; Cheon et al., 2002). Interestingly, TGF-β stimulation resulted in increased TNF-α secretion, but an increase of mRNA level was not observed. Previous experiments with TGF-β and synovial cell cultures did not result in increased TNF-α production, which indicates that our finding of increased TNF-α might be an indirect effect (Brennan et al., 1990). The most pronounced effect of long-term TGF-β stimulation was the strong increase of an α-SMA+ lining, which was not observed after stimulation with TNF-α. The increased α-SMA was not caused by hyperplasia, which did not occur after TGF-β stimulation.

The joint is considered a complex organ and joint diseases like RA and OA are thought to involve several cell types and tissues, including the synovium, but also the articular cartilage and (subchondral) bone (Mathiessen and Conaghan, 2017). These tissues are also dependent on the extracellular matrix for proper interactions and several in vitro studies have aimed at recreating 3D matrix mixed with specific cells. For example, chondrocytes can be co-suspended with gelatin microspheres in an alginate solution to obtain an articular cartilage-like structure (Su et al., 2012). This can be cultured in combination with FLS and in vitro differentiated macrophages to mimic OA cartilage pathology (Peck et al., 2018). In addition, FLS have been cultured in several scaffolds to generate 3D structures. In one study, FLS were combined with endothelial cells in a methylcellulose sphere that was subsequently incorporated in a collagen gel (Maracle et al., 2017). This method was used to study angiogenesis into a collagen scaffold. These studies highlight the added value of 3D models for joint tissues and the micromass adds a model to study the synovial membrane.

We propose that the synovial micromass model can be a solution for experiments that require a synovial membrane in which disease processes have not yet occurred, since the availability of healthy synovial tissue is very limited. The production of micromasses will result in a uniform composition of the starting material, which can also be easily manipulated with regard to its composition. In future applications, the micromasses may be co-cultured with different cell types or scaffolds, or in co-culture with cartilage explants to study effects on cartilage degeneration. Although no 3D models that include all cell types and tissues involved are available yet, the micromass model can be a good alternative to animal experiments studying RA and OA for fundamental research, target validation, as well as pre-clinical treatment testing. To our knowledge, this is the first human 3D culture model to study the interplay between macrophages and fibroblast-like synoviocytes in hyperplasia and synovial fibrosis-like pathology by stimulation with TNF-α and TGF-β, respectively.

References


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Conflict of interest

There are no conflicts of interest to report.

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