UNDERSTANDING THE EFFECTS OF MESENCHYMAL STROMAL CELL THERAPY FOR TREATING OSTEOARTHRITIS USING AN *IN VITRO* CO-CULTURE MODEL

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Abstract

Osteoarthritis (OA) is a leading cause of chronic pain and disability, for which there is no cure. Mesenchymal stromal cells (MSCs) have been used in clinical trials for treating OA due to their unique ability to generate paracrine anti-inflammatory and trophic signals. Interestingly, these studies have shown mainly short-term effects of MSCs in improving pain and joint function, rather than sustained and consistent benefits. This may reflect a change or loss in the therapeutic effects of MSCs after intra-articular injection. The present study aimed to unravel the reasons behind the variable efficacy of MSC injections for OA using an *in vitro* co-culture model.

Osteoarthritic human synovial fibroblasts (OA-HSFs) were co-cultured with MSCs to investigate their reciprocal effects on cell responses and whether a short-term exposure of OA cells to MSCs was sufficient for reducing their diseased characteristics in a sustained manner. Gene expression and histological analyses were performed.

OA-HSFs exposed to MSCs showed short-term downregulation of inflammatory markers. However, the MSCs showed upregulation of inflammatory markers and impaired ability to undergo osteogenesis and chondrogenesis in the presence of OA-HSFs. Moreover, short-term exposure of OA-HSFs to MSCs was found to be insufficient for inducing sustained changes to their diseased behaviour.

These findings suggested that MSCs may not provide long-term effects in correcting the OA joint environment due to them adopting the diseased phenotype of the surrounding tissues, which has important implications for the future development of effective stem-cell-based OA treatments with long-term therapeutic efficacy.

Keywords: Mesenchymal stromal cells, osteoarthritis, synovial fibroblasts, co-culture, inflammation.

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List of Abbreviations

ACAN	aggrecan
ADAMTS	a disintegrin and metalloproteinase with thrombospondin motifs
ANOVA	analysis of variance
BSP	bone sialoprotein
CCL2	C-C motif chemokine ligand 2
CD	cluster of differentiation
COL1A1	collagen type I alpha 1 chain
COL2A1	collagen type II alpha 1 chain
COX-2	cyclooxygenase-2
CXCL6	chemokine (C-X-C motif) ligand 6
DMEM	Dulbecco's modified Eagle medium
FBS	foetal bovine serum
HSF	human synovial fibroblast
IFN	interferon
IL	interleukin

MCP-1	monocyte chemoattractant protein-1
MMP	matrix metalloproteinase
MRI	magnetic resonance imaging
MSC	mesenchymal stromal cell
NBF	neutral buffered formalin
NSAID	non-steroidal anti-inflammatory drug
OA	osteoarthritis
OA-HSF	osteoarthritic human synovial fibroblast
OC	osteocalcin
OP	osteopontin
RT-PCR	reverse transcription polymerase chain reaction
RUNX2	runt-related transcription factor 2
SD	standard deviation
SOX9	SRY-box transcription factor 9
SPP1	secreted phosphoprotein 1
TIMP3	tissue inhibitor of metalloproteinase 3
TLR4	toll-like receptor 4
TNF	tumour necrosis factor

Introduction

OA is the most prevalent joint disease globally, affecting more than 18 % of women and 10 % of men over the age of 60 (Glyn-Jones *et al.*, 2015). While OA is generally characterised by the degradation of articular cartilage, disease pathogenesis affects and involves the entire joint, including the synovium and subchondral bone (He *et al.*, 2020; Loeser *et al.*, 2012). Due to irreversible damage to joint structures as OA progresses, patients experience joint stiffness, chronic pain, loss of mobility, reduction in quality of life and an increased risk of mortality due to other co-morbidities, such as cardiovascular disease (Nüesch *et al.*, 2011). OA occurs in articular joints, including the knee, hip, hand, spine and ankle, with knee OA having the highest prevalence globally (Cross *et al.*, 2014) and estimated to affect more than 650 million individuals aged 40 and over (Cui *et al.*, 2020). The high prevalence of OA and the need for chronic disease management, due to the absence of a cure, place a huge economic burden on healthcare systems worldwide, with direct healthcare costs for OA estimated at 1-2.5 % of national gross domestic product in developed countries (Safiri *et al.*, 2020). The global impacts of OA are anticipated to escalate in the coming years due to rising life expectancy, population ageing and increased obesity prevalence.

Current clinical treatments for OA aim at relieving pain and improve joint functionality in an attempt to delay joint replacement surgery (Cutolo *et al.*, 2015). Non-pharmacological options focus on patient education, weight loss and exercise programs, but have limited effects on early symptoms and long-term disease modification. Pharmacological treatments generally involve analgesics and NSAIDs but give rise to inappropriate polypharmacy and increased risk of dangerous side effects due to the high incidence of co-morbidities in OA patients. Intra-articular injections of corticosteroids or visco-supplements, such as hyaluronic acid, can be indicated for patients whose symptoms cannot be controlled with other treatments. These have yielded variable results, with some evidence supporting short-term effects (less than 6 months) on pain relief and functional improvement (Richards *et al.*, 2016). Surgical intervention is indicated for patients with severe OA who fail to benefit from conservative treatments, where the diseased joint is replaced with an implant to recover the joint junction. However, replacement surgery is only available for particular joints (*e.g.* hip, knee, shoulder) and is associated with increased risks of complications, such as infection, as well as limited implant lifetime of approximately 20 years (Ahmed and Hincke, 2010). The limitations of current OA treatments urge the development of new therapies that may help to stop or delay joint degradation and progression into an advanced disease.

MSCs have gained attention in OA research due to their potential to provide a disease-modifying and possibly regenerative therapy, owing to their unique immunomodulatory, anti-inflammatory, pro-regenerative, angiogenic, anti-fibrotic, anti-oxidative stress and multidirectional differentiation functions (Barry, 2019; Harrell *et al.*, 2019). Several clinical trials have been performed to assess the efficacy of intra-articular MSC injections to treat OA, mostly in the knee (Ha *et al.*, 2019; Jiang *et al.*, 2021). Interestingly, despite most trials reporting short-term symptomatic relief and some regaining of joint function, the therapeutic effects of MSCs diminishes within months after administration (Jiang *et al.*, 2021). Some studies have reported that repeated administration of MSCs could help maintain therapeutic efficacy (Matas *et al.*, 2019). It has been proposed that the effectiveness of MSC therapy may be limited by cell survival and engraftment, impeded by the excessive inflammatory immune response, oxidative stress and hypoxic microenvironment at the injury site (Fernández-Francos *et al.*, 2021). Experiments that traced the engraftment of injected MSCs in OA preclinical models observed that their retention rate is only 3 % (Barry, 2019). These findings suggest that the short-term benefits exerted by MSCs in an OA joint are mostly due to their paracrine effects rather than differentiation and replacement of joint cells and tissues, as initially believed.

A wide range of preclinical studies have been performed to elucidate the therapeutic effects and associated mechanisms of MSC activity in experimental models of OA (Wang *et al.*, 2022; Xing *et al.*, 2018). While there is ample evidence for the benefits of MSC injections in various OA animal models, only one-way analyses were performed in these studies. Since injected MSCs cannot be retrieved for analysis, all *in vivo* findings focus on the effects of MSCs on OA joint tissues and not the

other way around. Furthermore, the diversity of OA disease phenotypes and associated pathophysiology (endotype) in patients cannot be replicated by single animal models (Hunter and Little, 2016; Zaki *et al.*, 2022), which likely contributes to the poor translation of long-term disease-modifying therapeutic benefits of MSC injections observed in pre-clinical research to clinical studies, which mostly show only short-term effects.

Very limited evidence exists for exploring the effects of an OA joint environment on MSCs. In one of the only studies that established an "*in vitro* OA cell model", MSCs were co-cultured with macrophages and OA chondrocytes in "high" and "low" inflammatory environments to simulate different OA joint conditions (Diaz-Rodriguez *et al.*, 2019). Variable anti-inflammatory and immunomodulatory effects of MSCs were observed on the chondrocytes and macrophages depending on the inflammatory state of the culture environment. Coupled with other studies demonstrating that the exposure of MSCs to inflammatory cytokines, such as TNF-α, can lead to increased expression of inflammatory markers in the MSC secretome (Lee *et al.*, 2010), the present study hypothesised that the inhibitory environment created by diseased cells in an OA joint environment may be at least in part responsible for the reduced therapeutic benefits of MSCs seen in OA clinical studies.

While a range of studies have investigated the effects of MSCs on *in vitro* and *in vivo* OA models, there is an important knowledge gap with regard to: (1) the effects of OA joint-tissue cells on MSCs, (2) whether a short-term exposure of OA cells to MSCs (similar to the time of MSC retention after injection into an OA joint) is sufficient for reducing their diseased characteristics in a sustained manner and, thus, might improve their response to repeated MSC injections. To answer these questions, the present study established an *in vitro* co-culture model using human MSCs and primary OA-HSFs. OA-HSFs were chosen as the cell model due to their known involvement and contribution to OA pathogenesis (Li *et al.*, 2021), displaying the hallmarks of OA-related inflammation and synovial fibrosis (Maglaviceanu *et al.*, 2021) as well as relative ease of extraction and expansion while retaining diseased characteristics compared to other relevant cell types such as OA chondrocytes (Jackson *et al.*, 2014; Smith *et al.*, 2013). The findings suggested that the behaviour and therapeutic benefits of MSCs are significantly affected by the OA joint environment, which has important implications for developing effective stem-cell-based OA treatments.

Materials and Methods

Cell sources

OA-HSFs were isolated from the synovium of patients with knee OA undergoing joint replacement surgery at the Royal North Shore Hospital and North Shore Private Hospital, as previously described (Smith *et al.*, 2013). Briefly, tissue normally discarded as part of standard surgical care was collected, with written informed consent from OA patients undergoing total or partial knee replacement surgery (Northern Sydney Central Coast Health HREC Protocol LNR/15/HAWKE/494). De-identified tissues were provided to the laboratory and HSF were isolated by proteolytic digestion, grown to confluence in a T75 flask in DMEM supplemented with 10 % (v/v) FBS and 2 mmol/L glutamine, trypsinised and cryopreserved (aliquots of 1×10^7 cells/mL) (Melrose *et al.*, 2003; Smith and Ghosh, 1987). OA-HSFs from 2 patients (69 and 73 year old males, undergoing total knee replacement) were pooled and used at the 6th to 10th passage (split at a ratio of 1:2 in each passage) for all experiments.

Human bone-marrow-derived MSCs were purchased from RoosterBio (MSC-003; Frederick, MD, USA). Before use in the experiments, MSCs were expanded in RoosterNourish™-MSC medium (RoosterBio) according to the manufacturer's instructions. Briefly, MSCs were thawed and resuspended in RoosterNourish™-MSC medium and plated in T75 flasks at an average seeding density of 3.3×10^3 cells/cm^2. MSCs were grown until 70-80 % confluency before use. MSCs used for experiments were at passage 3 and their trilineage differentiation ability was confirmed by adipogenic, chondrogenic and osteogenic assays before use.

Co-culture experiments

MSCs were co-cultured with OA-HSFs in three types of culture media, simulating the types of conditions most relevant for OA. (1) Growth medium: DMEM supplemented with 10 % (v/v) FBS and 2 mmol/L L-glutamine; (2) osteogenic medium: StemPro™ Osteogenesis Differentiation Kit from Life Technologies; (3) chondrogenic medium: StemMACS™ ChondroDiff Medium from Miltenyi Biotec. MSCs were cultured in 12-well plates, seeded at a density of 4×10^4 cells/well in monolayer for growth and osteogenic cultures or seeded as a micromass containing 3×10^5 cells for chondrogenic cultures. For co-culture with OA-HSFs, the HSFs were seeded at a density of 2×10^4 cells/insert in 12-well Transwell® inserts with polycarbonate membrane and pore width of 0.4 μm (Corning). Then, inserts with seeded HSFs were placed in 12-well plates with growth, osteogenic or chondrogenic MSC cultures to establish the *in vitro* co-culture model. The permeable inserts allowed exchange of culture medium and metabolites between MSCs and OA-HSFs but not physical contact between them. All cultures were incubated at 37 °C in 5 % CO_2 and 50 % of the culture medium was replaced 3 times/week.

Three studies were conducted.

1. To confirm the short-term effects of MSCs on OA cells, OA-HSFs were cultured with (HSF + MSC) or without (HSF control) MSCs in growth medium for 3 and 7 d.

Table 1. Primer sequences for genes evaluated by real-time RT-PCR.

Gene	5′ to 3′ forward sequence	5′ to 3′ reverse sequence
18S	ACC ATA AAC GAT GCC GAC CG	CAA TCT GTC AAT CCT GTC CGT GTC
ADAMTS-4	AGA CAC AGG CAG GGA GAG ACA AAG	GGA GAA AAC TTA GTC CTT GGG CTT G
ADAMTS-5	AAC TCC CAG GAC AGA CCT ACG ATG	GCA GAT TCT CCC CTT TCC ACA AG
CCL2	GCT GAG ACT AAC CCA GAA ACA TCC	AAT GAA GGT GGC TGC TAT GAG C
CD44	AAA GGA GCA GCA CTT CAG GA	TGT GTC TTG GTC TCT GGT AGC
COX2	GAC AGT CCA CCA ACT TAC AAT GCT G	GCT GCT TTT TAC CTT TGA CAC CC
IL-6	GAA GAT TCC AAA GAT GTA GCC GC	GAA GGT TCA GGT TGT TTT CTG CC
IL-8	AAC TTT CAG AGA CAG CAG AGC ACA C	CAC AGT GAG ATG GTT CCT TCC G
MMP-2	TGA CGG AAA GAT GTG GTG TG	CTC CTG AAT GCC CTT GAT GT
MMP-13	TGA CGG AAA GAT GTG GTG TG	CTC CTG AAT GCC CTT GAT GT
MMP-14	CCA GGG TCT CAA ATG GCA ACA TAA TGA AA	CCA TGG AAG CCC TCG GCA AA
TIMP3	TGC CCT TCT CCT CCA ATA CA	CTT CCT TCC CTC CCT CAC TC
TLR4	GCT TTC ACT TCC TCT CAC CCT TTA G	CTG GCA TCA TCC TCA CTG CTT C
RUNX2	CCA GAT GGG ACT GTG GTT ACT GTC	CTGG GGA GGA TTT GTG AAG ACG
BSP	ATG GCC TGT GCT TTC TCA ATG	GGA TAA AAG TAG GCA TGC TTG
OP	ACG CCG ACC AAG GAA AAC TC	GGA GAT TCT GCT TCT GAG ATG GG
OC	ATG AGA GCC CTC ACA CTC CTC G	GTC AGC CAA CTC GTC ACA GTC C
SOX9	AAA CGG TGC TGC TGG GAA AC	CTC CTT TGC TTG CCT TTT ACC TC
COL2A1	CAG TTC GGA CTT TTC TCC CCT C	AGT TTC CTG CCT CTG CCT TGA C
ACAN	TCA CCA TCC CCT GCT ATT TCA TC	TCT CCT TGG ACA CAC GGC TC
COL1A1	ACA GGG CGA CAG AGG CAT AAA G	AAC AGG ACC AGC ATC ACC AGT G

2. To test whether OA cells could modify the behaviour of MSCs in the short and longer term, MSCs were cultured with (MSC + HSF) or without (MSC control) OA-HSFs in growth, osteogenic and chondrogenic medium for up to 21 d.
3. To test whether exposing OA cells to MSCs could cause sustained changes in their behaviour and have positive effects on tissue repair, OA-HSFs were either preconditioned by first co-culturing with MSCs for 3 d (cHSF) or not preconditioned and simply cultured in growth medium for 3 d (HSF). Subsequently, both groups were co-cultured with fresh MSCs in growth, osteogenic and chondrogenic medium for 3 and 7 d.

Gene expression analysis by quantitative RT-PCR

MSCs and OA-HSFs were harvested separately from each co-culture assay at the indicated time points for gene expression analysis. RNA was extracted from MSCs and OA-HSFs using the Isolate II RNA Micro Kit (Bioline, Canada) following the manufacturer's instructions. MSC chondrogenic micromasses were first snap-frozen in liquid nitrogen and minced before placing them in the lysis buffer, while lysis buffer was added directly to all monolayer MSCs or OA-HSFs in the well or Transwell® insert. RNA concentration of each sample was quantified using the Nanodrop 2000 (Thermo Fisher Scientific) and reverse transcription to cDNA (Omniscript, Qiagen) was conducted using 500 ng RNA per sample, using random pentadecamers (50 ng/mL, Sigma-Genosys) and RNase inhibitor (10 U per reaction, Bioline). The resulting cDNA was subjected to real-time PCR in a Rotorgene 6000 (Corbett Life Science, New South Wales, Australia) using Immomix (2× dilution; Bioline), SYBR Green I (10,000× dilution; Cambrex Bioscience) and 0.3 μmol/L human-specific primers (Sigma-Aldrich), as shown in Table 1. The gene expression levels were normalised to those of the *18S* housekeeping gene using the comparative Ct ($2^{-\Delta\Delta Ct}$) method and results were expressed as fold changes in gene expression relative to unstimulated control cells at day 0 (before the commencement of cultures). Pro-inflammatory and pro-catabolic responses in MSCs and OA-HSFs were evaluated using expression of OA-related genes (*IL-6*, *IL-8*, *MMP-2*, *MMP-13*, *ADAMTS4*, *ADAMTS5*, *CD44*, *TLR4*, *COX-2*, *CCL2*). MSC differentiation was evaluated using gene markers for osteogenesis (*RUNX2*, *BSP*, *SPP1*) and chondrogenesis (*SOX9*, *COL2A1*, *ACAN*).

Histological analysis

MSC differentiation was additionally evaluated by histology at 21 d. For osteogenic cultures, monolayer MSCs were fixed in 10 % NBF for 15 min, washed and stained using alizarin red S to visualise calcium deposition. For chondrogenic cultures, MSC micromasses were fixed in 10 % NBF for 4 h, then embedded in paraffin-wax, sectioned and stained using toluidine blue to visualise proteoglycan

deposition. Stained samples were visualised using light microscopy.

Statistical analysis

Data for all experiments were obtained from 4 independent samples, except for histological analyses that were performed on 2 independent samples. Gene expression data were expressed as mean ± SD. Statistical analyses were performed using GraphPad Prism V9.4.0 (GraphPad Software). For gene expression data in Fig. 1, Student's *t*-test was used to compare differences between two groups for the same gene. For gene expression data in Fig. 2, 3, 4 and 5, one-way ANOVA was used to compare differences among three or more groups at the same time point, followed by Sidak's multiple comparisons test, with $p < 0.05$ considered statistically significant.

Results

The reciprocal effects of human osteoarthritic synovial cells and MSCs on each other were investigated through short- and long-term co-culture to simulate the types of interactions present in an osteoarthritic joint following stem cell injection. Three co-culture studies were performed to investigate: (1) the effects of MSCs on OA-HSFs in growth medium; (2) the effects of OA-HSFs on MSCs in growth, osteogenic and chondrogenic medium; (3) the effects of preconditioning OA-HSFs with MSCs prior to co-culturing to mimic repeated injections of MSCs, in growth, osteogenic and chondrogenic medium.

OA-HSFs co-cultured with MSCs rapidly reduced pro-inflammatory and pro-catabolic molecule expression in the short-term

Expression of pro-inflammatory and pro-catabolic molecules implicated in OA pathogenesis was analysed for OA-HSFs cultured in growth medium, with or without MSCs in co-culture (Fig. 1). At 3 d, the OA-HSFs co-cultured with MSCs induced rapid reduction of the expression of several genes, particularly *ADAMTS-5*, *MMP-13* and *TLR4*, compared to the control group (Fig. 1**a**). Interestingly at 7 d, there were no differences in expression levels between OA-HSFs that were or were not co-cultured with MSCs for all inflammatory markers except *TLR4* and *COX-2* (Fig. 1**b**). The differences between groups also decreased at day 7 compared to day 3 for all genes. These findings suggested that the MSCs had paracrine anti-inflammatory and anti-catabolic effects on the OA-HSFs but these effects appeared to only persist in the short-term and were not retained in the OA-HSFs.

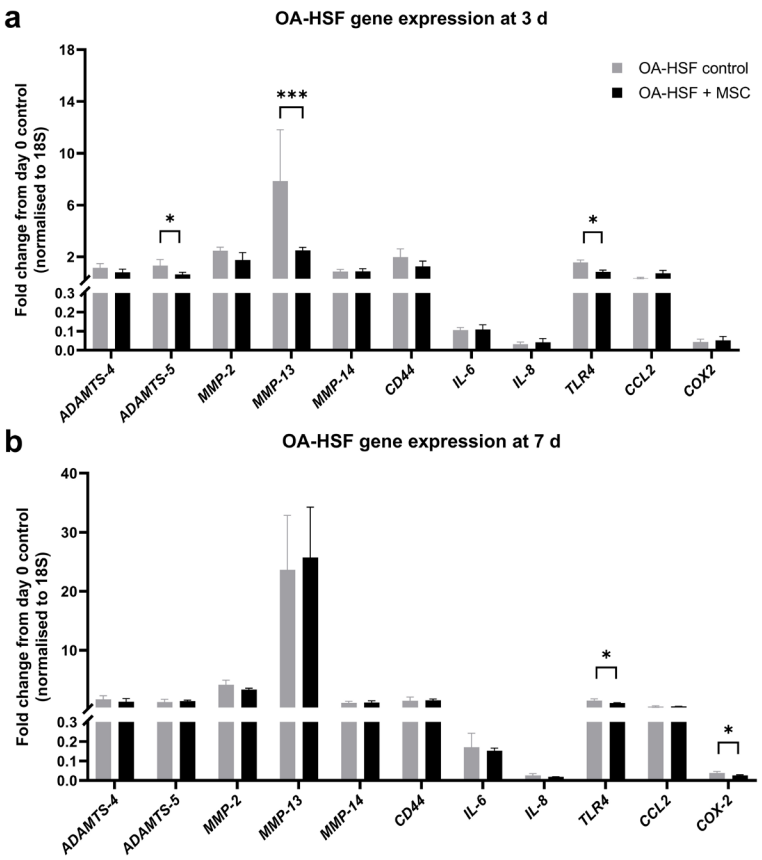

Fig. 1. Expression of OA pro-inflammatory and pro-catabolic genes in OA-HSFs. OA-HSFs were cultured in growth medium with or without MSCs in co-culture at (**a**) 3 and (**b**) 7 d. Expression levels were presented as fold change from OA-HSFs at day 0, normalised to the housekeeping gene *18S*. Data are represented as mean ± SD; $n = 4$; * $p < 0.05$, *** $p < 0.001$.

MSCs co-cultured with OA-HSFs showed increased expression of pro-inflammatory and pro-catabolic molecules and impaired differentiation over the long-term

MSCs were cultured in growth, osteogenic and chondrogenic media to mimic the types of conditions that are most relevant for joint repair. Gene expression of pro-inflammatory and pro-catabolic molecules implicated in OA pathogenesis was analysed for MSCs grown with or without OA-HSFs in co-culture over 21 d (Fig. 2). Gene expression and histological staining relevant to osteogenesis (in osteogenic medium) and chondrogenesis (in chondrogenic medium) were also analysed at 21 d for MSCs grown with or without OA-HSFs in co-culture (Fig. 3).

Several genes involved in inflammation and matrix/tissue degradation were found to be elevated in MSCs co-cultured with OA-HSFs compared to MSC controls, in different medium types and at both short- and long-term time points. Notably

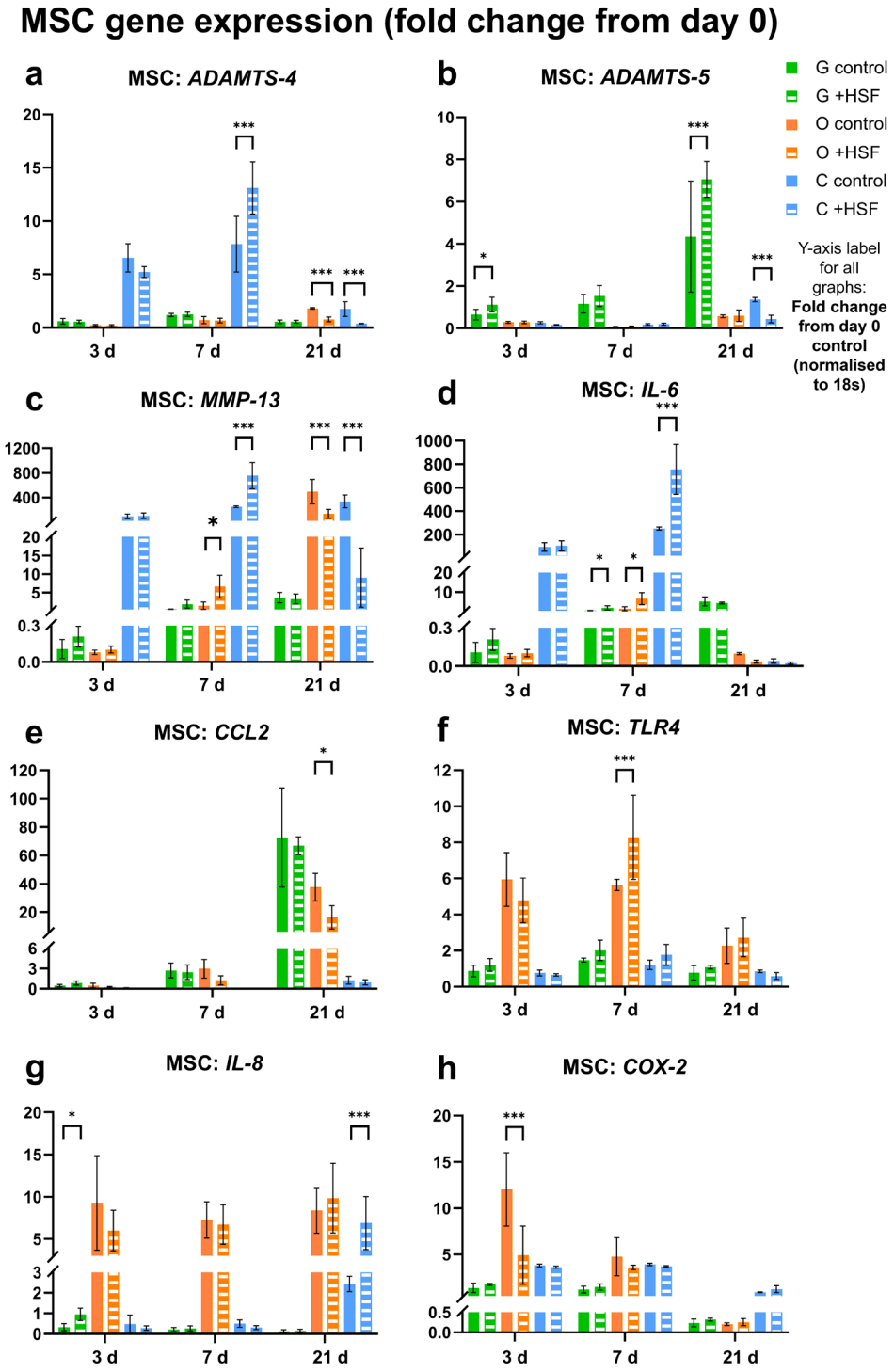

Fig. 2. Expression of OA pro-inflammatory and pro-catabolic genes in MSCs. MSCs were cultured in growth (G), osteogenic (O) and chondrogenic (C) medium, with or without OA-HSFs in co-culture for up to 21 d. Expression levels are presented as fold change from MSCs at day 0, normalised to the housekeeping gene *18S*. Data are represented as mean ± SD; $n = 4$; * $p < 0.05$, *** $p < 0.001$.

at 8 d, MSCs co-cultured with OA-HSFs showed significantly higher expression levels of *MMP-13* in all three medium types. Other genes such as *ADAMTS-4*, *ADAMTS-5*, *IL-6*, *IL-8* and *TLR4* were significantly more highly expressed in MSCs co-cultured with OA-HSFs at 4 or 8 d in at least one medium type. For some genes, their upregulation in MSCs co-cultured with OA-HSFs persisted until 21 d, notably for *ADAMTS-5* in growth medium and *IL-8* in chondrogenic medium. These findings suggested that osteoarthritic cells may have created an environment that acted on MSCs and increased their expression of pro-inflammatory and pro-catabolic factors, which might have dampened their therapeutic benefits. Interestingly, there were several inflammatory proteins with reduced expression at 21 d in MSCs co-cultured with OA-HSFs compared to controls, including ADAMTS-4, ADAMTS-5, MMP-13, IL-6 and CCL-2, but exclusively in osteogenic or chondrogenic medium, which may be associated with global changes in MSC gene expression at the later stages of differentiation.

MSCs co-cultured with OA-HSFs showed impaired ability to undergo osteogenic and chondrogenic differentiation at 21 d (Fig. 3). This was evidenced by markedly reduced gene expression of middle-late-stage markers for osteogenesis (*BSP*, *SPP1*) and chondrogenesis (*COL2A1*, *ACAN)* compared to control MSCs in differentiation medium. MSCs co-cultured with OA-HSFs also showed greatly attenuated histological features of differentiated bone (calcium deposition; Fig. 3**a**) and cartilage (proteoglycan deposition; Fig. 3**b**). These findings suggested that long-term exposure of MSCs to a diseased osteoarthritic environment may cause them to lose their defining characteristics, such as differentiation potential, and result in impaired regenerative ability.

Repeated exposure of OA-HSFs to MSCs did not change their inflammatory gene expression or influence on MSCs

MSCs were co-cultured with OA-HSFs in growth, osteogenic and chondrogenic media. OA-HSFs were either preconditioned (cHSF groups) or not (OA-HSF groups). The preconditioned OA-HSFs were pre-exposed to MSCs in co-culture for 3 d in growth medium and, subsequently, co-cultured with fresh MSCs, simulating repeated injections of MSCs. The not-preconditioned OA-HSFs were simply grown in growth medium for 3 d before being co-cultured with fresh MSCs. Gene expression of proteins implicated in OA pathogenesis was analysed for both OA-HSFs (Fig. 4) and MSCs (Fig. 5) over 7 d. An early differentiation marker was also analysed to look at any change specific to early stage osteogenic (*RUNX2*) or chondrogenic (*SOX9*) differentiation.

Preconditioning OA-HSFs did not have significant effects on modifying their expression of OA-related pro-inflammatory and pro-catabolic molecules (Fig.

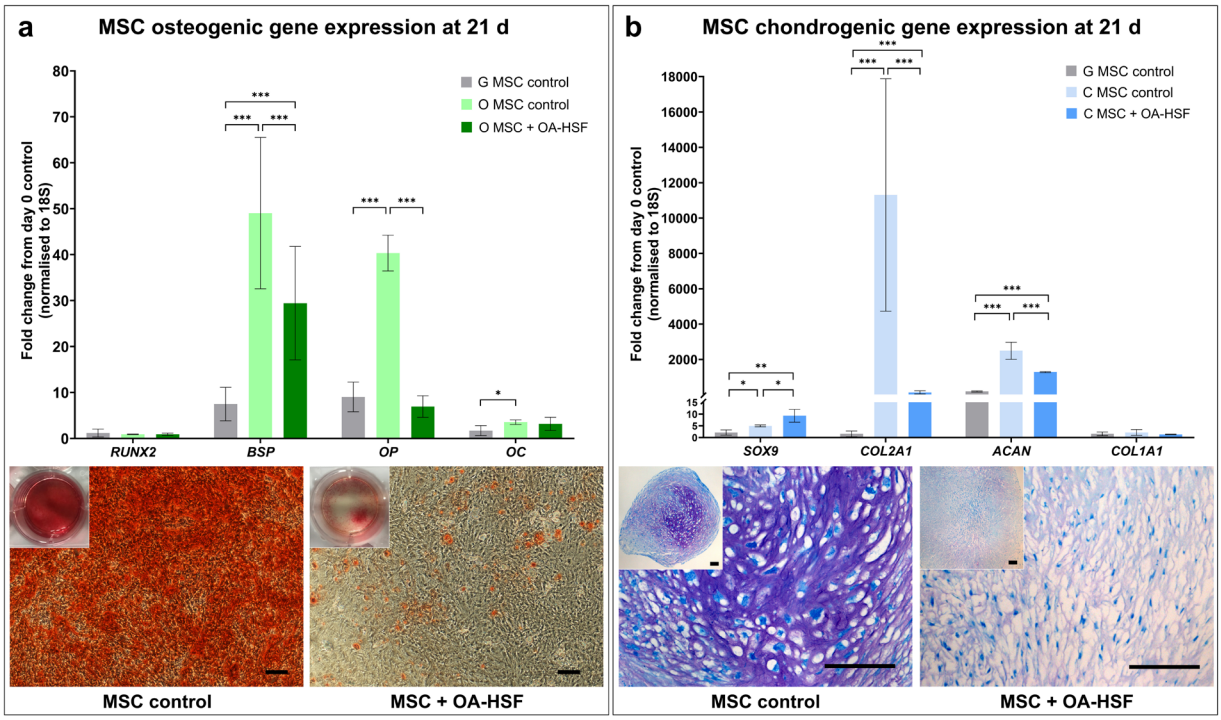

Fig. 3. Gene expression and histology of MSCs undergoing differentiation. (**a**) Osteogenic and (**b**) chondrogenic differentiation of MSCs when cultured in osteogenic (O) or chondrogenic (C) differentiation medium, compared to control growth (G) medium, with or without OA-HSFs in co-culture at 21 d. Gene expression levels were presented as fold change from MSCs at day 0, normalised to the housekeeping gene *18S*. Data are represented as mean ± SD; $n = 4$; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Calcium deposition in osteogenic cultures is indicated by alizarin red S staining (3**a**; scale bar = 500 μm). Proteoglycan deposition in chondrogenic cultures is indicated by toluidine blue staining (3**b**; scale bar = 200 μm).

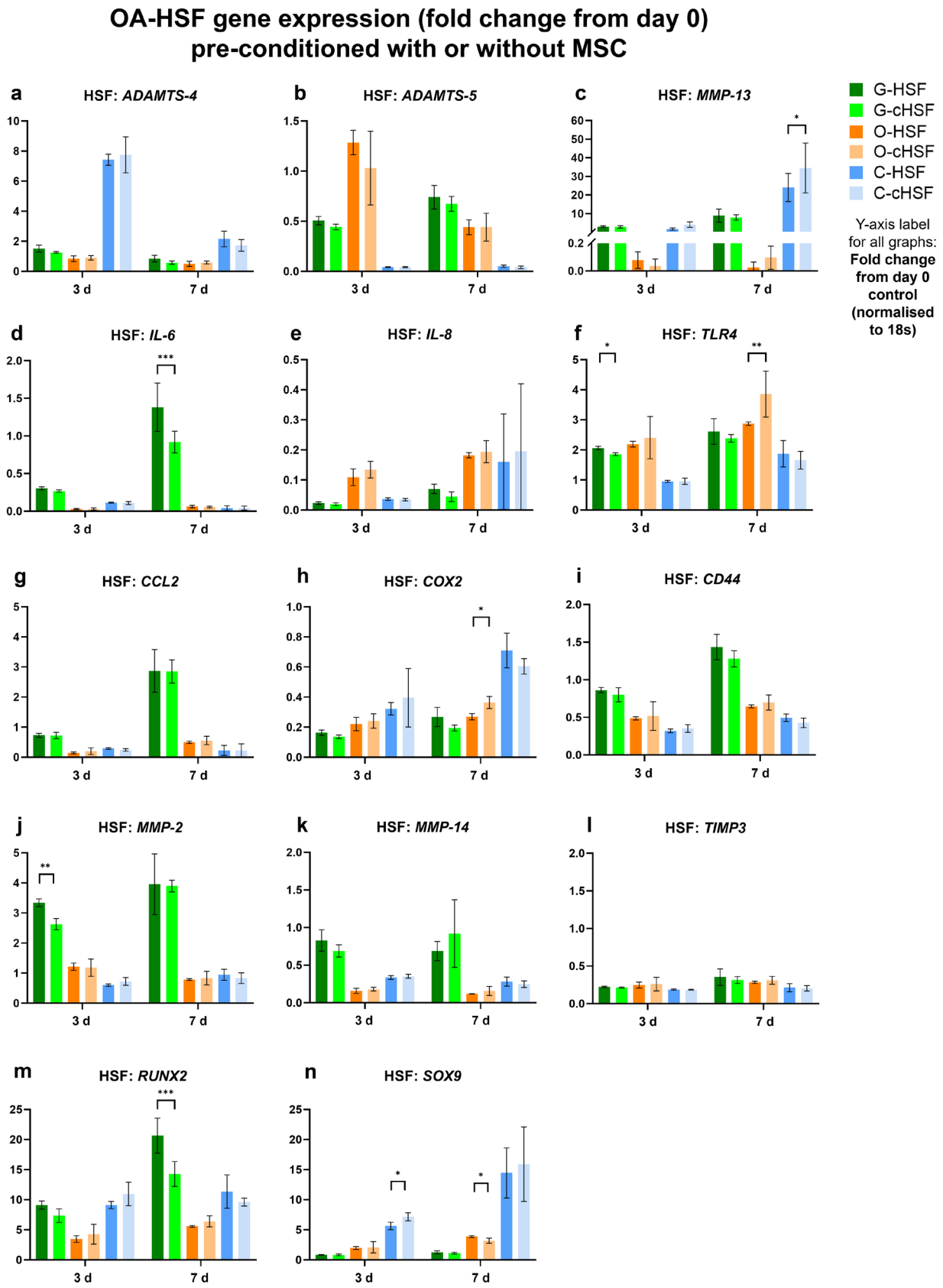

Fig. 4. Expression of OA pro-inflammatory and pro-catabolic genes and early-stage markers of osteogenic (*RUNX2*) and chondrogenic (*SOX9*) differentiation in OA-HSFs. OA-HSFs were co-cultured with fresh MSCs in growth (G), osteogenic (O) and chondrogenic (C) medium for 3 and 7 d. OA-HSFs were either preconditioned (cHSF) by exposure to MSCs in co-culture for 3 d in growth medium or not preconditioned (OA-HSF) and grown by themselves for 3 d in growth medium, before being introduced to fresh MSCs. Expression levels are presented as fold change from MSCs at day 0, normalised to the housekeeping gene *18S*. Data are represented as mean ± SD; $n = 4$; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

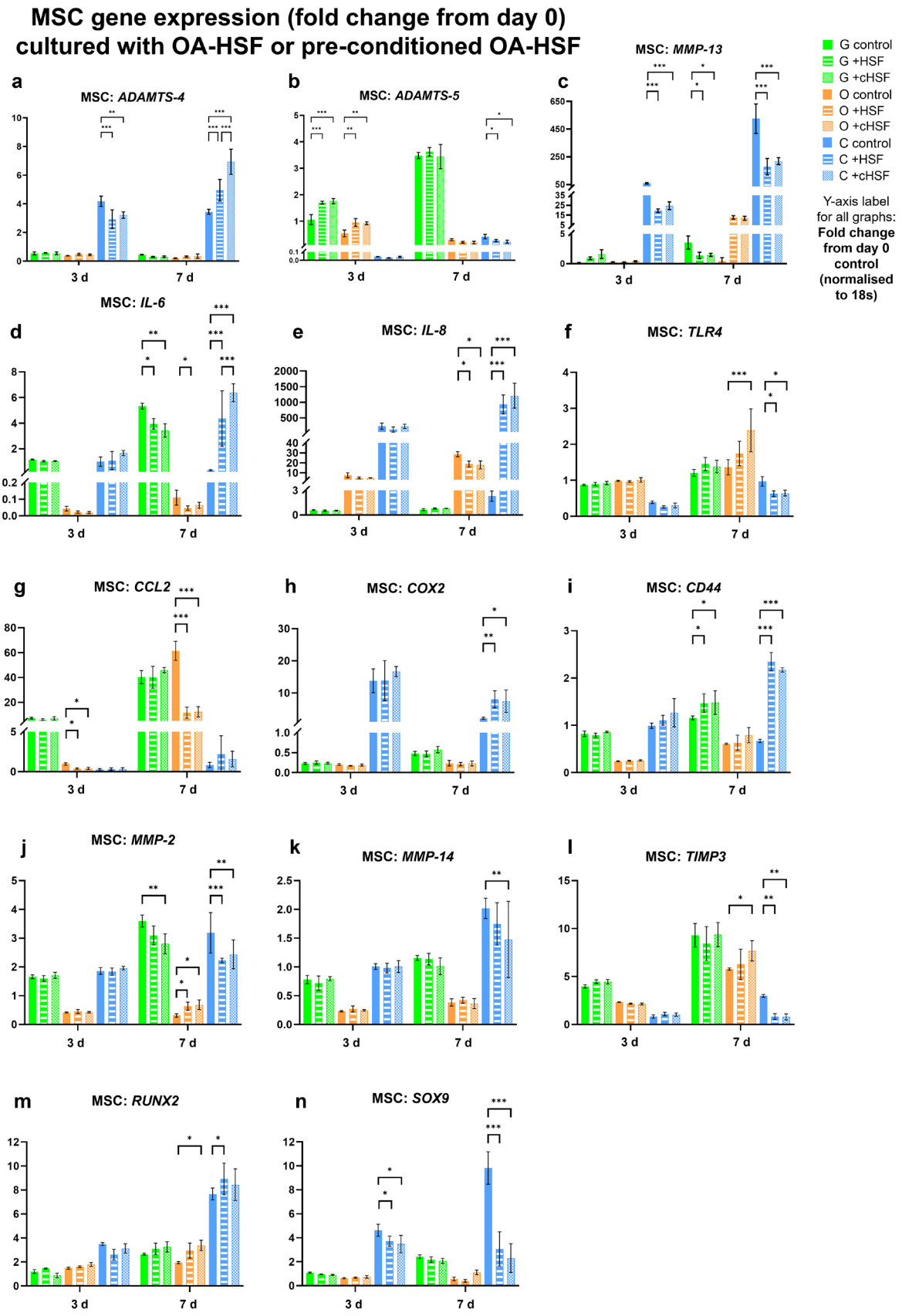

Fig. 5. Expression of OA pro-inflammatory and pro-catabolic genes and early-stage markers of osteogenic (*RUNX2*) and chondrogenic (*SOX9*) differentiation in MSCs. MSCs were grown by themselves or co-cultured with OA-HSFs in growth (G), osteogenic (O) and chondrogenic (C) medium for 3 and 7 d. OA-HSFs were either preconditioned (cHSF) by pre-exposure to MSCs in co-culture for 3 d in growth medium or not preconditioned (OA-HSF) and grown by themselves for 3 d in growth medium, before being introduced to fresh MSCs. Expression levels were presented as fold change from MSCs at day 0, normalised to the housekeeping gene *18S*. Data are represented as mean ± SD; $n = 4$; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

4). Regardless of whether they were pre-exposed to MSCs or not, both groups of OA-HSFs co-cultured with fresh MSCs for 3 and 7 d showed similar expression levels for most genes in all three medium types and at both time points. There were some exceptions for OA-HSFs co-cultured with MSCs in growth medium, where pre-exposure to MSCs led to a significant downregulation of *IL-6*, *TLR4* and *MMP-2* at 3 or 7 d. In chondrogenic medium, preconditioned OA-HSFs also showed significantly higher expression of the early chondrogenic marker *SOX9* compared to control OA-HSFs at 3 d. These findings suggested that some of the paracrine conditioning effects of MSCs may be retained in the preconditioned OA-HSFs in the short term. However, there were also other exceptions where preconditioned OA-HSFs showed higher expression of some pro-inflammatory/catabolic factors, such as *MMP-13*, *TLR4* and *COX-2* at 7 d but exclusively in differentiation medium, which may be a consequence of global gene expression changes induced by early osteogenic or chondrogenic differentiation. Overall, *in vitro* preconditioning with MSCs did not induce significant changes in the pro-inflammatory and catabolic phenotype of OA-HSFs, suggesting that repeated MSC injections in an OA joint are not likely to induce sustained changes to this diseased environment.

Looking at MSCs that were co-cultured for 3 and 7 d with OA-HSFs, preconditioned or not, expression levels for most genes were similar in all three medium types. Significant differences in MSC gene expression in all medium types were only seen when comparing the + OA-HSF or + cHSF group to the MSC control but not between these two MSC groups co-cultured with OA-HSFs. The only exception was *ADAMTS-4* at 7 d, where MSCs co-cultured with preconditioned OA-HSFs showed significantly higher expression of this gene compared to MSCs co-cultured with control OA-HSFs. For the osteogenic marker *RUNX2*, MSCs co-cultured with both OA-HSF groups showed higher expression at 7 d compared to the MSC control in osteogenic medium, possibly reflecting that early inflammatory priming is beneficial for kickstarting osteogenic induction. For the chondrogenic marker *SOX9*, MSCs co-cultured with both OA-HSF groups showed lower expression at 3 and 7 d compared to the MSC control in chondrogenic medium, reflecting the negative effects of an inflammatory environment on the induction of chondrogenic differentiation. For both early-stage differentiation markers, there were no differences in expression in the MSC groups co-cultured with preconditioned or control OA-HSFs. Overall, these findings indicated that MSCs, whether co-cultured with OA-HSFs that were preconditioned or not, did not change their expression levels of inflammatory or differentiation markers. In other words, the inhibitory effects of OA-HSFs on MSCs did not change, regardless of whether the OA-HSFs were previously exposed to MSC conditioning. Therefore, short-term exposure of osteoarthritic cells to MSCs may be insufficient for sustained modifications to their diseased phenotype and it may be unlikely that repeated MSC injections can improve therapeutic efficacy in OA.

Discussion

Through *in vitro* co-culture of human MSCs and OA-HSFs, the findings of the present study suggested that despite the anti-inflammatory and trophic functions of MSCs, they could not provide long-term effects in correcting the osteoarthritic joint environment due to them adopting the diseased behaviour of the surrounding cells. These findings have important implications for future OA therapies based on the use of stem cells. In addition to testing different cell sources, injection concentrations and administration frequencies in clinical trials, alternative approaches might be to work on correcting the catabolic environment within the osteoarthritic joint or utilise the biological derivatives of stem cells, which – unlike the living cell – will not respond in a negative way to the osteoarthritic environment.

In the first experiment, the MSCs provided a transient therapeutic effect on the OA-HSFs, resulting in an immediate suppression of pro-inflammatory and pro-catabolic gene expression. However, this effect trailed off at 7 d. This was consistent with the results of multiple clinical trials using intra-articular injections of MSCs for knee OA, which reported short-term improvement in most outcome measures post-administration but failure to maintain long-term therapeutic efficacy (Jiang *et al.*, 2021). It was also interesting to note that the short-term therapeutic effects of MSCs on OA-HSFs preferentially targeted genes directly involved in cartilage degradation, such as *ADAMTS-4* and *MMP-13*, which degrade aggrecan and type II collagen, respectively. These findings are consistent with clinical trials reporting short-term improvement of cartilage repair in knee OA treated with MSC injection (Ha *et al.*, 2019). It was previously hypothesised that the loss of therapeutic effect after MSC injection may be due to cell loss into the bloodstream or cell death at the defect site (Barry, 2019). The fact that the present study results demonstrated a decreased therapeutic effect of MSCs over time in co-culture with OA cells, where the MSCs remained localised and alive, suggested that the MSCs might be negatively responding to a diseased environment.

Based on the above findings, the second experiment was performed to specifically investigate, for the first time, the short- and long-term responses of MSCs when co-cultured with OA cells in different culture environments that are most relevant to an OA joint. It was interesting to find that in all cases where there were significant differences between groups cultured in growth medium, pro-inflammatory and pro-catabolic gene expression in MSCs was elevated when co-cultured with OA-HSFs at both short- and

long-term time points. These findings suggested that MSCs may begin to display similar diseased and pro-inflammatory behaviour as the surrounding cells when placed in an osteoarthritic environment, limiting their beneficial effects. Ultimately, this altered MSC expression profile may even contribute to ongoing and worsening matrix degradation and progression of OA. Intriguingly, there were several cases in long-term (21 d) co-culture where MSCs exposed to OA-HSFs downregulated inflammatory marker expression, but exclusively in osteogenic or chondrogenic medium. This may suggest an exciting prospect for long-term conditioning of the OA joint towards an environment that induces bone or cartilage regeneration to promote MSC paracrine functions. Further studies are needed to explore the feasibility of this approach.

It was interesting to explore the influence of OA cells on the differentiation capacity of MSCs in osteogenic and chondrogenic induction medium, which is one of the defining characteristics of MSCs (Chamberlain *et al.*, 2007). MSCs exposed to OA-HSFs in long-term co-culture showed significantly impaired ability to undergo osteogenic and chondrogenic differentiation, notably through the markedly reduced expression of mid-late-stage differentiation markers and prominent loss of staining area in histological analysis. The closest comparison to the results of the present study comes from an investigation of gene expression in synovial-fluid-derived MSCs from patients with advanced knee OA (Sanjurjo-Rodriguez *et al.*, 2020). These MSCs showed a decrease in osteogenic gene expression, specifically *BSP* and *RUNX2*, compared to control MSCs from subchondral bone. Interestingly, this study also described a 5- to 50-fold increase in the expression of pro-catabolic proteins, including MMP1 and ADAMTS-5, in the OA synovial fluid MSCs. Overall, the results from the second experiment suggested that the MSCs could lose their restorative and trophic characteristics under the influence of diseased OA cells, which may contribute to their short-lived therapeutic effects, as observed in clinical trials (Jiang *et al.*, 2021).

Some efforts have been made to unearth the mechanisms by which the secretion of paracrine factors in MSCs is affected by inflammatory conditioning. One study used proteomic analysis to identify 118 factors secreted by human adipose-derived MSCs in response to TNF-α exposure and found that several chemokines are secreted at significantly higher levels compared to quiescent MSCs, including IL-6, IL-8, CXCL6 and MCP-1 (Lee *et al.*, 2010). Since TNF-α is one of the key inflammatory cytokines and may be linked to OA progression, these findings suggest that the paracrine functions of MSCs can be converted to a pro-inflammatory state in an OA environment, complementing the results of the present study. In the only other study that established *in vitro* 3D models of OA to study MSC interactions, the OA models were tuned into high and low inflammatory environments (Diaz-Rodriguez *et al.*, 2019). In a high-inflammatory OA environment, MSCs induced osteoarthritic chondrocytes to produce markedly lower levels of IL-1β, IFN-γ, MMP-9 and MMP-13 as well as reduced macrophage activation, which were different from the responses seen in a low-inflammatory OA environment. Together with the findings of the present study, there is emerging evidence to suggest that the immunomodulatory and therapeutic effects of MSCs are influenced by the OA environment and may be further dependent on the severity of joint inflammation.

The third experiment simulated a repeated administration of MSCs to an OA joint. If repeated exposure of OA cells to MSCs could induce a greater beneficial effect, differences in inflammatory gene expression would be expected between the control and preconditioned OA-HSF groups, for both the OA-HSFs and MSCs co-cultured with OA-HSFs. However, overall trends suggested that re-exposure of OA-HSFs to MSCs, even when conducted over a short time frame of a few days, was not sufficient to induce sustained changes to their diseased phenotype. In the OA-HSFs, there were some transient effects where MSC preconditioning led to downregulation of certain inflammatory markers in growth medium, which perhaps justifies some of the increased therapeutic benefits seen with repeated MSC injections for knee OA in clinical trials (Matas *et al.*, 2019; Song *et al.*, 2018). No significant differences in gene expression were observed for MSCs co-cultured with OA-HSFs, whether preconditioned or not, suggesting that repeated exposure of OA-HSFs to MSCs does not reduce their negative influence on the paracrine activities of MSCs. These findings may implicate that single or even multiple MSC injections will not lead to a sustained therapeutic effect in OA joints.

The outcomes of published clinical studies reporting the use of MSC injections to treat OA, particularly for repeated administrations, have been inconsistent due to large variations in the tissue sources of MSCs, isolation and expansion protocols, injection doses and frequencies, delivery vehicles, patient cohorts, outcome measures, length of follow-up and so on (Ha *et al.*, 2019; Jiang *et al.*, 2021; McIntyre *et al.*, 2017). The number of patients recruited to most existing clinical studies is also relatively small. For instance, two trials testing repeated administration of MSCs derived from umbilical cord (Matas *et al.*, 2019) and adipose tissue (Song *et al.*, 2018), respectively, each involved 6 to 9 patients per treatment group. In both trials, improvements were seen in outcome measures related to pain and quality of life. However, when compared to repeated injections of hyaluronic acid, there was no improvement in MRI score, as an objective measure of cartilage repair at 12 months (Matas *et al.*, 2019). Song *et al.* (2018) compared low, medium and high doses of MSCs (10-50 million cells) all administered with 3 injections. Although cartilage volume increased over the follow-up period, this

returned to baseline by 96 weeks. The findings of the present study complement the existing clinical evidence for multiple MSC injections for knee OA. There are also interesting contradictions regarding the optimal dose of MSCs in the clinical treatment of knee OA. Some studies show the greatest benefits at the highest dose, ranging from 50 (Song *et al.*, 2018) to 100 million cells (Jo *et al.*, 2017), although this may also give rise to additional risks of pain, joint infection and swelling (Gupta *et al.*, 2016). Others have reported greater therapeutic benefits of low-dose MSC injections, where 10 million bone-marrow-derived MSCs outperformed 100 million (Lamo-Espinosa *et al.*, 2016) or 2 million adipose-derived MSCs outperformed 10 and 50 million (Pers *et al.*, 2016). The array of inconsistent clinical results raises uncertainty concerning the long-term benefits of periodic MSC treatment for OA.

Whether the failure of some clinical trials of MSC injections for OA is potentially due to their inability to alter the recruitment of inflammatory cell types is an interesting area for future investigation. Previous work suggests that intra-articular injection of MSCs in a mouse model of post-traumatic OA does alter the local inflammatory cell profile in the affected joint (Shu *et al.*, 2020). Interestingly, the effects of MSCs on structural OA pathology were not correlated with specific changes in synovial inflammatory cell populations but rather with the expression of synovial MMPs and ADAMTS. Separately, the changes in inflammatory cell populations including synovial macrophages and T-cells were associated with changes in pain response. Whether similar effects of MSCs on synovial inflammatory cells might be seen when administered in chronic or later-stage disease or in other OA phenotypes such as age-associated or metabolic OA requires further investigation.

It is important to keep in mind when interpreting the results of the present study that the experiments were conducted *in vitro* using isolated cell types in controlled biochemical environments and this may not recapitulate the complex *in vivo* milieu of an OA joint. Resident cell types, including synovial fibroblasts, articular chondrocytes, osteoblasts and other bone cells, immune cells as well as neurons, among others, all exert paracrine functions and act on each other in complex ways to modulate the OA joint environment. However, it is not possible to perform co-cultures with all relevant cell types in a single *in vitro* study. This limitation points to the need for physiologically relevant joint-on-a-chip models that may capture some of the features of a dynamic joint environment (Banh *et al.*, 2022). Moreover, due to the limited availability of primary patient-derived cells, the OA-HSFs used in the study were subjected to repeated passaging before use. Findings from a previous study show that OA-HSFs do retain a 'diseased' phenotype, with elevated expression of inflammatory cytokines, even after repeated passaging (Smith *et al.*, 2013). Despite the lack of other studies in the literature that compare phenotype changes in OA-HSFs between early and late passages, there is some limited evidence on the impact of repeated passaging on HSFs derived from joints with rheumatoid arthritis, with studies noting constitutive production of inflammatory and mitogenic cytokines after ~10 population doublings (Bucala *et al.*, 1991; Hirth *et al.*, 2001) as well as increased production of IL-6, IL-8, VEGF and PGE2 after repeated passaging (from passage 3 to 7 at 1:4 division) (Park *et al.*, 2012). The possible phenotypic changes in HSFs derived from joint diseases following *in vitro* culture should be considered in future studies.

The present study has the prospect of providing an exciting opportunity of improving the therapeutic efficacy of MSCs through priming techniques – such as by exposing the MSCs to hypoxia (Pattappa *et al.*, 2020) or compounds, including vitamin E (Bhatti *et al.*, 2017) – which have led to improved treatment effects in OA *in vivo* models. It is worth mentioning that *ex vivo* priming of MSCs with inflammatory mediators has also been shown to enhance the expression of immunomodulatory proteins in MSCs (Najar *et al.*, 2018). However, inflammatory priming typically exposes the MSCs only to an initial period of inflammatory stimulus. The present study data suggested that when MSCs were chronically exposed to the inflammatory milieu instead of short-term priming, their expression profile changed to a chronic inflammatory or catabolic state, which caused the MSCs to lose their therapeutic benefits in the long term. An additional consideration is that *in vitro* priming with one or even a few cytokines is different from the *in vivo* OA milieu, which presents a plethora of pro-inflammatory but also anti-inflammatory mediators. The co-culture model established using OA-HSFs and MSCs in the present study allowed for a better reflection of the complex multi-cytokine stimuli to which MSCs are exposed *in vivo* and the two-way crosstalk between cells that may cause chronic changes in the expression profiles of both cell types.

Conclusion

By investigating the responses of MSCs and OA cells in an *in vitro* co-culture model, the present study provided novel insights into how the paracrine functions of MSCs might be affected by the OA environment and the implications of this on the long-term therapeutic efficacy of single or multiple MSC injections for clinical OA treatment. The study findings suggested that the behaviour and therapeutic benefits of MSCs were significantly affected by the OA joint environment, with important implications for developing effective stem-cell-based OA treatments. The present study is one of a very selected few in OA research that has established an *in vitro* OA cell model to study the reciprocal effects of MSCs and OA tissue cells.

Availability of data and materials

The datasets used and/or analysed are available from the corresponding author on reasonable request.

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Editor's note: All the questions raised by the reviewers were answered in the manuscript, therefore there is no Discussion with Reviewers section for this paper.

The Scientific Editor responsible for this paper was Brian Johnstone.