European Cells and Materials Vol. 26 2013 (pages 80-90) DOI: 10.22203/eCM.v026a06 ISSN 1473-2262

INHIBITION OF ONCOSTATIN M IN OSTEOARTHRITIC SYNOVIAL FLUID ENHANCES GAG PRODUCTION IN OSTEOARTHRITIC CARTILAGE REPAIR

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Abstract

Mediators in the synovial fluid are thought to play a major role in osteoarthritic cartilage turnover. The purpose of the current study was to investigate the role of oncostatin M (OSM) in osteoarthritis (OA) by evaluating the presence of the cytokine and its receptors in the OA joint and interfering with its activity in synovial fluid co-cultured with cartilage explants. OSM levels were increased in the synovial fluid of osteoarthritic patients compared to healthy donors. Immunohistochemistry confirmed the presence of both the leukaemia inhibitory factor (LIF) and OSM receptors for OSM throughout the whole depth of osteoarthritic cartilage and synovial tissue, whereas in healthy cartilage their presence seemed more restricted to the superficial zone. Blocking OSM activity, using an activity inhibiting antibody, in 25 % osteoarthritic synovial fluid added to OA cartilage explant cultures increased glycosaminoglycan (GAG) content from 18.6 mg/g to 24.3 mg/g ($P < 0.03$) and total production from 7.0 mg/g to 11.9 mg/g ($P < 0.003$). However, OSM exogenously added to cartilage explant cultures reflecting low and high concentrations in the synovial fluid (5 and 50 pg/mL) did not affect cartilage matrix turnover, suggesting that factors present in the synovial fluid act in concert with OSM to inhibit GAG production. The current study indicates the potential to enhance cartilage repair in osteoarthritis by modulating the joint environment by interfering with OSM activity.

Keywords: Oncostatin M; cartilage; osteoarthritis.

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Introduction

One of the key processes in osteoarthritis (OA) is loss of the articular cartilage extracellular matrix due to a shift of the balance between production and degradation. Increased synthetic activity is evident by clonal expansion of resident chondrocytes and increased intensity of the territorial matrix surrounding them. However, this cannot compensate for the generalised loss of proteoglycans in the extraterritorial matrix, leading to a net loss of cartilage matrix (Martel-Pelletier *et al.*, 2008). Soluble factors secreted from cartilage, synovial tissue and bone are known to play a role in the turnover of cartilage matrix, and many have been shown to both increase cartilage degeneration and inhibit the production of the extracellular matrix, which has been documented most extensively for interleukin-1 (IL-1) and tumour necrosis factor-α (TNFα) (Fernandes *et al.*, 2002; Goldring and Marcu, 2009; Goldring and Goldring, 2004; Martel-Pelletier *et al.*, 1999). Another soluble factor that may be important in affecting cartilage matrix integrity in OA is the pro-inflammatory cytokine oncostatin M (OSM). OSM, a cytokine from the interleukin-6 (IL-6) family, is secreted by osteoarthritic synovial tissue (Beekhuizen *et al.*, 2011) and is found in rheumatoid synovial fluid (Hui *et al.*, 1997). OSM is associated with multiple processes in the body, e.g. haematopoiesis, neural and liver development and cell proliferation (Miyajima *et al.*, 2000; Tanaka *et al.*, 2003; Tanaka and Umesaki, 2003) and in rheumatoid arthritis (RA), OSM is associated with bone erosion, synovial inflammation and fibrosis, and cartilage degeneration (Sims and Walsh, 2010; Walker *et al.*, 2010). The potential of OSM to degenerate cartilage and induce catabolic processes in chondrocytes was shown in different studies (Barksby *et al.*, 2006; Durigova *et al.*, 2011), although nothing is known about its effect on cartilage synthesis. In addition, OSM is known to synergise with catabolic cytokines such as IL-1 and TNFα in the induction of cartilage degeneration *in vitro* (Barksby *et al.*, 2006; Catterall *et al.*, 2001; Gilbert *et al.*, 2012; Hui *et al.*, 2001; Hui *et al.*, 2003). However, most pro-inflammatory cytokines, including OSM, have been demonstrated only at low levels in osteoarthritic synovial fluid (Hui *et al.*, 1997; Manicourt *et al.*, 2000; Okamoto *et al.*, 1997; Rutgers *et al.*, 2009), and it is not clear whether such interactions are relevant in OA-associated cartilage degeneration *in vivo*. Moreover, therapies targeting IL-1 and TNFα were shown to be ineffective in inhibiting

osteoarthritic cartilage loss, in contrast to their application in rheumatoid arthritis.

OSM signals through two different receptor complexes, the GP130/LIFRβ (LIFR) or the GP130/OSMRβ (OSMR) complex (Gearing *et al.*, 1992; Mosley *et al.*, 1996; Thoma *et al.*, 1994). The OSMR was demonstrated in a chondrocyte cell line and in synovial fibroblasts. In addition, OSM signalling is mediated through the protein kinase R signalling pathway in bovine chondrocytes (Gilbert *et al.*, 2012). Although both receptors for OSM have been shown in multiple tissues, e.g. liver and prostate with different expression in healthy and pathologic conditions (Royuela *et al.*, 2004; Znoyko *et al.*, 2005), the presence of the different receptors for OSM has not been studied in healthy or in osteoarthritic cartilage tissue *in vivo* (Rowan *et al.*, 2001).

The purpose of this study was to elucidate the role of OSM in OA and more specifically the effect of inhibiting OSM on cartilage integrity in OA. To this end, the levels of OSM in the synovial fluid of osteoarthritic patients were compared to healthy subjects, and the presence of receptors for OSM was evaluated in healthy and osteoarthritic joint tissue. Furthermore, the role of OSM in cartilage repair was studied by culture of OA cartilage explants with OA synovial fluid in the presence or absence of a blocking antibody against OSM. Finally, to determine whether OSM could directly affect cartilage repair, the effect of recombinant OSM on cartilage explants at concentrations found in OA synovial fluid was studied.

Materials and Methods

Harvesting of cartilage, synovial tissue and synovial fluid

Osteoarthritic cartilage was collected under sterile conditions from patients undergoing total knee arthroplasty for OA. Cartilage explants were harvested from the femoral condyles, cut into square pieces of approximately 1 x 1 mm and the wet weight was determined so glycosaminoglycan (GAG) release, content and production could be corrected for cartilage weight (Mastbergen *et al.*, 2005). No difference was made between damaged or more intact cartilage, for histology, tissue analysis nor explant cultures. Only the almost completely denuded bone areas were not used. From each donor, 6 cartilage explants were fixed immediately in 4 % formaldehyde for histological evaluation and 6 were wet weighted and stored at -20 °C for biochemical analysis at t = 0. The rest of the explants were used for culturing or cell isolation.

Osteoarthritic synovial fluid and synovial tissue was obtained during total knee arthroplasty, healthy synovial fluid and tissue were collected from *post mortem* donors within 24 h after death, only if the knee cartilage was macroscopically healthy. The synovial tissue was immediately fixed in 4 % formaldehyde for immunohistochemical analysis. Synovial fluid was stored at -80 °C.

To obtain OA chondrocytes, articular cartilage was cut and digested in collagenase 0.15 % (Worthington, Lakewood, NJ, USA) overnight at 37 °C. Subsequently, the chondrocytes were seeded in a monolayer at a cell density of 5000 cells/cm^2 and cultured in expansion medium containing Dulbecco's Modified Eagle Medium (DMEM), 10 % foetal bovine serum (FBS), 1 % penicillin/streptomycin (pen/strep) and 10 ng/mL basic fibroblast growth factor (bFGF; R&D Systems, Minneapolis, MN, USA). Chondrocytes were cultured until passage 2. Anonymous use of redundant tissue for research purposes is part of the standard treatment agreement with patients in our university hospital and according to the national guidelines 'code of conduct for the proper secondary use of human tissue' (van Diest, 2002).

Multiplex ELISA

The presence of OSM in synovial fluid was determined using multiplex ELISA, as part of a multiplex ELISA for several cytokines, as previous described (Beekhuizen *et al.*, 2011). Validation of the Multiplex ELISA was previously done by de Jager *et al*., showing excellent sensitivity and specificity (de Jager *et al.*, 2005; de Jager *et al.*, 2003). Synovial fluid was pre-treated with hyaluronidase for 15 min at 37 °C. Samples were spun down in a X-column (Costar 8169; Corning Inc, Corning, NY, USA) and dissolved in 0.1375 % high performance ELISA buffer (HPE)-Tween-20. The pre-treated synovial fluid was incubated with the coupled beads. After incubation with the biotinylated antibodies, samples were incubated with streptavidin-phycoerythrin (PE). The samples were measured and analysed using the Bio-Plex suspension system (Bio-Rad Laboratories, Hercules, CA, USA). The concentration of OSM in the synovial fluid was calculated using the standard curve and is expressed as pg/mL.

(Immuno)histochemistry

For (immuno)histochemistry, the cartilage and synovial tissue explants were fixed overnight in 4 % formaldehyde, embedded in paraffin and cut into 5 µm sections. To evaluate the cartilage degeneration grade (Pritzker *et al.*, 2006), a safranin-O and fast-green staining was used (Rosenberg, 1971). For LIFR staining (sc-659, C-19; Santa Cruz Biotechnologies, Santa Cruz, CA, USA), sections were incubated with polyclonal anti-rabbit LIFRβ (both cartilage and synovial tissue; concentration 0.67 µg/mL) for 1 h at room temperature (Royuela *et al.*, 2004; Znoyko *et al.*, 2005). The sections were incubated with anti-rabbit–horseradish peroxidase (HRP) (1.25 µg/mL; Dako, Glostrup, Denmark). Sections were developed by incubations with 3,3'diaminobenzidine (DAB) for 5 min. For OSMR staining, sections were incubated with polyclonal anti-goat OSMRβ (sc-8496, C-20; SantaCruz; for cartilage sections at 2.0 µg/mL and synovial tissue at 4.0 µg/mL) overnight. Sections were incubated with anti-goat-HRP (2.5 µg/mL, Dako) for 1 h. Finally, sections were developed by incubation with DAB. For the negative controls, adjacent sections of both healthy and osteoarthritic cartilage and synovial tissue were incubated with an isotype anti-rabbit IgG (Dako) for the LIFR or isotype anti-goat IgG (Santa Cruz Biotechnologies) for OSMR. As a positive control, human thymus sections were used.

To quantify the LIFR and OSMR staining, the percentage of positive cells in both cartilage and synovial

tissue sections was determined using semi-automatic software (Leica Microsystems, Rijswijk, The Netherlands). The cells were counted with a grid distance of 17.6 µm. All sections were blinded and at least 100 cells were counted per slide. In total at least 5 healthy and 5 OA donors were used for both cartilage and synovial tissue. For the cartilage explants, only the positive cells in the middle and deep layer were counted, due to the loss of the superficial layer in OA cartilage explants. For synovial tissue, all cells throughout the entire tissue were counted.

Culture of cartilage explants in OA synovial fluid

To evaluate the effect of OSM present in synovial fluid on cartilage metabolism, the activity of OSM in OA synovial fluid was blocked with an activity-inhibiting antibody. For these experiments the synovial fluid from multiple (± 8) OA donors were pooled to obtain sufficient amounts of synovial fluid for a total culture period of 14 d. In total 3 different pooled synovial fluids were used, at OSM concentrations between 25 and 40 pg/mL. The pooled synovial fluid was aliquoted and stored at -80 °C until use. Cartilage explants from 5 different donors (n = 6 per donor/per condition) were cultured in the presence or absence of 25 % pooled OA synovial fluid, at a final OSM concentration of 6.25 or 11.25 pg/mL (25 % OA synovial fluid dissolved in medium), in DMEM (Gibco BRL Life Technologies, Darmstadt, Germany) containing penicillin and streptomycin (Gibco), ascorbate-2-phosphate (Sigma-Aldrich, St Louis, MO, USA), Insulin Transferrin Selenium-X (ITS-X) (Gibco) and 50 µg/mL L-proline (Sigma) for 14 d. To block the effect of OSM, an activity-inhibiting antibody against OSM was used at a concentration of 10 µg/mL (MAB295; R&D, Minneapolis, MN, USA). Isotype IgG antibody (MAB002; R&D) and cultures with anti-OSM antibody only served as controls. The culture medium was renewed 3 times per week. For each medium change, fresh synovial fluid (25 %) and anti-OSM or isotype antibody was added to the culture medium. The supernatant was stored at -80 °C for further analysis. After 14 d of culture, one explant was fixed for histochemical analysis. The rest of the explants were stored at -20 °C for biochemical analysis.

Effect of OSM inhibition on PGE2 production by OSM stimulated OA chondrocytes

To assess whether the anti-OSM antibody was effectively blocking OSM activity, OA chondrocytes were cultured in the presence or absence of 2 ng/mL recombinant OSM, or 10 µg/mL OSM activity-inhibiting antibody (anti-OSM). As a control, unstimulated cells and an isotype IgG were used. The OA chondrocytes were seeded in a density of 42,000 cells per well for a total culture period of 3 d. After 3 d, the conditioned medium was stored. The inhibiting effect of anti-OSM was determined using a prostaglandin E2 (PGE2) ELISA. In total 1 OA donor was used with n = 4 per condition**.**

PGE2 analysis

PGE2 concentration in the conditioned medium of OSM stimulated OA chondrocytes was determined using a competitive PGE2 ELISA (ENZO Life Sciences, Antwerpen, Belgium), according to the manufacturer's protocol.

Culture of cartilage explants in the presence of recombinant OSM

OA cartilage explants from 4 different donors (n = 6 per donor/per condition) were cultured for 14 d in the presence or absence of recombinant OSM (295-OM; R&D) at 5 and 50 pg/mL, representing the lowest and the average concentration of OSM found in OA synovial fluid. As a positive control a concentration of 10 ng/mL, previously shown to have an effect on cartilage metabolism (Durigova *et al.*, 2008a; Durigova *et al.*, 2008b), was also included. Culture medium was renewed 3 times a week and stored at -80 °C for further analysis. One explant was fixed for further histochemical analysis. The remaining explants (n = 5) were stored at -20 °C for biochemical analysis.

Biochemistry

GAG release and total GAG content was quantified using the dimethylmethylene-blue (DMMB) spectrophotometric analysis described by Farndale *et al.* (1986). Cartilage explants were digested in 400 µL 2 % papain (Sigma) in 50 mM phosphate buffer, 2 mM N-acetylcysteine, and 2 mM Na_2-EDTA (pH 6.5) at 60 °C overnight. Two hundred µL of DMMB solution and 100 µL of medium sample or papain digest were mixed and absorbance read at 540 nm and 595 nm using a spectophotometer (Bio-Rad Laboratories). As reference, chondroitin sulphate C (Sigma) was used. Calculation of GAG production was performed using the following formula:

Table 1. Results of LIFR and OSMR staining in osteoarthritic and healthy cartilage and synovial tissue.

	Healthy (% ± SD)	Osteoarthritic (%± SD)	*P* value
Cartilage			
LIFR	73.0 ± 10.4	72.3 ± 21.9	1.00
OSMR	19.4 ± 16.5	24.4 ± 16.8	0.73
Synovial tissue			
LIFR	71.2 ± 4.3	86.3 ± 9.0	* 0.04
OSMR	0 ± 0	10.3 ± 12.9	* 0.04

Per condition 5 samples were scored using semi-automatic software. At least 100 cells were counted per section. Data is given in % ± SD of positive cells**.**

$$GAG_{production} = (GAG_{content\ day\ 14} - GAG_{content\ day\ zero}) + GAG_{cumulative\ release}$$

given in mg GAG/g cartilage.

Statistical analysis

Data are expressed as mean ± SD. SPSS 16.0 software (SPSS Inc. Chicago, IL, USA) was used for the statistical analysis. The data was tested for normality and Gaussian distribution. A Student's *t*-test was used to assess difference between the healthy and osteoarthritic synovial fluid samples. Univariate analysis of variance (ANOVA), with a randomised block design and a *post-hoc* Bonferroni was used to assess differences in the glycosaminoglycan parameters. Differences with a *P*-value less than 0.05 were considered statistically significant. Differences in the percentage of receptor-positive cells in cartilage and synovial tissue scored after immunohistochemistry were evaluated by a Mann-Whitney U test.

Results

Presence of OSM in OA synovial fluid

In the OA synovial fluid samples OSM was detected in 10 of the 32 samples (range 0-570 pg/mL; mean 39 pg/mL), whereas in the healthy samples only one of the 23 samples contained OSM (range 0-25 pg/mL; mean 1 pg/mL; $P < 0.001$) (Fig. 1).

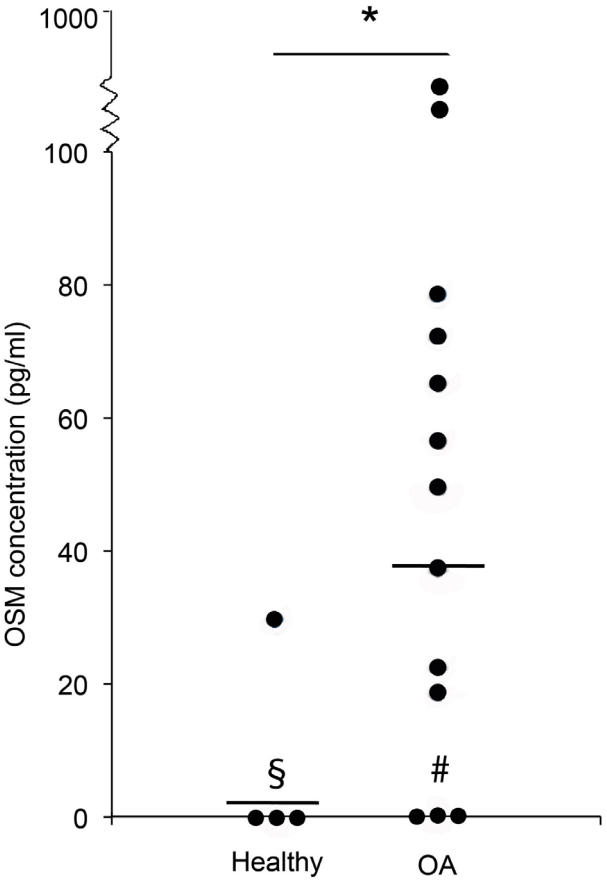

Fig. 1. OSM concentration in healthy (n = 24) and osteoarthritic (n = 32) synovial fluid samples. In healthy synovial fluid OSM was detected in only one sample (1.5 ± 6.8 pg/mL). In osteoarthritic synovial fluids OSM was detected in 10 out of 32 samples (39.7 ± 108.0 pg/mL; *$P < 0.001$). § = 24 samples, # = 22 samples.

Presence of receptors for OSM in joint tissue

The LIFR was present throughout the entire cartilage depth in both osteoarthritic and healthy cartilage (Fig. 2). In healthy cartilage, the OSMR was mainly expressed in chondrocytes in the superficial layer and some chondrocytes in the middle and deep layer. However, in osteoarthritic cartilage, positive chondrocytes were found throughout the entire cartilage (Fig. 3). As the superficial layer in OA cartilage was lost, only the middle and deep zones of healthy and OA tissues were quantified for the presence of LIF and OSM receptor-positive cells. No statistically significant difference between healthy and OA cartilage for LIFR nor OSMR staining was found (Table 1).

Synovial tissue of both healthy and osteoarthritic joints expressed the LIFR (Fig. 4), with positive cells mainly expressed in the synovial tissue lining. The expression of LIFR in OA synovial tissue was slightly higher compared with healthy synovial tissue ($P < 0.04$; Table 1). Furthermore, the OSMR was not expressed in any of the healthy synovial tissue donors (Fig. 4 and Table 1), whereas in the osteoarthritic synovial tissue multiple cells in the synovial lining expressed the OSMR ($P < 0.04$; Fig. 4 and Table 1).

Effect of blocking OSM in synovial fluid on cartilage

To study the role of OSM in osteoarthritic cartilage degeneration, OA cartilage explants were cultured in the presence of OA synovial fluid (the OSM concentration in the pooled synovial fluids ranged between 30 and 40 pg/mL; data not shown), in the presence or absence of an activity-inhibiting antibody against OSM (anti-OSM). Although addition of OA synovial fluid to cartilage explants for 14 d did not affect GAG content, release or production in cartilage explants (Fig. 5), blocking OSM in OA synovial fluid increased GAG content from 18.6 mg/g to 24.3 mg/g ($P < 0.03$) and production from 7.0 mg/g to 11.9 mg/g ($P < 0.003$) in cartilage explants (Fig. 5a,c). Blocking OSM in synovial fluid did not have an effect on GAG release (Fig. 5b). Anti-OSM did not affect any of the turnover parameters in cartilage explants in the absence of synovial fluid. The effectiveness of the antibody was shown in OSM-stimulated OA chondrocytes, where the effect of recombinant OSM on PGE2 production was completely abrogated by the anti-OSM antibody but not by the isotype control (Fig. 5d; $P < 0.001$). There was a basal production of PGE2 by the OA chondrocytes as shown by the unstimulated control.

Effect of recombinant OSM on OA cartilage

The effect of OSM alone on OA cartilage explants was studied by addition of recombinant OSM to OA cartilage explants. Only at a supraphysiological concentration of 10 ng/mL, but not at 5 or 50 pg/mL, GAG content decreased and GAG release increased (Fig. 6a/b; $P < 0.05$ and $P < 0.001$). No effect on total GAG production was noted irrespective of the concentration used (Fig. 6c).

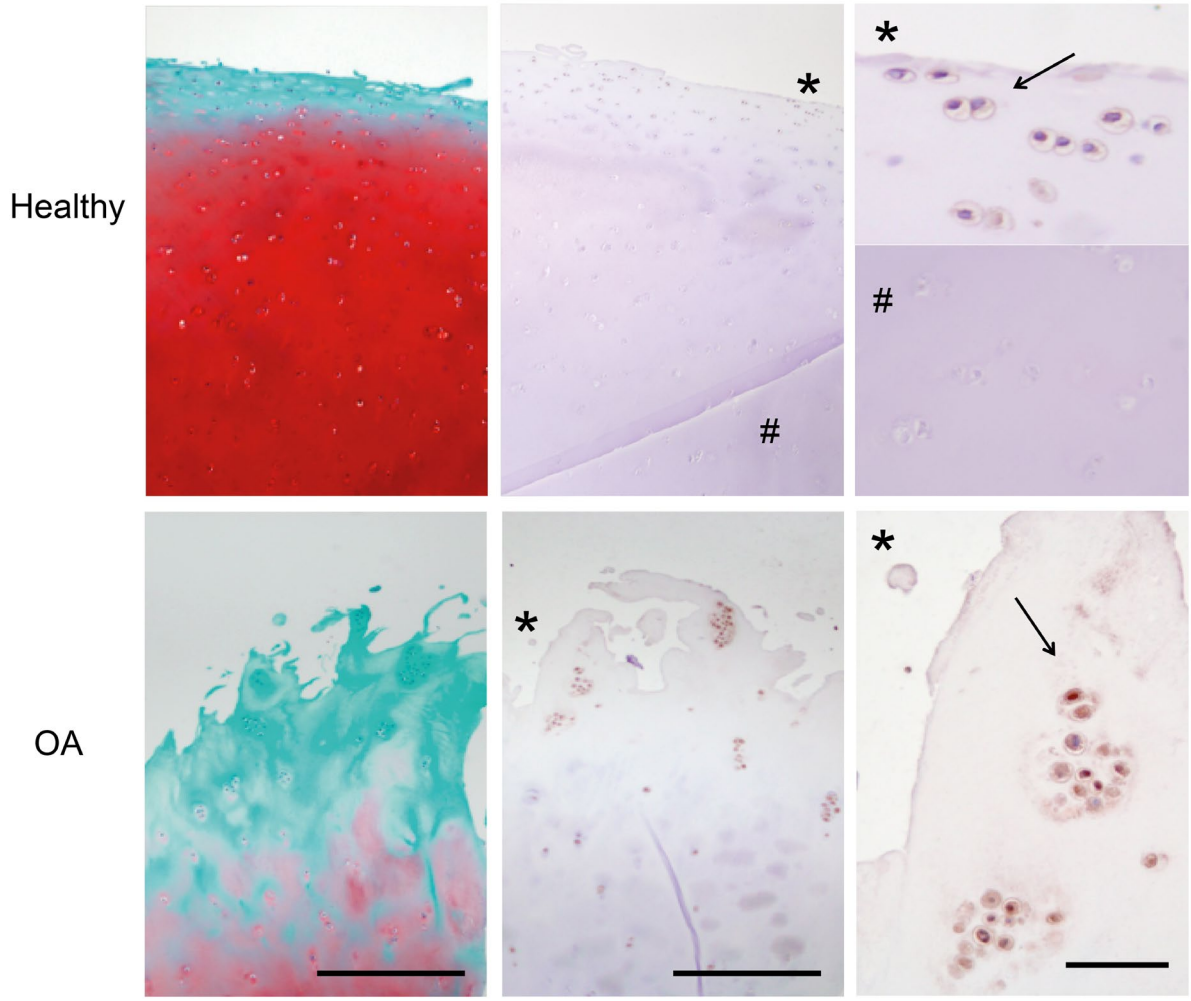

Fig. 2. Immunohistochemistry for LIFR in healthy (upper panels) and osteoarthritic cartilage (lower panels). LIFR is present in osteoarthritic cartilage throughout the entire cartilage (middle and right lower panels). In the healthy cartilage, only some cells in the superficial layer showed positive staining (middle and right upper panels). The left panels show the safranin-O staining of the healthy (upper left) and osteoarthritic cartilage (lower left). Positive cells stained brown by DAB and indicated by the arrow; scale bar represents 500 μm, or 40 μm (magnified right panels).

Discussion

The current study showed increased levels of OSM in osteoarthritic synovial fluid and expression of both receptors for OSM in cartilage and synovial tissue. Although the receptors were found both in healthy and OA joint tissue, their expression seemed to be more widespread in OA tissue. Addition of OA synovial fluid to cartilage explant cultures did not affect matrix turnover. However, when OSM was blocked in OA synovial fluid, cartilage repair was clearly enhanced. In contrast, recombinant OSM added to cartilage explants at the concentrations found in OA synovial fluid did not affect chondrocyte metabolism.

The role of OSM in rheumatoid arthritis and joint tissues other than cartilage has been extensively studied (Cawston *et al.*, 1998; Hui *et al.*, 2005; Nowell *et al.*, 2006; Walker *et al.*, 2010). OSM in combination with IL-1 or TNFα induced bone destruction *via* the RANK/RANKL pathway (Hui *et al.*, 2005). Moreover, OSM triggered angiogenesis and cell migration in synovial tissue (Fearon *et al.*, 2006). These changes are also known to occur in OA in synovial tissue (Felson, 2006). In RA, however, the levels of the pro-inflammatory cytokines IL-1, TNFα and OSM are high, in contrast to OA where almost no IL-1 or TNFα is present. Possibly, this might cause the different effects of OSM in RA compared to OA.

Receptors for OSM are found in multiple cell types in the joint, such as osteoblasts, synovial fibroblasts and chondrocytes, indicating that OSM could exert an effect on all tissues in the joint. Rowan *et al.* (2001) showed the presence of GP130/OSMRß complex, but failed to detect the GP130/LIFRß complex, in an immortalised chondrocyte cell line. Other studies showed the presence of the OSMR and LIFR in both osteoblasts (Allan *et al.*, 1990; Walker *et al.*, 2010) and synovial fibroblasts (Nowell *et al.*, 2006). However, OSM receptor expression in healthy and diseased joint tissue has never been reported before, let alone the existence of differences in expression between healthy and diseased tissue. Although OSM receptor expression was not evidently different in cartilage tissue,

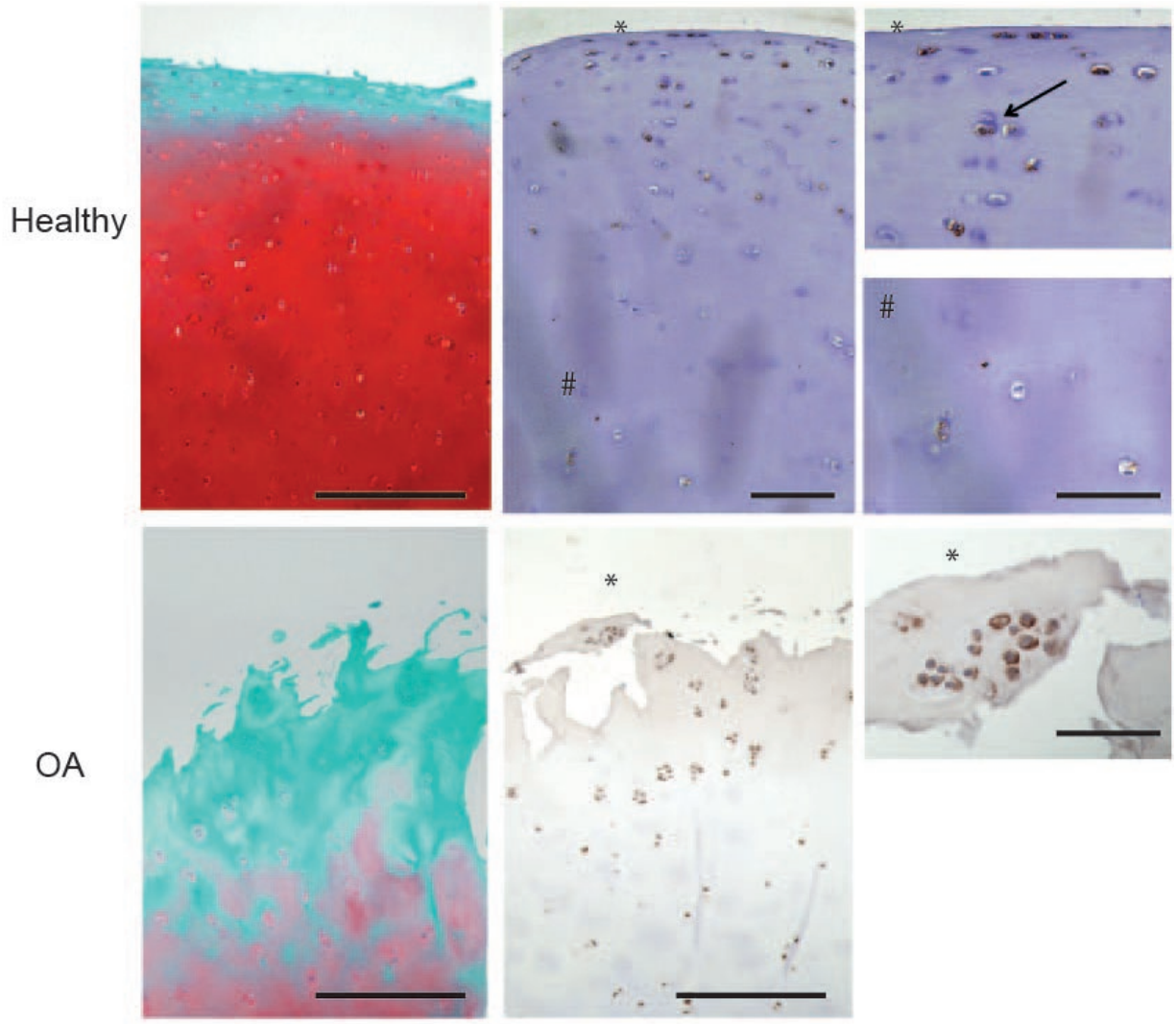

Fig. 3. Immunohistochemistry for OSMR in healthy (upper panels) and osteoarthritic cartilage (lower panels). OSMR is present in osteoarthritic cartilage throughout the entire cartilage (middle and right lower panels). In the healthy cartilage cells in the superficial layer showed positive staining and some cells in the middle/deep layer (middle and right upper panels). The left panels show the safranin-O staining of the healthy (upper left) and osteoarthritic cartilage (lower left). Positive cells stained brown by DAB and indicated by the arrow; scale bar represents 500 μm, or 40 μm (magnified right panels).

in synovial tissue the expression of the receptors for OSM was up-regulated in osteoarthritic samples, in contrast to healthy tissue where almost no cells were positive. This indicates an increased expression of the OSMR in diseased joint tissue, which may further enhance the effects induced by OSM. In addition, the LIFR was upregulated in osteoarthritic synovial tissue.

Studies have showed that gene activation through the LIFR and the OSMR are different. Whereas OSM normally signals through the LIFR and activates JAK/STAT transcriptional activators, OSM can also act *via* OSMR. However, the OSMR can also activate specific pathways through activation of the MAPK signalling cascade (Halfter *et al.*, 1999; Mosley *et al.*, 1996), suggesting that dependent on receptor expression, different responses, e.g. MMP activity and chondrocyte metabolism, could be generated in osteoarthritis. OSM is the only cytokine from the IL-6 family that can induce cartilage degeneration, albeit at non-physiologically high concentrations (Hui *et al.*, 2000; Hui *et al.*, 2003). This is most likely *via* the induction of matrix degrading proteases, e.g. MMPs and ADAMTS, which are known to be increased in OSM-stimulated cartilage explants or chondrocyte cultures (Durigova *et al.*, 2008a; Durigova *et al.*, 2008b; Durigova *et al.*, 2011; Gilbert *et al.*, 2012). Although the proteases involved downstream of OSM signalling were not further identified in the current study, stimulation of GAG release by the addition of a high concentration of OSM is in line with previous data.

In RA, OSM levels are high and in concert with high concentrations of IL-1 and TNFα known to induce degeneration and GAG release (Manicourt *et al.*, 2000). The use of neutralising OSM antibodies in two different models of murine RA was shown to partially prevent

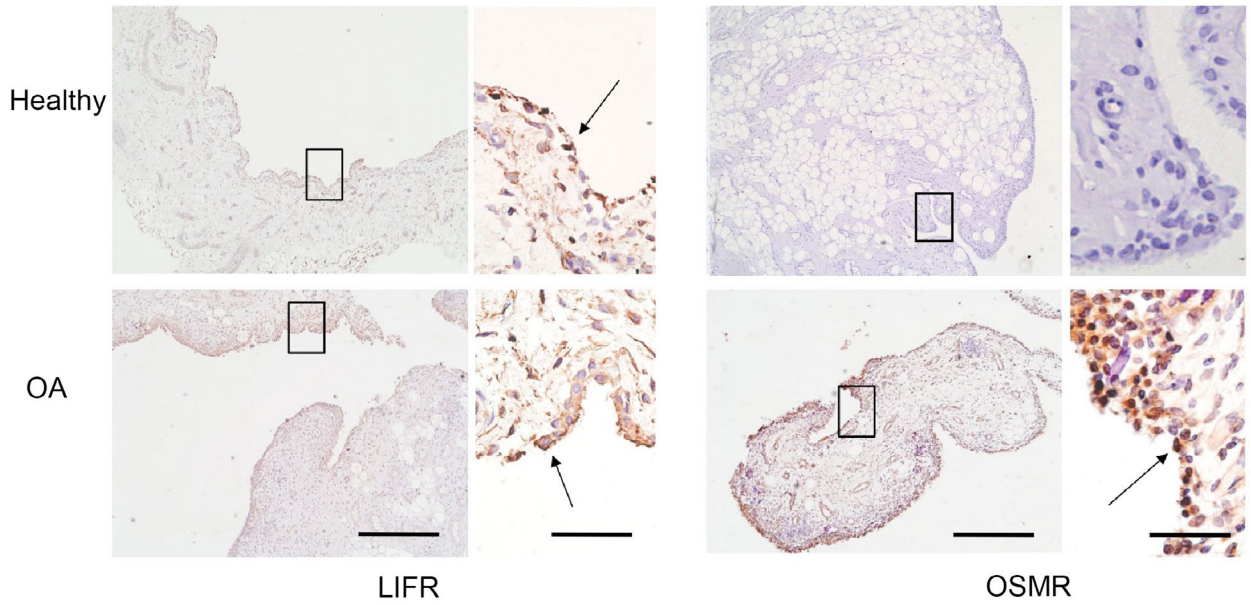

Fig. 4. Immunohistochemistry for LIFR and OSMR in healthy and osteoarthritic synovial tissue. LIFR is present in both healthy and osteoarthritic synovial tissue (left pictures). OSMR was abundantly expressed in osteoarthritic synovial tissue, however not in healthy synovial tissue (right pictures; positive cells stained brown by DAB and indicated by the arrow; scale bar represents 500 μm, or 20 μm in magnified panel).

cartilage destruction (Plater-Zyberk *et al.*, 2001). In addition, blockers of several cytokines binding the GP130 family of receptors, such as IL-6, are currently used in the clinic (Maini *et al.*, 2006; Nishimoto *et al.*, 2007). In particular, tocilizumab, a monoclonal antibody against the IL-6 receptor, was found to have a positive effect on disease symptoms and to decrease the levels of markers of bone and cartilage degeneration in patients with RA (Garnero *et al.*, 2010; Hashimoto *et al.*, 2011).

Despite the clear degenerative effect of OSM at high levels, its mode of action in osteoarthritis seems to be somewhat different. Levels of OSM, but also of TNFα and IL-1 are low in OA synovial fluid (Vignon *et al.*, 1993). Moreover, at the levels found in OA synovial fluid, OSM does not induce cartilage breakdown, which is in line with the observation that inhibition of the OSM present in OA synovial fluid does not inhibit GAG release. In contrast, the increased production and deposition of extracellular matrix found upon inhibition of OSM activity rather suggests an inhibitory effect of OSM on an anabolic factor(s) in the synovial fluid. In other studies, the precise role and effect of OSM on extracellular matrix production should be further investigated.

As this is an artificial *in vitro* model with only cartilage explants, we cannot exclude that the culture method (e.g. culture medium with high concentration insulin, no bone or synovial tissue present) may have partly masked the effect of inhibiting OSM. Future *in vivo* experiments will show if inhibiting OSM could stop OA degeneration in a more challenging and less controlled environment.

It should be borne in mind that in almost 70 % of the OA synovial fluid samples OSM was undetectable, and hence its role may be limited to those patients in which OSM is produced. The presence of OSM in some patients may suggest different (stages of) pathology. We have recently shown that OSM clusters with several other cytokines, such as IL-1β, IL-8 and IFNγ, suggesting it is part of a common pathway (Beekhuizen *et al.*, 2013), which should be further investigated. We observed, however, no association of OSM production with other aspects of pathology, such as synovial inflammation. However, we did not have access to the patient data and did not obtain synovial tissue of all OA patients, so we cannot state with certainty that this association did not exist. Still, as inhibition of these low concentrations already resulted in such clear effects on cartilage repair, it cannot be excluded that very low, but undetectable OSM levels were present in all donors and would still have resulted in increased matrix production upon inhibition. Even if this would not hold true, a 30 % reduction in OA disease morbidity would already have a huge impact on society in terms of disease burden. It is important to note that the synovial fluid could only be used at 25 %, due to its limited availability, suggesting that the enhancement of cartilage matrix production may have been even more pronounced with higher concentrations of synovial fluid.

Conclusions

This study shows that OSM plays a role in cartilage matrix turnover during osteoarthritis. By blocking OSM in the synovial fluid of OA patients, cartilage repair is improved in osteoarthritic cartilage. Supported by the lack of effect found for exogenously added OSM, these data indicate that OSM indirectly inhibits repair mechanisms in osteoarthritis. Moreover, the presence of receptors for OSM in both cartilage and synovial tissue suggests that blocking OSM could have an effect on multiple joint tissues and not just cartilage. The current study is one of the first that show that cartilage repair can be improved by altering the joint environment in osteoarthritis.

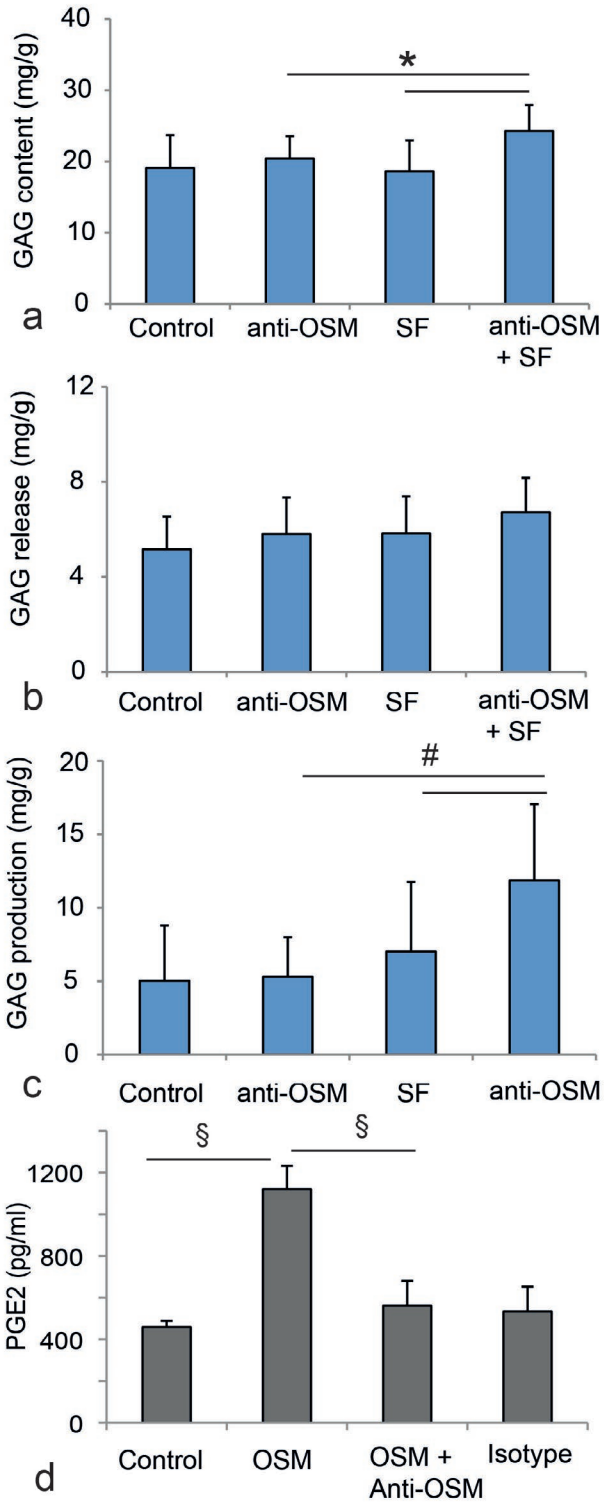

Fig. 5. Effect of blocking OSM in synovial fluid on osteoarthritic cartilage explants cultured for 14 d. Blocking OSM in osteoarthritic synovial fluid increased GAG content in cartilage (**a**; * $P < 0.03$) and increased GAG production (**c**; $^{\#}P < 0.003$), however, did not have an effect on GAG release (**b**). Addition of only synovial fluid did not have an effect on GAG content, release or production in osteoarthritic cartilage (mean ± SD of 5 different OA donors; 5 explants per donor/condition in mg GAG/g cartilage). Anti-OSM, at a concentration of 10 µg/mL, completely decreased the production of PGE2 induced by 2 ng/mL of recombinant OSM by OA chondrocytes (**d**; § $P < 0.001$; mean +/- SD of 1 donor; $n = 4$ per condition).

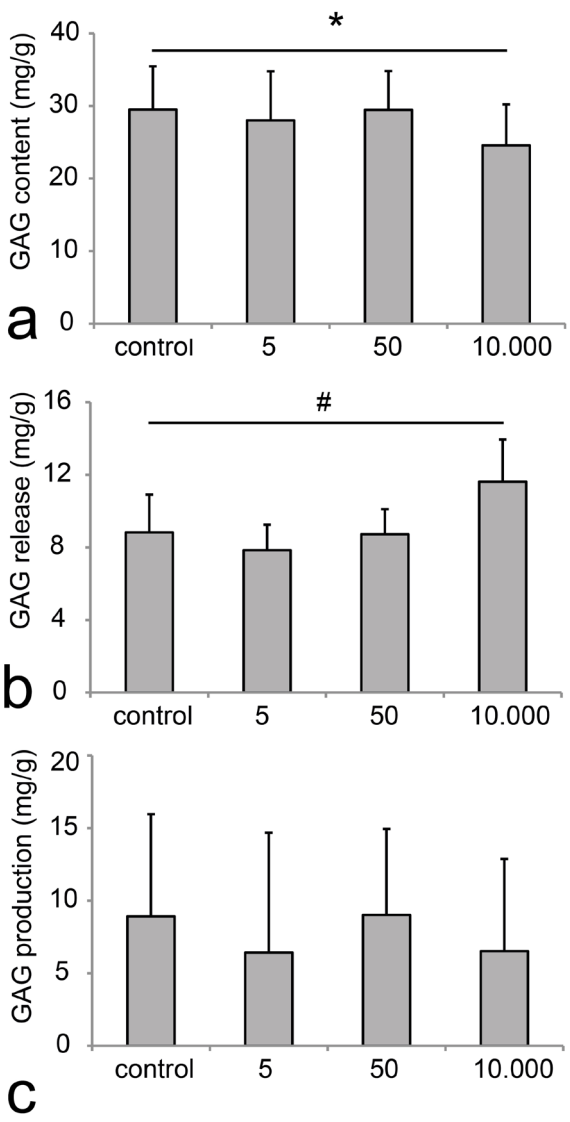

Fig. 6. Effect of recombinant OSM on osteoarthritic cartilage explants cultured for 14 d. At 10 ng/mL, recombinant OSM increased GAG release and decreased GAG content (**a,b**; * $P < 0.05$, $^{\#}P < 0.001$). However, no effect was seen at concentrations in the range of those found in OA synovial fluid (5 and 50 pg/mL). None of the concentrations of OSM had an effect on GAG production (**c**; mean +/- SD of 4 different OA donors; 5 explants/donor per condition in mg GAG/g cartilage).

Acknowledgements

This research forms part of the Project P2.02 OAcontrol of the research program of the BioMedical Materials institute, co-funded by the Dutch Ministry of Economic Affairs, Agriculture and Innovation. L.B. Creemers is funded by a research grant of the Dutch Arthritis Association. We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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Discussion with Reviewers

Reviewer I: How does a positive effect on GAG production and release *in vitro* equate to a positive effect on cartilage regeneration *in vivo*? Surely, cartilage regeneration depends on more than GAG production.

Authors: Our focus has been on GAG turnover as one of the major outcome parameters of cartilage repair, a commonly used readout parameter in cartilage research. We agree with the reviewer that there is more to cartilage repair than only GAG production. Collagen II production would also be very informative, but turnover of collagen is slow and hence differences in collagen content will be difficult to detect in biochemical analysis in such a short culture period. Whether any effect *in vitro* can be reproduced *in vivo* is always a matter of debate and we certainly agree that given the promising results *in vitro*, it would be interesting as a next step to investigate the effect of intra-articular anti-OSM in an experimental model of osteoarthritis *in vivo*.

Reviewer I: Does OA cartilage regenerate or repair?

Authors: The reviewer raises an important issue that possibly should also be raised in the cartilage community in general. According to the Biology online definition, regeneration is "The natural renewal of a structure, as of a lost tissue or part" and to the Oxford Dictionary "The formation of new animal or plant tissue". As cartilage tissue is present before start of the culturing of the explants (see Fig. 2), the correct term should indeed be repair of the osteoarthritic cartilage. Restoration of the articular surface *in vivo* would then involve both repair, i.e. of the existing cartilage, and regeneration, i.e. of the surfaces completely devoid of cartilage. Suboptimal or scar tissue could be considered as repair tissue as stated in the definition above, however scar tissue in the end is not functional. As the current study involves pre-existent OA cartilage, we refer to our findings as "repair".