Osteoarthritis and Cartilage 22 (2014) 264–274

Osteoarthritis and Cartilage

Pro-inflammatory stimulation of meniscus cells increases production of matrix metalloproteinases and additional catabolic factors involved in osteoarthritis pathogenesis

A.V. Stone †, R.F. Loeser ‡, K.S. Vanderman †, D.L. Long ‡, S.C. Clark †, C.M. Ferguson †*

† Department of Orthopaedic Surgery, Wake Forest School of Medicine, Medical Center Boulevard, Winston-Salem, NC 27157, USA
‡ Department of Internal Medicine, Section on Molecular Medicine, Wake Forest School of Medicine, Medical Center Boulevard, Winston-Salem, NC 27157, USA

ARTICLE INFO

Article history:
Received 27 February 2013
Accepted 9 November 2013

Keywords:
Meniscus
Osteoarthritis
Cytokine
Matrix metalloproteinase
MMP

SUMMARY

Objective: Meniscus injury increases the risk of osteoarthritis; however, the biologic mechanism remains unknown. We hypothesized that pro-inflammatory stimulation of meniscus would increase production of matrix-degrading enzymes, cytokines and chemokines which cause joint tissue destruction and could contribute to osteoarthritis development.
Design: Meniscus and cartilage tissue from healthy tissue donors and total knee arthroplasties (TKAs) was cultured. Primary cell cultures were stimulated with pro-inflammatory factors [IL-1β, IL-6, or fibronectin fragments (FnF)] and cellular responses were analyzed by real-time PCR, protein arrays and immunoblots. To determine if NF-κB was required for MMP production, meniscus cultures were treated with inflammatory factors with and without the NF-κB inhibitor, hypoestoxide.
Results: Normal and osteoarthritic meniscus cells increased their MMP secretion in response to stimulation, but specific patterns emerged that were unique to each stimulus with the greatest number of MMPs expressed in response to FnF. Meniscus collagen and connective tissue growth factor (CTGF) gene expression was reduced. Expression of cytokines (IL-1α, IL-1β, IL-6), chemokines (IL-8, CXCL1, CXCL2, CSF1) and components of the NF-κB and tumor necrosis factor (TNF) family were significantly increased. Cytokine and chemokine protein production was also increased by stimulation. When primary cell cultures were treated with hypoestoxide in conjunction with pro-inflammatory stimulation, p65 activation was reduced as were MMP-1 and MMP-3 production.
Conclusions: Pro-inflammatory stimulation of meniscus cells increased matrix metalloproteinase production and catabolic gene expression. The meniscus could have an active biologic role in osteoarthritis development following joint injury through increased production of cytokines, chemokines, and matrix-degrading enzymes.

Introduction

Meniscus injury is known to increase the risk of osteoarthritis. Untreated meniscus tears have an odds ratio of 5.7 for the development of radiographic osteoarthritis[1]. Even after partial meniscectomy, the relative risk (RR) for osteoarthritis increases following both degenerative tears (RR 7.0) and traumatic tears (RR 2.7)[2,3]. Successful repairs may lead to resumption of sports activity and decreased incidence of osteoarthritis[4]; however, many tears are not amenable to repair secondary to the tissue's minimal vasculature. This increased risk is historically attributed to changes in knee biomechanics due to meniscus deficiency[3,5,6].

The impact of cytokine stimulation on articular cartilage and subsequent extracellular matrix degradation is well documented[7–9]; however, the role of the meniscus in this process is unclear. The knee joint functions as an organ with a shared environment comprised of cartilage, synovium, ligaments and the meniscus. The meniscus is consequently exposed to inflammatory factors produced by knee tissues in response to acute or chronic injury and this exposure likely impacts meniscus biology. Certain aspects of meniscus biology are pathologically altered in meniscus injury and in the development of osteoarthritis[10–18]. Thus, the meniscus likely also has a biologic role in osteoarthritis development through the production of matrix-degrading enzymes and inflammatory

* Address correspondence and reprint requests to: C.M. Ferguson, Department of Orthopaedic Surgery, Wake Forest School of Medicine, Medical Center Boulevard, Winston-Salem, NC 27157, USA. Tel: 1-336-716-0000.
E-mail addresses: ausstone@wakehealth.edu (A.V. Stone), rloeser@wakehealth.edu (R.F. Loeser), kvanderm@wakehealth.edu (K.S. Vanderman), dllong@wakehealth.edu (D.L. Long), ferguson@wakehealth.edu (C.M. Ferguson).

1063-4584/$ – see front matter
http://dx.doi.org/10.1016/j.joca.2013.11.002

factors. We hypothesized that inflammatory factors associated with joint injury would stimulate menisci to increase production of matrix-degrading enzymes, cytokines and chemokines which could contribute to joint tissue destruction and subsequent development of osteoarthritis.

Materials and methods

Knee tissue acquisition

Our institutional review board approved this protocol. Normal human meniscus specimens ($n = 18$ menisci from $n = 18$ donors 25–65 years old) were procured through the National Disease and Research Interchange (NDRI, Philadelphia, PA) or the Gift of Hope Organ and Tissue Donor Network (Elmhurst, IL) while osteoarthritic menisci were obtained from patients undergoing total knee arthroplasty (TKA) for osteoarthritis ($n = 36$ menisci from $n = 36$ donors 44–83 years old). Synovial tissue was removed. Meniscus tissue was macroscopically graded according to a modified International Cartilage Research Society Cartilage Morphology Score (Table SI). All normal meniscus specimens were a grade zero or one, while all but one osteoarthritic meniscus was a grade three or four (one osteoarthritic meniscus received a morphology grade two). Articular cartilage from TKA bone cuts was processed as previously described[7]. All comparisons between chondrocytes and meniscus cells used tissue from the same donor.

Cell culture

Normal and osteoarthritic human meniscus and articular chondrocytes were isolated using our laboratory's tissue digestion and processing methods and primary cells cultured to confluence as described[7]. Prior to stimulation, primary cultures were incubated overnight in serum-free media (DMEM/F12) and then treated for either 6 or 24 h with one of the following: 10 ng/ml IL-1β; 10 ng/ml IL-6 with 25 ng/ml soluble IL-6 receptor; or TGF-α 20 ng/ml (all from R and D Systems) or fibronectin fragments (FnF), a recombinant fragment of fibronectin protein containing domains 7–10 of full length fibronectin (at 1 μM; gift from Harold Erickson, Duke University). For the NF-κB time course study, cells were stimulated with FnF (1 μM) for 15, 30, 45, 60, or 90 min with and without 30 min pretreatment with the NF-κB inhibitor, hypoestoxide (25 μM, Sigma). Cell lysates of nuclear and cytoplasmic fractions collected. Nuclear preparations were processed using the NE-PER fraction kit (Pierce Scientific) according to the manufacturer's instructions. For additional NF-κB studies, cell cultures were stimulated with cytokines at the aforementioned concentration with and without hypoestoxide (25 μM, Sigma) and cell lysates were collected and analyzed using immunoblot. Media was collected for MMP analysis and cells were harvested by scraping in either Trizol (Invitrogen) for RNA isolation or lysis buffer [lysis buffer (Cell Signal Technologies) plus Phosphatase Inhibitor Cocktail 2 (Sigma) and phenylmethanesulfonyl fluoride (Sigma)] for protein analysis.

Gene and protein analysis

RNA was quantified (Nanodrop, ThermoScientific) and verified (BioAnalyzer Chip, Agilent) to ensure high quality RNA (RIN > 6). The reverse-transcription PCR generated cDNA (RetroScript Kit, Ambion). Real-time PCR was performed using the Applied Biosystems 7900HT thermocycler with TaqMan Universal PCR MasterMix and TaqMan Gene Assay (Applied Biosystems: mmp1 Hs00899658_m1; mmp3 Hs00968305_m1; GAPDH Hs02758991_g1). Data was analyzed using the ΔΔCT method in Microsoft Excel (Microsoft).

For quantitative real-time PCR arrays, RNA was harvested as above and purified using the RNEasy Mini kit (Qiagen, #74104). The purified RNA was then used for the extracellular matrix and adhesion PCR array (SABiosciences, #PAHS-013ZA-12) or NF-κB target gene PCR array (SABiosciences, #PAHS-225ZA-12) and the manufacturer's optimized master-mix (SABiosciences, #330522) for the Applied Biosystems 7900HT thermocycler according to the manufacturer's protocol.

For protein analysis, cell media was loaded in equal volumes (1:1 in Lamelli Sample Buffer, 5% β-mercaptoethanol; BioRad), separated by SDS-PAGE (BioRad), transferred to nitrocellulose (Odyssey, Invitrogen) and probed with the primary antibody [anti-MMP1 (PAB12708, Abnova); anti-MMP3 (AB2963, Millipore); anti-MMP8 (MAB3316, Millipore); anti-MMP13 (AB84594, Abcam)] and secondary antibody (CellSignal). Blots were visualized with chemiluminescence (Amersham ECL, GE Life Sciences). Since no known control exists for meniscus secreted proteins, loading was controlled by loading an equal volume of media from wells that had equivalent cell numbers verified by total protein content. Media was analyzed with an MMP Protein Array (#AAH-MMP-1, RayBiotech) or the Cytokine Array (#AAH-CYT-5, RayBiotech). For the NF-κB experiments, immunoblots were probed for phosphorylated-p65, then stripped and probed for total-p65, and then finally β-actin as the loading control. For nuclear preparations, blots were also probed for Lamin B (a nuclear protein) and lactate dehydrogenase (a cytoplasmic protein) to demonstrate the integrity of the fractions. Processed films were imported into Photoshop v7.0 (Adobe) and labeled. Densitometry was completed with ImageJ 1.44p (NIH).

Statistical analysis

Statistical analysis was performed with SigmaPlot v10.0 (Systat Software) and Prism v5.02 (GraphPad Software, Inc.). Real-time PCR arrays were analyzed in Microsoft Excel (Microsoft) using the standard ΔΔCt method normalized to endogenous housekeeping genes in array-specific analysis templates (SABiosciences, http://www.sabiosciences.com/pcrarraydataanalysis.php). The template employed the Student's t test for replicates of four individual donors with significance of $P \leq 0.05$. We accepted this analysis method with the understanding that we did not account for multiple comparisons. A small number of genes may have been found to be significantly different because of the total number of genes analyzed; however, this limitation was accepted because we chose to analyze related genes of either extracellular matrix proteins or the NF-κB family and the arrays were used for hypothesis generation within targeted gene families rather than hypothesis testing for any individual gene.

The effects of cytokine stimulation on MMP-1 and MMP-3 gene expression in normal and osteoarthritic menisci were compared using a multivariate analysis of variance (MANOVA). *Post-hoc* tests were performed when group effects were found to be significant. A *post-hoc* two-tailed Dunnett's test was performed when appropriate to compare cytokine treatments to the unstimulated control, since we did not attempt to rank cell response to the different cytokine treatments.

Immunoblot densitometry was reported with the 95% confidence intervals and analyzed using ANOVAs. We reported Bonferroni corrections for multiple comparisons. Significance was set at $P \leq 0.05$.

Results

Response of normal meniscus to pro-inflammatory factors

Normal meniscus cell cultures were stimulated with pro-inflammatory factors to evaluate alterations in extracellular matrix

gene expression. Meniscus cells were stimulated with IL-1β, IL-6, or FnF. FnFs are found in the synovial fluid and extracellular matrix of arthritic joints and are known to induce cartilage degradation but have not been studied with meniscus[19–22]. The pro-inflammatory stimuli significantly increased expression of multiple matrix-degrading enzymes, including many of the primary MMPs responsible for degradation of both meniscus and cartilage matrix; however, the specific MMPs expressed varied according to the stimulus (Table I). All three stimuli increased expression of MMP-1, while IL-1β also stimulated MMP-2 and MMP-10 expression and IL-6 stimulated MMP-3 and ADAMTS1 expression. FnF produced the most significant increase in MMP-1 as well as MMP-2, MMP-3, MMP-8, MMP-10, and MMP-13. FnF also stimulated expression of the cell adhesion molecules VCAM-1 and α_1- and α_2-integrins, while IL-1β stimulated α_1- and β_1-integrin expression (Table I). IL-6 uniquely stimulated β2-integrin expression. Matrix proteins decreased by FnF include collagen VIα1, versican, thrombospondins-1 and -3 and connective tissue growth factor (CTGF; Table I) while collagen VIIα1 and lamininβ3 were increased. In contrast, IL-1β increased expression of catenins including α_1, β_1, and δ_2 as well as hyaluronan synthase-1 which was also increased by FnF. IL-6 uniquely downregulated collagen XVIα1 and versican and similar to FnF decreased thrombospondin-1. Genes on the array which did not have a significant change in response are shown in Table SII.

After identifying alterations in extracellular matrix gene expression, we examined changes in expression and production of selected MMPs that could be secreted and cause local tissue destruction. For this set of experiments, we also included stimulation with TGF-α. TGF-α is a less well studied cytokine in osteoarthritis pathogenesis, but is implicated in articular cartilage degradation[23,24]. We compared the effects of cytokine stimulation on MMP-1 and MMP-3 expression in normal and osteoarthritic meniscus cell cultures. Cytokine stimulation significantly increased mean MMP-1 ($P < 0.001$) and MMP-3 ($P = 0.006$) gene expression in meniscus cultures [Fig. 1(A)]. MMP-1 and MMP-3 gene expression was significantly greater at 24 h than 6 h (respectively $P = 0.014$ and $P = 0.005$), and for clarity, the 24 h time points are shown [Fig. 1(A)]. MMP-1 expression was significantly increased by IL-1β ($P = 0.020$), IL-6 ($P = 0.044$), and FnF ($P < 0.001$). FnF ($P = 0.001$) significantly increased MMP-3 expression, while the effects of IL-1β trended toward significance ($P = 0.061$). At the concentration tested, TGF-α did not significantly increase MMP-1 ($P = 0.998$) or MMP-3 ($P = 0.992$) gene expression. Normal meniscus cells demonstrated a greater increase in mean MMP-1 expression than osteoarthritic cells ($P = 0.007$). The increase in MMP-3 expression did not differ significantly between the two groups ($P = 0.135$). Osteoarthritic cell cultures secreted more MMP-1, MMP-2, and MMP-3 than normal meniscus cell cultures [Fig. 1(B)].

Matrix-degrading protein production in normal and osteoarthritic meniscus cells

Protein production of selected MMPs was evaluated by immunoblotting. The first set of normal primary meniscus cell cultures were stimulated with IL-1β, IL-6, or TGF-α (Fig. 2). Meniscus cells significantly increased MMP-1 production following stimulation by IL-1β [18.3 fold (−8.65 to 45.2)], IL-6 [24.1 fold (−8.61 to 56.7)], and TGF-α [5.78 fold (1.71–9.86)] (Fig. 2, $P = 0.0091$). MMP-3 was also significantly increased by stimulation with IL-1β [5.24 fold (−2.56 to 13.0)], IL-6 [3.70 fold (−0.47 to 7.86)], and TGF-α [2.46 fold (−0.59 to 5.52)] [Fig. 1(B), $P = 0.021$]; MMP-2 was used as a gel loading control since its levels in conditioned media were not found to change with stimulation.

Similar to the first set of experiments, FnF treated meniscus cultures exhibited increased MMP-1 and MMP-3 [Fig. 1(B)]. MMP-1 production significantly increased in response to IL-1β, IL-6 and FnF stimulation with respective fold increases of 17.1 (−21.7 to 55.9), 21.4 (−10.7 to 53.5), and 21.9 (−5.58 to 49.4) [Fig. 1(B), $P = 0.013$]. Stimulation increased MMP-3 as well: IL-1β, 2.76 fold (0.96–4.56); IL-6, 3.41 fold (0.52–6.31); and FnF, 3.45 fold (0.66–5.30) (Fig. 2, $P = 0.027$). Normal meniscus cells also produced MMP-13; however, the response only trended to statistical significance ($P = 0.095$).

Immunoblot analysis of osteoarthritis meniscus cell MMP production demonstrated significant responses to cytokine

Table I
Quantitative real-time PCR array for selected extracellular matrix related genes

Gene	Gene product	Fragmin		IL-1β		IL-6	
		P value	Fold change	*P* value	Fold change	*P* value	Fold change
ADAMTS1	ADAM metallopeptidase with thrombospondin type 1 motif, 1	0.7073	1.06	0.7195	−1.13	0.0152	2.81
COL16A1	Collagen, type XVI, alpha 1	0.0654	−1.61	0.9925	−1.04	0.0450	−2.06
COL6A1	Collagen, type VI, alpha 1	0.0398	−2.84	0.4808	−1.99	0.2551	−2.51
COL7A1	Collagen, type VII, alpha 1	0.0355	20.90	0.0686	19.37	0.2131	6.80
VCAN	Versican	0.0002	−5.35	0.4973	−1.28	0.0421	−1.89
CTGF	Connective tissue growth factor	0.0239	−12.85	0.2830	−5.55	0.1364	−5.31
CTNNA1	Catenin (cadherin-associated protein), alpha 1, 102 kDa	0.2049	1.18	0.0044	1.49	0.1672	1.36
CTNNB1	Catenin (cadherin-associated protein), beta 1, 88 kDa	0.8528	−1.01	0.0335	1.64	0.7983	−1.27
CTNND2	Catenin (cadherin-associated protein), delta 2 (neural plakophilin-related arm-repeat protein)	0.1555	2.87	0.0441	−2.44	0.9589	−1.38
HAS1	Hyaluronan synthase-1	0.0283	4.58	0.0374	7.64	0.2945	1.78
ITGA1	Integrin, alpha 1	0.0033	1.86	0.0309	2.12	0.9016	−1.30
ITGA2	Integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)	0.0452	4.90	0.0547	3.11	0.3196	1.46
ITGB1	Integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)	0.1927	1.36	0.0452	1.86	0.3471	1.27
ITGB2	Integrin, beta 2 (complement component 3 receptor 3 and 4 subunit)	0.2652	4.34	0.0839	5.92	0.0196	7.27
LAMB3	Laminin, beta 3	0.0279	4.98	0.0910	9.54	0.1558	2.39
MMP-1	Matrix metallopeptidase 1 (interstitial collagenase)	0.0000	27.56	0.0204	11.95	0.0064	15.42
MMP-10	Matrix metallopeptidase 10 (stromelysin 2)	0.0077	36.91	0.0234	18.83	0.0917	4.96
MMP-13	Matrix metallopeptidase 13 (collagenase 3)	0.0058	3.53	0.0856	4.08	0.1497	2.65
MMP-2	Matrix metallopeptidase 2 (gelatinase A, 72 kDa gelatinase, 72 kDa type IV collagenase)	0.0055	3.30	0.0290	3.09	0.0965	1.75
MMP-3	Matrix metallopeptidase 3 (stromelysin-1, progelatinase)	0.0000	11.92	0.0805	3.75	0.0344	4.84
MMP-8	Matrix metallopeptidase 8 (neutrophil collagenase)	0.0068	8.19	0.1595	3.46	0.3536	10.27
THBS1	Thrombospondin-1	0.0104	−4.14	0.1186	−2.89	0.0474	−2.70
THBS3	Thrombospondin 3	0.0021	−6.29	0.2002	−1.77	0.1384	−2.20
VCAM-1	Vascular cell adhesion molecule 1	0.0131	2.23	0.4385	1.50	0.3202	1.95

Highlighted cells indicate $P < 0.05$.

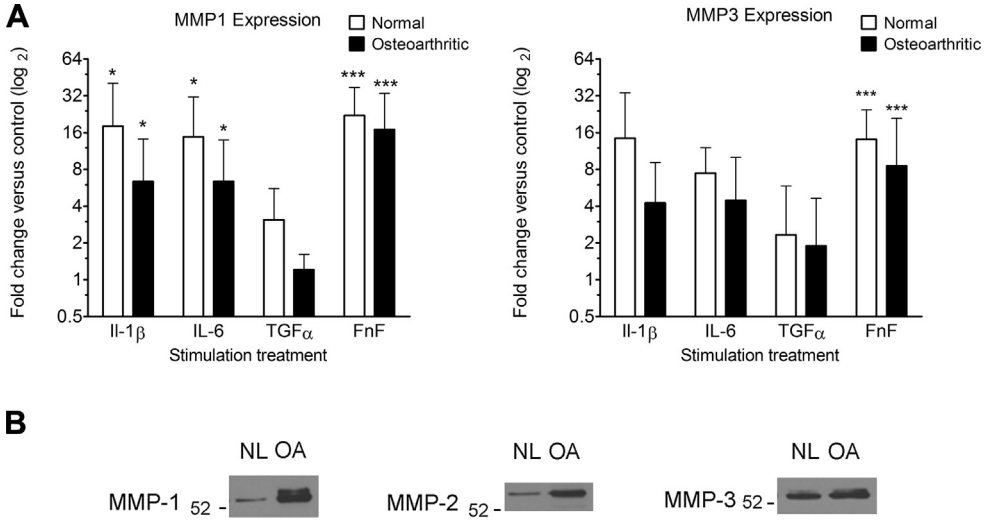

Fig. 1. Response of normal and osteoarthritic meniscus cells to pro-inflammatory stimulation. (A) *MMP-1 and MMP-3 gene expression in meniscus cells*. Primary normal and osteoarthritic cell cultures were stimulated with IL-1β (10 ng/ml), IL-6 (10 ng/ml plus 25 ng/ml sIL6R), TGF-α (20 ng/ml) or FnF (1 μM) and cells were harvested 24 h after stimulation (MMP-1, $n = 6$ normal and osteoarthritic unique donors; MMP-3, $n = 4$ normal and $n = 5$ osteoarthritic unique donors) [MMP-1: $^{*}P = 0.020$ (IL-1β), $P = 0.044$ (IL-6), $^{***}P < 0.001$ (FnF); MMP-3: $^{***}P < 0.001$ (FnF) significant increases vs unstimulated control]. All real-time PCR data was normalized to internal control (unstimulated) for accurate full change comparisons. Error bars represent 95% confidence intervals. (B) *MMP-1 and MMP-3 immunoblots from normal and osteoarthritic meniscus primary cultures* (representative blots from $n = 4$ unique donors). Conditioned media from unstimulated control samples from normal and osteoarthritic meniscus cultures was probed for MMP-1, MMP-2, and MMP-3.

stimulation. Densitometry measurements demonstrated significant MMP-1 increases of 1.43 (0.72–2.14), 1.65 (1.00–2.29), 1.40 (0.59–2.22) and 4.54 (−5.85 to 14.9) for IL-1β, IL-6, TGF-α and FnF stimulation, respectively ($P = 0.007$, $n = 5$ unique donors). MMP-3 increased significantly with 2.67 (0.42–4.93) change for IL-6 and 1.58 (1.03–2.14) for IL-1β, and increases of and 1.86 (0.81–2.91) for TGF-α and 1.13 (1.01–1.25) for FnF ($P = 0.001$, $n = 5$ unique donors). Subgroup analysis identified IL-6 as a more potent stimulus for MMP-1 and MMP-3 at the concentration tested ($P < 0.05$). MMP-8 production responded to cytokine stimulation but was more variable ($P = 0.108$) than MMP-1 and -3. All osteoarthritic menisci produced some MMPs without stimulation, but some severely osteoarthritic meniscus cultures were unable to be further stimulated to increase MMP production and were not included in the densitometry analysis ($n = 3$, grade 4; data not shown). Normal menisci increased their MMP-1 production in response to cytokine stimulation more than osteoarthritic menisci ($P = 0.003$), but MMP-3 production did not reach statistical significance ($P = 0.068$). Unlike normal menisci, cytokine stimulation did not increase MMP-13 production in osteoarthritic meniscus cells (Fig. 3).

Osteoarthritic meniscus cells were also compared to osteoarthritic chondrocytes obtained from the same donor to determine if the two cell types differed in their response to cytokine stimulation. As shown in the MMP protein arrays [Fig. 3(A)], human osteoarthritic meniscus cultures responded to cytokine stimulation with qualitative increases in secretion of MMP-1, MMP-3 and MMP-8. Osteoarthritic chondrocytes demonstrated a different MMP profile with greater MMP-13 production [Fig. 3(A)]. The array results were confirmed with immunoblots, which demonstrated that osteoarthritic menisci responded to IL-1β, IL-6 and TGF-α with increased MMP-1 and MMP-3 secretion [Fig. 3(B)]. While both osteoarthritic chondrocytes and menisci produced MMP-1 and MMP-3, chondrocytes qualitatively secreted more MMP-13 and ADAMTS-5 than osteoarthritic meniscus cells [Fig. 3(B)].

NF-κB pathway associated expression in normal meniscus cells

Since FnF increased the greatest number of genes in the extracellular-matrix array (Table I) and we previously demonstrated that FnF stimulated NF-κB pathway genes in chondrocytes[21], we selected FnF stimulation to evaluate the NF-κB family in meniscal cells. Twenty-six genes out of 84 on the NF-κB family array were significantly increased by FnF and only one, AGT, was decreased (Table II). FnF stimulation increased expression of NF-κB components (NFκB1, NFκB1A, and Rel) and many target genes, including cytokines (IL-1α and -1β, IL-6, and IL-8) and chemokines (CSF1, CXCL1, and CXCL2). FnF additionally increased the expression of both receptors and ligands in the tumor necrosis factor (TNF)-α family (CD40, Fas, LTB, TNFSF10 and TRAF2) as well as CD80 and CD83.

Treatment with FnF in the presence of the NF-κB inhibitor hypoestoxide significantly altered the expression of a number of genes. The chemokines C4A and CCL2 were decreased as were the transcription factors STAT3 and EGR2. FnF with hypoestoxide decreased expression of the enzymes MAP2K6, NQO1, NR4A2, and PLAU. The receptor expression for IL1R2 was decreased while IL2RA was increased. Additional gene alterations that were not statistically significant may be found in Table SIII.

Since FnF increased cytokine and chemokine gene expression in the NF-κB arrays, we used a protein array and tested conditioned media from FnF and cytokine treated cells to examine meniscus cytokine and chemokine production. Two different donors and exposures are shown to highlight the differences (Fig. 4). All three pro-inflammatory stimuli increased production of CXCL1, CXCL2, CXCL3 (identified by the GRO antibody), CXCL5, CCL8 (MCP-2), CCL7 (MCP-3), GM-CSF, and MIP-3α. FnF and IL-1β increased IL-6 and CCL2 production. FnF and IL-6 increased IL-1β, and MIP-1β. FnF increased IL-1α while IL-1β uniquely increased MIF, and finally IL-6 increased IL-7. Since the arrays contained antibodies to detect IL-1β and IL-6, it is unclear if they increased their respective production or the blots were detecting the cytokines added to stimulate the cells.

To further examine FnF stimulation of the NF-κB pathway, we assessed p65 phosphorylation following stimulation by FN-F as well as IL-1β + IL-6. Phosphorylation of p65 increased following treatment with the pro-inflammatory factors and the addition of the NF-κB inhibitor hypoestoxide reduced p65 phosphorylation following stimulation with FnF [Fig. 5(A)]. The overall level of

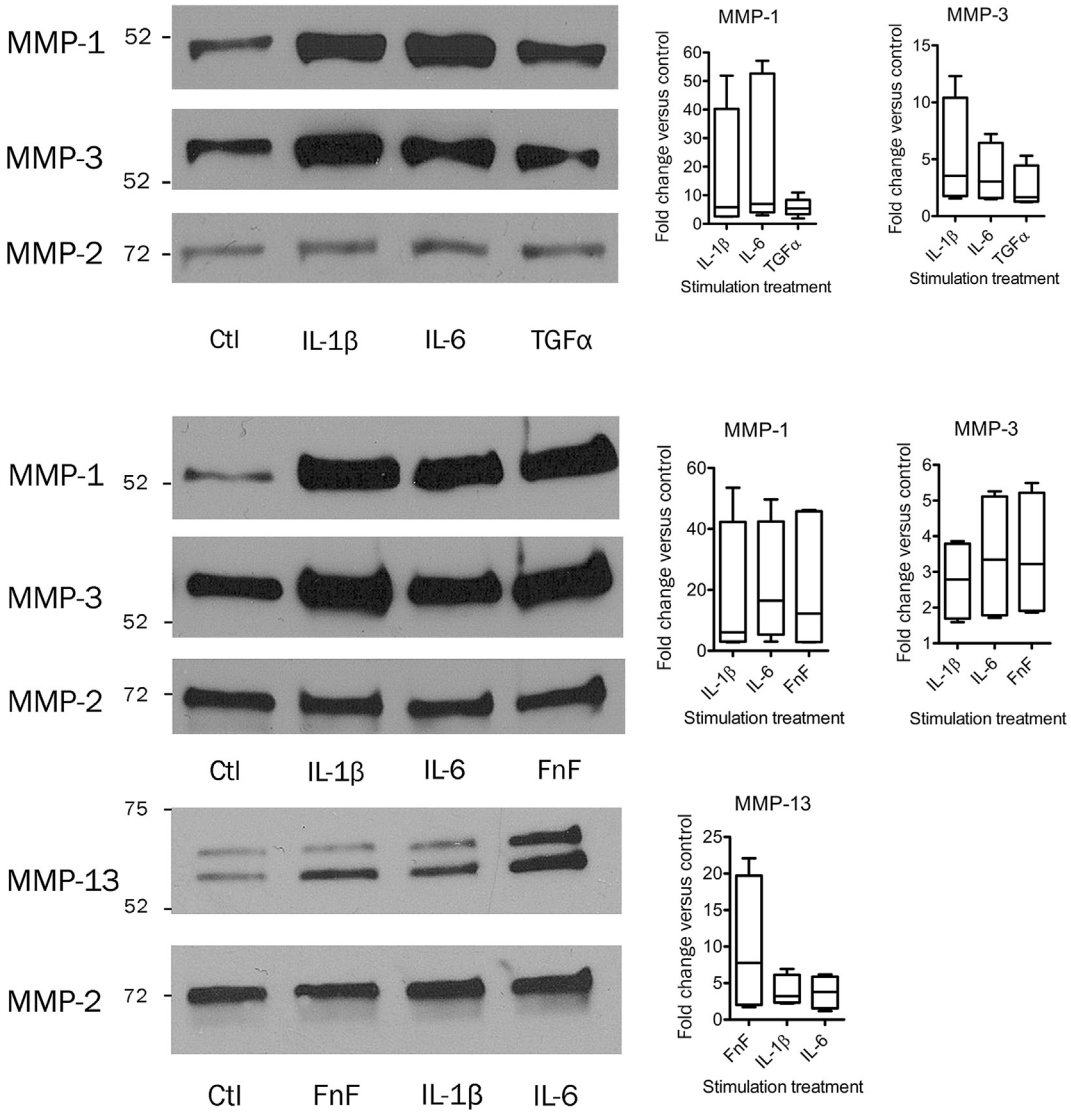

Fig. 2. MMP secretion from normal meniscus cells in response to cytokine stimulation. Normal meniscus primary cell cultures were stimulated with IL-1β (10 ng/ml), IL-6 (10 ng/ml plus 25 ng/ml sIL6R), TGF-α (20 ng/ml) or FnF (1 μM) ($n = 4$ unique donors) [mean increase in MMP-1 ($P = 0.013$); MMP-3 ($P = 0.013$)]. Cells were harvested 24 h after stimulation. Conditioned media was collected at 24 h after stimulation and immunoblotted for MMP-1, -3, or -13. MMP-2 levels did not change and served as an additional loading control. Densitometry analysis is shown at the right. Error bars represent 95% confidence intervals.

phosphorylated-p65 was statistically significant ($P = 0.007$), but hypoestoxide's effect on IL-1β in combination with IL-6 stimulation was more variable. Additional cell cultures were also harvested for RNA and conditioned media analysis after cytokine stimulation and hypoestoxide inhibition. Stimulation treatment significantly altered MMP-1 ($P < 0.001$) and MMP-3 ($P = 0.001$) expression [Fig. 5(B)]. Within the group, treatment with FnF or IL-1β + IL-6 significantly increased expression (MMP-1 $P < 0.01$ for both hypoestoxide groups; MMP-3 $P < 0.05$ for both hypoestoxide groups), while mean change following treatment in combination with hypoestoxide did not significantly differ from unstimulated control. The same trend was identified for MMP-1 and MMP-3 protein production [Fig. 5(C)].

The effect of pro-inflammatory mediators on stimulation of p65 was further characterized by performing a nuclear translocation analysis (Fig. 6). A time course experiment demonstrated a time dependent increase in FnF stimulated p65 phosphorylation in the cytosol and nucleus (Fig. 6). Importantly, the amount of phosphorylated-p65 in the nucleus increased over control cells peaking at 30 min and declining to basal levels by 90 min. Pretreatment with hypoestoxide reduced p65 phosphorylation and nuclear translocation (Fig. 6).

Discussion

The clinical importance of the meniscus in osteoarthritis development is well documented[1–6]; however, meniscus pathology in osteoarthritis is largely attributed to mechanically mediated loss of structural integrity[5,12,17,25,26]. These biomechanical stress factors may lead to "osteoarthritis in the meniscus" which is proposed to be responsible for meniscus MRI changes observed during the early osteoarthritis development[27]. Recent evidence suggests that the meniscus may have a more biologically active role in the complicated whole joint pathology of osteoarthritis[11,15,18,28,29]. Many of these studies use animal meniscus specimens and are limited in their translation to human osteoarthritis pathogenesis[30]. Our data using cultured human meniscal tissue expands upon previous gene expression reports[10,11,18] using RNA isolated from

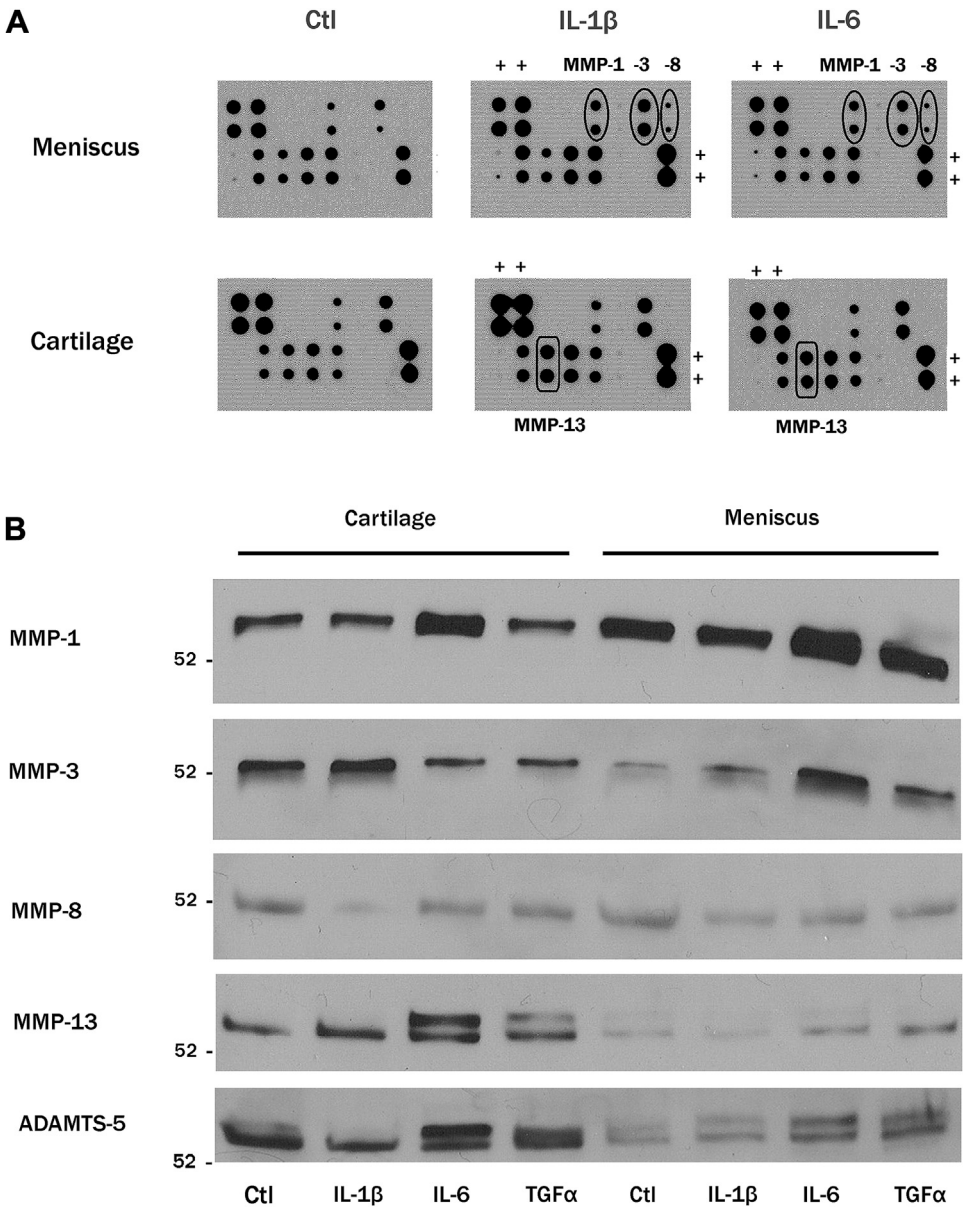

Fig. 3. Comparison of osteoarthritic meniscus and cartilage cells in response to cytokine stimulation. (A) Antibody MMP Array with conditioned media from osteoarthritic meniscal cells and chondrocytes following 24 h stimulation with *IL-1β (10 ng/ml) or IL-6 (10 ng/ml plus 25 ng/ml sIL6R)*. All protein arrays were developed simultaneously to enable direct comparisons and each protein on the array is presented in duplicate. ($n = 1$, + indicates positive control) (B) *MMP-1, -3, -8, and -13 and ADAMTS-5 production in osteoarthritic chondrocytes and meniscus cells*. Immunoblot analysis of conditioned media from unstimulated controls (Ctl) vs with IL-1β (10 ng/ml), IL-6 (10 ng/ml plus 25 ng/ml sIL6R), TGF-α (20 ng/ml) stimulated cultures ($n = 4$ matched donors).

normal and osteoarthritic human meniscus and further support a role for meniscus involvement in osteoarthritis pathogenesis.

The first objective was to identify extracellular matrix and MMP expression patterns in normal meniscus following pro-inflammatory stimulation. Early aberrations in cytokine signaling are believed to be responsible for propagating the reactive and degradative responses in joint tissues that ultimately lead to osteoarthritis[7–10,18,28,31,32]. Normal meniscus cells stimulated with pro-inflammatory factors vigorously increased their catabolic factor expression and protein production. The pattern of normal meniscus cell MMP production was consistent with that of osteoarthritic meniscus cells, although the diseased cells were less dynamic in their response. Normal meniscus cells were highly responsive to FnF, IL-1β and IL-6, while osteoarthritic menisci were more responsive to IL-6 then IL-1β at the concentrations tested. Normal meniscus cells also responded more quickly to stimulation than osteoarthritic meniscus cells, as evidenced by significantly greater increases in MMP expression at 6 h after stimulation than the osteoarthritic cells. This difference could be related to alterations in receptor density or inflammatory pathways in osteoarthritic cells. Meniscus cells produced a complementary pattern of MMP production to osteoarthritic chondrocytes in response to pro-inflammatory stimulation.

Alterations of MMP expression are important in osteoarthritis development and progression. MMP-1 degrades collagen type I which is the primary constituent of meniscal extracellular matrix[33]. Increased MMP-1 activity may damage the structural integrity of the meniscus. MMP-3 (stromelysin-1) production is similarly important because it is upregulated in articular cartilage in early osteoarthritis[9,31]. MMP-3 cleaves multiple matrix proteins and

Table II
Quantitative real-time PCR array for NF-κB family genes and targets

Gene	Gene product	FnF		FnF + HE		FnF vs FnF + HE	
		P value	Fold change	*P* value	Fold change	*P* value	Fold change
AGT	Angiotensinogen (serpin peptidase inhibitor, clade A, member 8)	0.0106	−3.12	0.0015	−5.16	0.2088	−1.65
BCL2A1	BCL2-related protein A1	0.0142	11.18	0.3721	1.40	0.0289	−7.99
BIRC3	Baculoviral IAP repeat containing 3	0.0364	38.12	0.4267	1.65	0.0393	−23.12
C4A	Complement component 4A (Rodgers blood group)	0.1233	−1.56	0.0006	−3.03	0.1131	−1.95
CCL2	Chemokine (C−C motif) ligand 2	0.6597	1.27	0.0097	−23.34	0.0002	−29.67
CCND1	Cyclin D1	0.0050	2.25	0.3924	−1.58	0.0024	−3.56
CD40	CD40 molecule, TNF receptor superfamily member 5	0.0308	1.73	0.4231	1.30	0.9873	−1.33
CD80	CD80 molecule	0.0039	6.51	0.3328	1.79	0.0089	−3.63
CD83	CD83 molecule	0.0019	5.50	0.1925	1.72	0.0048	−3.20
CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	0.0272	1.39	0.0003	2.59	0.0026	1.86
CFB	Complement factor B	0.0393	2.07	0.3574	−1.54	0.0044	−3.20
CSF1	Colony stimulating factor 1 (macrophage)	0.0378	6.08	0.6138	1.08	0.0410	−5.61
CXCL1	Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	0.0348	6.11	0.0882	−23.97	0.0276	−146.25
CXCL2	Chemokine (C-X-C motif) ligand 2	0.0295	13.10	0.0636	−17.20	0.0260	−225.32
EGFR	Epidermal growth factor receptor	0.5638	1.05	0.0004	−3.19	0.0250	−3.35
EGR2	Early growth response 2	0.0565	2.51	0.0118	4.55	0.2146	1.81
F3	Coagulation factor III (thromboplastin, tissue factor)	0.0065	14.89	0.0205	1.76	0.0082	−8.46
FAS	Fas (TNF receptor superfamily, member 6)	0.0136	2.64	0.9615	1.04	0.0120	−2.53
ICAM-1	Intercellular adhesion molecule 1	0.0015	8.72	0.0004	2.67	0.0052	−3.26
IL1A	Interleukin 1, alpha	0.0103	151.33	0.5790	1.17	0.0107	−129.68
IL1B	Interleukin 1, beta	0.0107	115.81	0.5342	−1.43	0.0145	−165.80
IL1R2	Interleukin 1 receptor, type II	0.2322	−2.48	0.0084	−6.14	0.2782	−2.48
IL1RN	Interleukin 1 receptor antagonist	0.0008	46.84	0.6667	1.07	0.0008	−43.62
IL2RA	Interleukin 2 receptor, alpha	0.1029	3.26	0.0250	2.44	0.3611	−1.34
IL-6	Interleukin 6 (interferon, beta 2)	0.0140	9.58	0.1011	−19.62	0.0095	−188.06
IL-8	Interleukin 8	0.0180	58.84	0.2360	4.10	0.0262	−14.38
IRF1	Interferon regulatory factor 1	0.0513	12.02	0.0067	2.51	0.0740	−4.80
LTB	Lymphotoxin beta (TNF superfamily, member 3)	0.0001	8.27	0.0067	−11.02	0.0000	−90.92
MAP2K6	Mitogen-activated protein kinase kinase 6	0.1348	−1.85	0.0103	−10.14	0.0493	−5.48
NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	0.0054	3.24	0.7798	−1.00	0.0094	−3.25
NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	0.0427	5.49	0.8604	−1.01	0.0400	−5.53
NQO1	NAD(P)H dehydrogenase, quinone 1	0.2707	1.85	0.0195	3.31	0.2403	1.79
NR4A2	Nuclear receptor subfamily 4, group A, member 2	0.1557	1.70	0.0089	−6.08	0.0066	−10.33
PDGFB	Platelet-derived growth factor beta polypeptide	0.5847	1.20	0.0576	−7.60	0.0208	−9.14
PLAU	Plasminogen activator, urokinase	0.1960	1.97	0.0380	−9.97	0.0115	−19.59
REL	V-rel reticuloendotheliosis viral oncogene homolog (avian)	0.0463	3.13	0.7859	1.06	0.0572	−2.95
RELA	V-rel reticuloendotheliosis viral oncogene homolog A (avian)	0.0526	2.51	0.2633	−1.46	0.0196	−3.67
SOD2	Superoxide dismutase 2, mitochondrial	0.0034	2.92	0.4788	1.16	0.0144	−2.51
STAT3	Signal transducer and activator of transcription 3 (acute-phase response factor)	0.8243	−1.13	0.0346	−2.20	0.1204	−1.94
TNFSF10	TNF (ligand) superfamily, member 10	0.0274	1.70	0.0013	−2.95	0.0024	−5.00
TRAF2	TNF receptor-associated factor 2	0.0201	3.73	0.0913	2.39	0.3195	−1.56

Highlighted cells indicate $P < 0.05$.

activates other MMPs, including MMP-1[31]. The disease processes we observed through up-regulation and production of MMPs are likely present in the intact meniscus. This conclusion is supported by studies demonstrating increased MMP-3 and aggrecanase production in immunohistochemical analysis of partial menisectomy specimens[15], increased MMP-1 activity, proteoglycan release and nitric oxide release following IL-1β treatment in healthy pig meniscus explants[34], and increased expression of ADAMTS and MMPs in ovine meniscus following cytokine stimulation[18]. Additional catabolic changes were identified with extracellular matrix analysis. A more dynamic gene response for MMP-8 was identified in normal meniscus cells, along with MMP-10. MMP-10 was reported in the fibrocartilaginous nucleus pulposus and was associated with increased gross and histological degeneration, pain, and increased IL-1 and substance P[35].

Pro-inflammatory stimulation also increased MMP-13 gene expression and production in normal meniscus cells. Our findings are consistent with a report of increased MMP-13 following IL-1α treatment in normal inner meniscus and articular chondrocytes[18]. Increased MMP-13 gene expression in stimulated normal meniscus cells is also congruent with reported MMP-13 expression in partial meniscectomy specimens[11], and the inner region of the meniscus would be expected to constitute the majority of cells in partial meniscectomy. The meniscus cell phenotype is reported to become increasingly chondrocytic in the inner zones of the meniscus[18,33,36]. The inner, avascular, region is likely the first section to deteriorate during the development of osteoarthritis and may explain in part why we did not see significant increases in MMP-13 production in our osteoarthritic meniscus cells which would likely be mainly from the outer region where MMP-1 predominates over MMP-13.

Pro-inflammatory factors also altered expression of cell adhesion proteins. Alterations in the meniscus integrin receptor expression would be expected to alter cell−matrix interactions as previously shown for chondrocytes[37] and is implicated in osteoarthritis pathogenesis[10]. Cell adhesion markers VCAM-1, ICAM-1 and E-selectin were also increased and were previously demonstrated to be present in hypertrophic and early osteoarthritic synovium and is involved in inflammatory cell recruitment to the synovium[32,38]. ICAM-1 was specifically identified as increased in early osteoarthritis, while VCAM-1 was shown to be predictive of joint replacement for severe arthritis[32,39]. Pharmacologic reductions of these molecules for early to mid-stage osteoarthritis of the knee was associated with improvements of pain and function[40].

Lymphotoxin β and GM-CSF were both increased and although they are primarily linked to rheumatoid arthritis, they have also been noted in the osteoarthritic synovium[41–43]. Future investigation

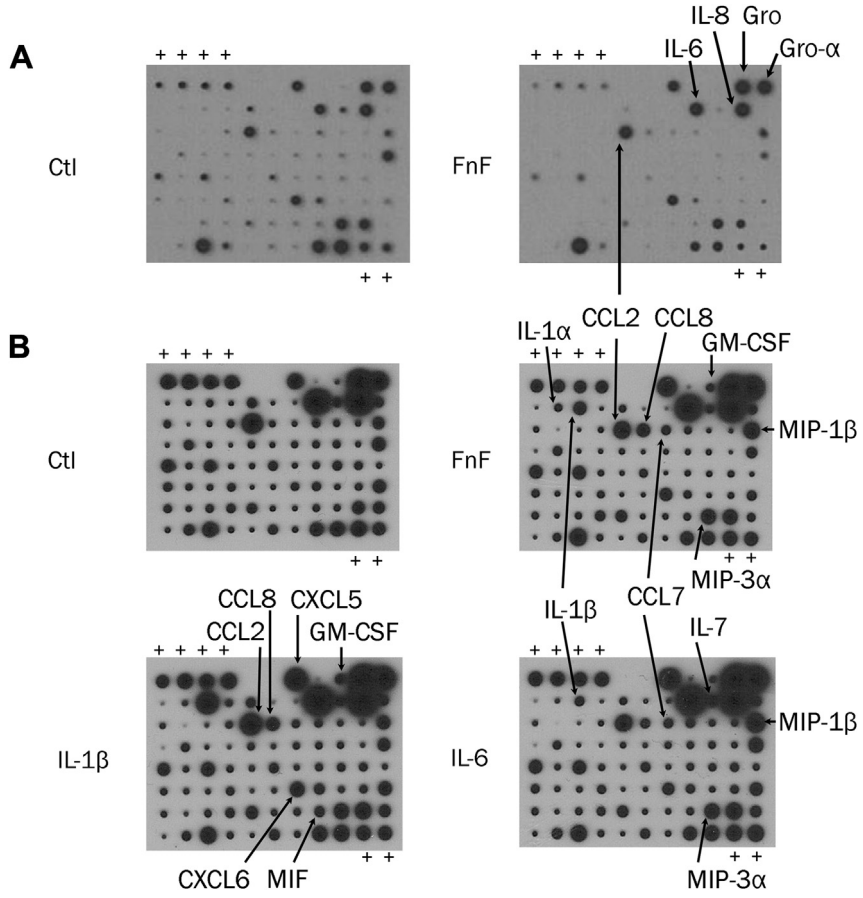

Fig. 4. Protein array of conditioned media from normal meniscus following pro-inflammatory stimulation. Conditioned media from normal meniscus cells 24 h after stimulation with either FnF (1 μM), IL-1β (10 ng/ml) or IL-6 (10 ng/ml plus 25 ng/ml sIL6R). The first donor is shown with shorter (A) exposure and the second donor with a longer exposure (B) to detect less abundant cytokines.

may link these cytokines to the more fibroblastic cell phenotype in the meniscus or to inflammatory cell recruitment. Catabolic expression was accompanied by a notable decrease in expression of the anabolic factor CTGF. CTGF was recently indentified in a rabbit model for promoting collagen production and healing of meniscus defects[44]. The combination of abnormal cell recruitment and decreased anabolic factors could easily compromise wound healing.

Meniscus cells can be stimulated to produce matrix-degrading enzymes which could impact neighboring cartilage matrix, but the tissue interaction is likely part of a more dynamic signaling network. In addition to the catabolic factors above, meniscus responded to pro-inflammatory factors with increases in cytokine and chemokine expression and production in a manner similar to chondrocytes[22]. Multiple interleukins, including IL-1β and IL-6 that were used in our stimulation experiments, were increased in both expression and production. IL-1β was recently reported to be increased in osteoarthritic synovial fluid[45]. Additionally, treatment of articular chondrocytes and meniscus explants with IL-1α and IL-1β was found to increase cartilage and meniscus catabolic activity through increased MMP activity and nitric oxide release[45]. Chemokines CXCL1, CXCL2, CXCL3, CCL8 (MCP-2), CCL7 (MCP-3), and CXCL6 (GCP-2) were increased and may contribute to the development of inappropriate inflammatory cycles after injury[9,28]. Our results support recently reported findings in an analysis of gene expression in meniscus tears, which found increased expression of IL-1β, ADAMTS-5, MMP-1, MMP-9, MMP-13, and NFkB2 in patients with meniscus tears younger than 40[11]. Cytokine and chemokine expression (including IL-1β, TNF-α, MMP-13, CCL3, and CCL3L1) were greater in patients with a meniscus tear and concomitant ACL tear which indicates a more severe injury[11]. Furthermore, we identified a more expansive list of cytokine and chemokine alterations and proposed that these alterations are at least in part mediated by the NF-κB pathway.

The NF-κB pathway is well studied in osteoarthritic chondrocytes. FnF stimulation of NF-κB increases chondrocyte cytokine and chemokine production[9,22,28,46]. In meniscus cells, FnF and cytokine directed p65 phosphorylation suggests that the NF-κB pathway may be responsible for increased cytokine and chemokine production. Injured meniscus previously demonstrated elevated NF-κB phosphorylation identified by immunohistochemistry[16].

Increased production of inflammatory factors may act in both autocrine and paracrine fashion, but these may also act on surrounding tissues through the synovial fluid. This mechanism for joint destruction is supported by a number of studies identifying these factors as increased in the disease state and detailing their deleterious effects on cartilage, bone and the synovium[9,28,32], which would likely suppress reparative cell functions and propagate a loss of matrix integrity. Additionally, these findings may better explain the higher failures in meniscus repair in older patients[4,11,47]. Older patients with a previous meniscus injury are likely producing increased matrix-degrading enzymes as a function of both the initial injury and age, and both factors are likely to contribute to disease progression.

Our study carries common limitations of laboratory models. Primary cell culture was the most efficient and precise model to analyze both protein and RNA responses to stimulation; however,

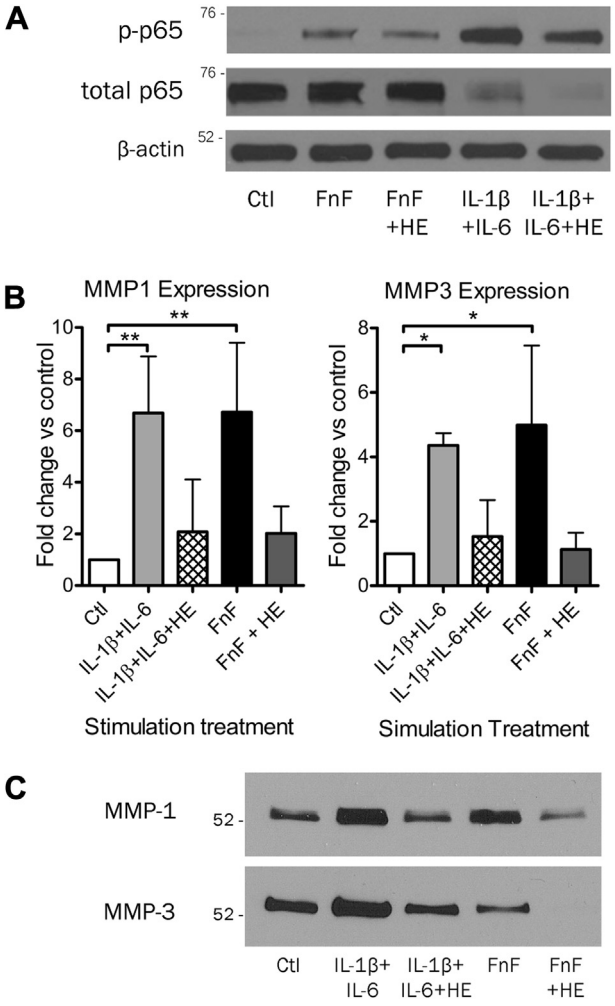

Fig. 5. Response of normal meniscus cells to pro-inflammatory factors with and without the NF-kB inhibitor hypoestoxide. Cells were stimulated for 30 min with either FnF (1 μM) or IL-1β (10 ng/ml) and IL-6 (10 ng/ml plus 25 ng/ml sIL6R) with or without hypoestoxide (HE, 25 μM), and cell lysates prepared. Lysates were then probed for phosphorylated-p65 (active form). Immunoblots were then probed for total-p65 and followed by β-actin as the loading control ($n = 5$ individual donors). Densitometric analysis identified significant increases in p65 phosphorylation ($P = 0.007$). The blots were stripped and re-probed for total-p65 and β-actin. Total-p65 was present in lanes with minimal phospho-p65. (B) Cells were harvested for RNA collection 24 h after stimulation with either with IL-1β (10 ng/ml) and IL-6 (10 ng/ml plus 25 ng/ml sIL6R) or FnF (1 μM) with and without the inhibitor hypoestoxide (HE, 25 μM). All real-time PCR data normalized to internal control (unstimulated cells) for accurate fold change comparisons. MMP-1 and MMP-3 expression significantly changed with treatment groups [MMP-1 overall $P < 0.001$, $^{**}P = 0.01$; MMP-3 overall $P = 0.001$; $^{*}P = 0.04$]. Error bars represent 95% confidence intervals ($n = 5$ unique donors). (C) The conditioned media was also probed for MMP production ($n = 5$ unique donors).

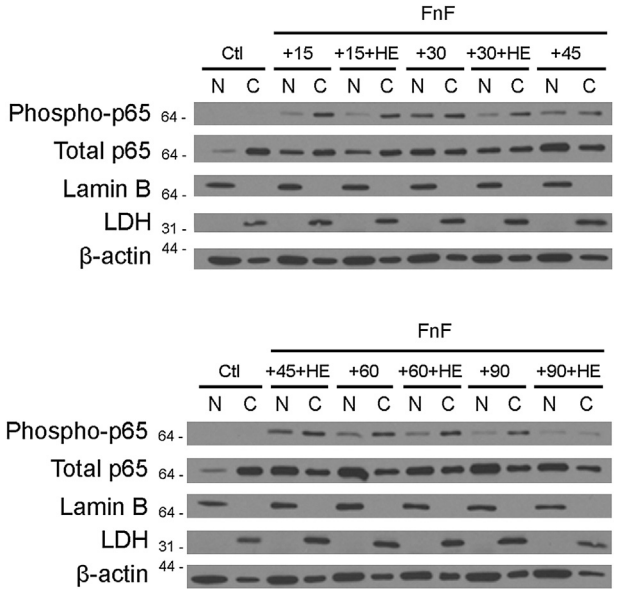

Fig. 6. Effect of fibronectin fragment stimulation on nuclear translocation of p65 in normal meniscus cell culture. Time course analysis at 15, 30, 45, 60, and 90 min demonstrating nuclear (N) and cytoplasmic (C) fractions of FnF (1 μM) treated cells with and without hypoestoxide (HE) pretreatment. Nuclear and cytoplasmic cell fractions were immunoblotted for phosphorylated-p65 (active form), total-p65, Lamin B (nuclear protein marker), lactate dehydrogenase (LDH, cytosolic marker) and β-actin (total protein marker found in both fractions). Blots shown are representative of $n = 4$ unique donors.

our findings should be interpreted with the understanding that cell cultures may not directly mimic *in vivo* cell behavior. This study sought to identify cell alterations in normal meniscus tissue that may lead to the development of osteoarthritis. Future studies may further explore the NF-κB pathway as well as the role of MAP kinases and disease progression in an animal model, which was beyond the scope of this manuscript. Another limitation of the study is the inherent variability in the state of the meniscus disease at the time of specimen acquisition. TKAs are most frequently performed for the indication of pain and functional limitation from osteoarthritis, but the indication encompasses a range of tissue destruction ranging from moderate to severe cartilage eburnation and meniscus degradation. The larger standard deviation in MMP expression and production in osteoarthritic tissue relative to normal may be partially attributable to the varied disease state. We opted to examine the entire cell population in the meniscus to elucidate differences between the normal meniscus and the osteoarthritis disease state. Additional studies have examined the differences in meniscus cell type[18,30], so we believe our characterization of normal and osteoarthritis human meniscus may add to a better understanding of osteoarthritis pathogenesis following meniscal injury.

The role of the meniscus in osteoarthritis likely extends beyond the mechanical compromise of the meniscus structure to encompass biologic interactions. Meniscus secretion of inflammatory factors and matrix-degrading enzymes likely contributes to the development of pathology. While the full cell mechanism was not characterized, we believe that the increased expression of MMPs, cytokines, and chemokines in response to pro-inflammatory factors contributes to osteoarthritis pathogenesis in the meniscus and articular cartilage. The ultimate goal of this research is to identify factors contributing to early pathology in an effort to prevent, or at least attenuate, the development of osteoarthritis.

Author contributions

Stone: Conception and design, analysis and interpretation of the data, drafting of the article, critical revision of the article for important intellectual content, final approval of the article, obtaining of funding, collection and assembly of data.

Loeser: Conception and design, analysis and interpretation of the data, critical revision of the article for important intellectual content, final approval of the article, obtaining of funding.

Vanderman: Critical revision of the article for important intellectual content, final approval of the article, collection and assembly of data.

Long: Conception and design, analysis and interpretation of the data, critical revision of the article for important intellectual content, final approval of the article.

Clark: Critical revision of the article for important intellectual content, final approval of the article, collection and assembly of data.

Ferguson: Conception and design, analysis and interpretation of the data, critical revision of the article for important intellectual content, final approval of the article, obtaining of funding.

Sources of funding

This study was funded by the Young Investigator Grant from the American Orthopaedic Society for Sports Medicine (Stone) and the Clinician Scientist grant from the Orthopaedic Research and Education Foundation (Stone). Additional support was received from the NIH/NIAMS K08AR059172 (Ferguson) and NIH/NIAMS R37 AR049003 (Loeser).

Conflict of interests

The authors have no competing interests to report. Funding sources disclosed above.

Acknowledgments

The authors would like to expressly thank Michael Callahan, PhD, Raghunatha Yammani, PhD, and Mark Van Dyke, PhD for their contributions to this project. We would also like to thank the Wake Forest School of Medicine Orthopaedic Surgery Joint Service for their assistance in meniscus specimen acquisition. We would like to thank the National Disease and Research Interchange (NDRI) and Drs Susan Chubinskaya and Arkady Margulis at Rush Medical Center for procuring meniscus from the Gift of Hope Organ and Tissue Donor Network.

Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.joca.2013.11.002.

References

1. Englund M, Guermazi A, Roemer FW, Aliabadi P, Yang M, Lewis CE, *et al*. Meniscal tear in knees without surgery and the development of radiographic osteoarthritis among middle-aged and elderly persons: the Multicenter Osteoarthritis Study. Arthritis Rheum 2009;60(3):831–9.
2. Roos H, Lauren M, Adalberth T, Roos EM, Jonsson K, Lohmander LS. Knee osteoarthritis after meniscectomy: prevalence of radiographic changes after twenty-one years, compared with matched controls. Arthritis Rheum 1998;41(4):687–93.
3. Englund M, Roos EM, Roos HP, Lohmander LS. Patient-relevant outcomes fourteen years after meniscectomy: influence of type of meniscal tear and size of resection. Rheumatology (Oxford) 2001;40(6):631–9.
4. Stein T, Mehling AP, Welsch F, von Eisenhart-Rothe R, Jäger A. Long-term outcome after arthroscopic meniscal repair versus arthroscopic partial meniscectomy for traumatic meniscal tears. Am J Sports Med 2010;38(8):1542–8.
5. Krause WR, Pope MH, Johnson RJ, Wilder DG. Mechanical changes in the knee after meniscectomy. J Bone Joint Surg Am 1976;58(5):599–604.
6. Englund M, Guermazi A, Lohmander LS. The meniscus in knee osteoarthritis. Rheum Dis Clin North Am 2009;35(3):579–90.
7. Long D, Blake S, Song XY, Lark M, Loeser RF. Human articular chondrocytes produce IL-7 and respond to IL-7 with increased production of matrix metalloproteinase-13. Arthritis Res Ther 2008;10(1):R23.
8. Sandell LJ, Xing X, Franz C, Davies S, Chang LW, Patra D. Exuberant expression of chemokine genes by adult human articular chondrocytes in response to IL-1β. Osteoarthritis Cartilage 2008;16(12):1560–71.
9. Goldring MB, Otero M. Inflammation in osteoarthritis. Curr Opin Rheumatol 2011;23(5):471–8.
10. Sun Y, Mauerhan D, Honeycutt P, Kneisl J, Norton J, Hanley E, *et al*. Analysis of meniscal degeneration and meniscal gene expression. BMC Musculoskelet Disord 2010;11(1):19.
11. Brophy RH, Farooq Rai M, Zhang Z, Torgomyan A, Sandell LJ. Molecular analysis of age and sex-related gene expression in meniscal tears with and without a concomitant anterior cruciate ligament tear. J Bone Joint Surg Am 2012;94(5):385–93.
12. Gupta T, Zielinska B, McHenry J, Kadmiel M, Haut Donahue TL. IL-1 and iNOS gene expression and NO synthesis in the superior region of meniscal explants are dependent on the magnitude of compressive strains. Osteoarthritis Cartilage 2008;16(10):1213–9.
13. Hellio Le Graverand MP, Sciore P, Eggerer J, Rattner JP, Vignon E, Barclay L, *et al*. Formation and phenotype of cell clusters in osteoarthritic meniscus. Arthritis Rheum 2001;44(8):1808–18.
14. Hellio Le Graverand MP, Vignon E, Otterness IG, Hart DA. Early changes in lapine menisci during osteoarthritis development: part II: molecular alterations. Osteoarthritis Cartilage 2001;9(1):65–72.
15. Ishihara G, Kojima T, Saito Y, Ishiguro N. Roles of metalloproteinase-3 and aggrecanase 1 and 2 in aggrecan cleavage during human meniscus degeneration. Orthop Rev (Pavia) 2009;1(2):e14.
16. Papachristou DJ, Papadakou E, Basdra EK, Baltopoulos P, Panagiotopoulos E, Papavassiliou AG. Involvement of the p38 MAPK-NF-kappaB signal transduction pathway and COX-2 in the pathobiology of meniscus degeneration in humans. Mol Med 2008;14(3–4):160–6.
17. Zielinska B, Killian M, Kadmiel M, Nelsen M, Haut Donahue TL. Meniscal tissue explants response depends on level of dynamic compressive strain. Osteoarthritis Cartilage 2009;17(6):754–60.
18. Fuller ES, Smith MM, Little CB, Melrose J. Zonal differences in meniscus matrix turnover and cytokine response. Osteoarthritis Cartilage 2012;20(1):49–59.
19. Xie DL, Meyers R, Homandberg GA. Fibronectin fragments in osteoarthritic synovial fluid. J Rheumatol 1992;19(9):1448–52.
20. Zack MD, Arner EC, Anglin CP, Alston JT, Malfait AM, Tortorella MD. Identification of fibronectin neoepitopes present in human osteoarthritic cartilage. Arthritis Rheum 2006;54(9):2912–22.
21. Long DL, Loeser RF. p38[gamma] mitogen-activated protein kinase suppresses chondrocyte production of MMP-13 in response to catabolic stimulation. Osteoarthritis Cartilage 2010;18(9):1203–10.
22. Pulai JI, Chen H, Im HJ, Kumar S, Hanning C, Hegde PS, *et al*. NF-kappa B mediates the stimulation of cytokine and chemokine expression by human articular chondrocytes in response to fibronectin fragments. J Immunol 2005;174(9):5781–8.
23. Appleton CTG, Usmani SE, Bernier SM, Aigner T, Beier F. Transforming growth factor α suppression of articular chondrocyte phenotype and Sox9 expression in a rat model of osteoarthritis. Arthritis Rheum 2007;56(11):3693–705.
24. Appleton CTG, Usmani SE, Mort JS, Beier F. Rho/ROCK and MEK/ERK activation by transforming growth factor-[alpha] induces articular cartilage degradation. Lab Invest 2009;90(1):20–30.

25. Bennett L, Buckland-Wright J. Meniscal and articular cartilage changes in knee osteoarthritis: a cross-sectional double-contrast macroradiographic study. Rheumatology 2002: 41917–23.
26. Englund M. The role of biomechanics in the initiation and progression of OA of the knee. Best Pract Res Clin Rheumatol 2010;24(1):39–46.
27. Englund M, Felson DT, Guermazi A, Roemer FW, Wang K, Crema MD, *et al*. Risk factors for medial meniscal pathology on knee MRI in older US adults: a multicentre prospective cohort study. Ann Rheum Dis 2011 Oct;70(10):1733–9.
28. Loeser RF, Goldring SR, Scanzello CR, Goldring MB. Osteoarthritis: a disease of the joint as an organ. Arthritis Rheum 2012;64(6):1697–707.
29. Loeser RF, Olex AL, McNulty MA, Carlson CS, Callahan M, Ferguson C, *et al*. Disease progression and phasic changes in gene expression in a mouse model of osteoarthritis. PLoS One 2013;8(1):e54633.
30. Son M, Levenston ME. Discrimination of meniscal cell phenotypes using gene expression profiles. Eur Cell Mater 2012: 23195–208.
31. Troeberg L, Nagase H. Proteases involved in cartilage matrix degradation in osteoarthritis. Biochim Biophys Acta (BBA) – Prot Proteomics 2012;1824(1):133–45.
32. Benito MJ, Veale DJ, FitzGerald O, van den Berg WB, Bresnihan B. Synovial tissue inflammation in early and late osteoarthritis. Ann Rheum Dis 2005;64(9):1263–7.
33. Makris EA, Hadidi P, Athanasiou KA. The knee meniscus: structure-function, pathophysiology, current repair techniques, and prospects for regeneration. Biomaterials 2011;32(30):7411–31.
34. McNulty AL, Miller MR, O'Connor SK, Guilak F. The effects of adipokines on cartilage and meniscus catabolism. Connect Tissue Res 2011;52(6):523–33.
35. Richardson S, Doyle P, Minogue B, Gnanalingham K, Hoyland J. Increased expression of matrix metalloproteinase-10, nerve growth factor and substance P in the painful degenerate intervertebral disc. Arthritis Res Ther 2009;11(4): R126.
36. Verdonk PC, Forsyth RG, Wang J, Almqvist KF, Verdonk R, Veys EM, *et al*. Characterisation of human knee meniscus cell phenotype. Osteoarthritis Cartilage 2005;13(7):548–60.
37. Loeser RF, Sadiev S, Tan L, Goldring MB. Integrin expression by primary and immortalized human chondrocytes: evidence of a differential role for alpha1beta1 and alpha2beta1 integrins in mediating chondrocyte adhesion to types II and VI collagen. Osteoarthritis Cartilage 2000;8(2):96–105.
38. Ulbrich H, Eriksson EE, Lindbom L. Leukocyte and endothelial cell adhesion molecules as targets for therapeutic interventions in inflammatory disease. Trends Pharmacol Sci 2003;24(12):640–7.
39. Schett G, Kiechl S, Bonora E, Zwerina J, Mayr A, Axmann R, *et al*. Vascular cell adhesion molecule 1 as a predictor of severe osteoarthritis of the hip and knee joints. Arthritis Rheum 2009;60(8):2381–9.
40. Karatay S, Kiziltunc A, Yildirim K, Karanfil RC, Senel K. Effects of different hyaluronic acid products on synovial fluid levels of intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 in knee osteoarthritis. Ann Clin Lab Sci 2004;34(3): 330–5.
41. Farahat MN, Yanni G, Poston R, Panayi GS. Cytokine expression in synovial membranes of patients with rheumatoid arthritis and osteoarthritis. Ann Rheum Dis 1993;52(12):870–5.
42. Shlopov BV, Gumanovskaya ML, Hasty KA. Autocrine regulation of collagenase 3 (matrix metalloproteinase 13) during osteoarthritis. Arthritis Rheum 2000;43(1):195–205.
43. O'Rourke KP, O'Donoghue G, Adams C, Mulcahy H, Molloy C, Silke C, *et al*. High levels of Lymphotoxin-Beta (LT-Beta) gene expression in rheumatoid arthritis synovium: clinical and cytokine correlations. Rheumatol Int 2008;28(10):979–86.
44. He W, Liu YJ, Wang ZG, Guo ZK, Wang MX, Wang N. Enhancement of meniscal repair in the avascular zone using connective tissue growth factor in a rabbit model. Chin Med J (Engl) 2011;124(23):3968–75.
45. McNulty AL, Rothfusz NE, Leddy HA, Guilak F. Synovial fluid concentrations and relative potency of interleukin-1 alpha and beta in cartilage and meniscus degradation. J Orthop Res 2013 Jul;31(7):1039–45.
46. Pelletier JP, Martel-Pelletier J, Abramson SB. Osteoarthritis, an inflammatory disease: potential implication for the selection of new therapeutic targets. Arthritis Rheum 2001;44(6):1237–47.
47. Eggli S, Wegmuller H, Kosina J, Huckell C, Jakob RP. Long-term results of arthroscopic meniscal repair. An analysis of isolated tears. Am J Sports Med 1995;23(6):715–20.