Molecular mechanisms of histone deacetylases in rheumatoid arthritis fibroblast-like synoviocytes
Angiolilli, C.

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Chapter 6

JNK-DEPENDENT DOWNREGULATION OF FOXO1 IS REQUIRED TO PROMOTE THE SURVIVAL OF FIBROBLAST-LIKE SYNOVIOCYTES IN RHEUMATOID ARTHRITIS.

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*AMG and CA contributed equally to this work.

ABSTRACT

**Background:** Forkhead box O (FoxO) transcription factors integrate environmental signals to modulate cell proliferation and survival, and alterations in FoxO function have been reported in rheumatoid arthritis (RA).

**Objectives:** To examine the relationship between inflammation and FoxO expression in RA, and to analyse the mechanisms and biological consequences of FoxO regulation in RA fibroblast-like synoviocytes (FLS).

**Methods:** RNA was isolated from RA patient and healthy donor (HD) peripheral blood and RA synovial tissue. Expression of FoxO1, FoxO3a and FoxO4 was measured by quantitative PCR. FoxO1 DNA binding, expression and mRNA stability in RA FLS were measured by ELISA-based assays, immunoblotting and quantitative PCR. FLS were transduced with adenovirus encoding constitutively active FoxO1 (FoxO1ADA) or transfected with small interfering RNA targeting FoxO1 to examine the effects on cell viability and gene expression.

**Results:** FoxO1 mRNA levels were reduced in RA patient peripheral blood compared with HD blood, and RA synovial tissue FoxO1 expression correlated negatively with disease activity. RA FLS stimulation with interleukin 1β or tumour necrosis factor caused rapid downregulation of FoxO1. This effect was independent of protein kinase B (PKB), but dependent on c-Jun N-terminal kinase (JNK)-mediated acceleration of FoxO1 mRNA degradation. FoxO1ADA overexpression in RA FLS induced apoptosis associated with altered expression of genes regulating cell cycle and survival, including BIM, p27Kip1 and Bcl-XL.

**Conclusions:** Our findings identify JNK-dependent modulation of mRNA stability as an important PKB-independent mechanism underlying FoxO1 regulation by cytokines, and suggest that reduced FoxO1 expression is required to promote FLS survival in RA.
JNK-dependent downregulation of FoxO1 is required to promote RA FLS survival

INTRODUCTION

Aberrant regulation of genes and pathways involved in cellular proliferation and survival makes pivotal contributions to development and perpetuation of inflammation in chronic immune-mediated inflammatory diseases (IMIDs), including systemic lupus erythematosus (SLE), type I diabetes and rheumatoid arthritis (RA). In RA, macrophages and stromal fibroblast-like synoviocytes (FLS) display increased resistance to apoptotic stimuli, which promotes their accumulation in the inflamed joint, as well as release of products responsible for bone and cartilage damage. Because of their ability to promote cellular survival and proliferation, and evidence of their enhanced activation in RA synovial tissue, phosphatidylinositol 3-kinases (PI3Ks) have emerged as potential therapeutic targets. Protein kinase B (PKB, also known as Akt), a downstream target of PI3K, modulates cell fate choices through multiple mechanisms, among which is regulation of forkhead box O (FoxO) transcription factors. FoxO transcriptional targets include genes regulating cellular metabolism, response to oxidative stress, proliferation and apoptosis. FoxO-dependent gene expression profiles are cell type- and stimulus-specific, and unique combinations of environmental signals trigger transcriptional programmes that either promote cell cycle arrest and survival in response to stress or induce apoptosis.

The activity of three ubiquitously expressed FoxO family members, FoxO1, FoxO3a and FoxO4, is regulated by a complex interplay between phosphorylation, acetylation, ubiquitilation and other post-translational modifications. PKB-mediated FoxO phosphorylation disrupts FoxO interaction with target DNA sequences and facilitates nuclear export, leading to FoxO inactivation. Reciprocally, FoxO phosphorylation by mitogen-activated protein kinases (MAPKs) promotes FoxO nuclear retention and transcriptional activity. Also, reversible FoxO acetylation modulates FoxO DNA-binding affinity and gene target specificity. FoxO proteins are regulated not only through post-translational mechanisms, but also transcriptionally. PI3K–PKB signaling downregulates FoxO1 levels in B cells and fibroblasts, and involvement of other signalling pathways in the regulation of FoxO expression has been reported.

Persistent activation of the PI3K–PKB axis in the inflamed synovium, observed in both animal arthritis models and RA synovial tissue, might contribute to RA pathology partly through inactivation of FoxO transcription factors. PKB-inactivated FoxO proteins are detected in RA synovial tissue, and synovial macrophages express elevated levels of inactive FoxO4 compared with disease controls. Peripheral blood mononuclear cells (PBMCs) of patients with RA express lower levels of FoxO1 than healthy individuals, and hypermethylation of the FoxO1 gene has been reported in RA FLS. Reduced FoxO4 expression has been reported in colonic epithelial cells of patients with inflammatory bowel disease compared with healthy controls. The importance of FoxO proteins in maintaining immune system homeostasis is further supported by animal studies. FoxO3a-deficient mice develop spontaneous autoimmunity due to persistent T-cell proliferation and survival, while deletion of both FoxO1 and FoxO3a in T cells leads to defective regulatory T-cell development and multifocal inflammatory disease. Finally, mice lacking FoxO4 develop more severe mucosal inflammation than wild-type mice in a colitis model. Despite this, the molecular mechanisms leading to changes in
FoxO expression and function in chronic IMIDs have not been characterized. The present study was undertaken to understand the relationships between FoxO expression and disease activity in RA, to identify mechanisms underlying inflammation-mediated regulation of FoxO proteins, and to assess the biological consequences of alterations in FoxO function in RA FLS.

MATERIALS AND METHODS

Subjects, peripheral blood and synovial tissue samples
Peripheral blood was collected from a previously described cohort of 10 methotrexate-naïve RA patients and 15 healthy individuals. Synovial biopsy specimens were obtained by arthroscopy from actively inflamed joints of 19 patients with RA fulfilling the 1987 revised criteria of the American College of Rheumatology as described previously, embedded in TissueTek OCT (Miles Diagnostics, Elkhart, Indiana, USA), snap-frozen and stored in liquid nitrogen. The study was approved by the institutional review board, and all patients provided written informed consent. Clinical characteristics of patients are detailed in table 1.

<table>
<thead>
<tr>
<th>Characteristic</th>
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<tr>
<td>Age (years)</td>
<td>60 (38–67)</td>
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<tr>
<td>Male:female (n)</td>
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<td>Disease duration (months)</td>
<td>86.5 (1–409)</td>
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<tr>
<td>ESR (mm/h)</td>
<td>19 (4–119)</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>9.1 (1–122.6)</td>
</tr>
<tr>
<td>DAS28</td>
<td>4.64 (1.87–7.49)</td>
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</tbody>
</table>

CRP, C-reactive protein; DAS28, disease activity score in 28 joints; ESR, erythrocyte sedimentation rate.

Cell culture and stimulations
FLS were isolated from synovial biopsy specimens of patients with RA and cultured as previously described. After overnight culture in medium containing 1% fetal bovine serum (Invitrogen, Breda, The Netherlands), FLS between passages 4 and 9 were stimulated with interleukin (IL)-1β, platelet-derived growth factor (PDGF) (both from R&D Systems, Minneapolis, Minnesota, USA), tumor necrosis factor (TNF; Biosource International, Camarillo, California, USA) or lipopolysaccharide (LPS; Sigma-Aldrich). Activation of signalling pathways was blocked using PKB inhibitor VIII, SB203580, U0126, SP600125, AS601245 or JNK inhibitor IX (all from Calbiochem/EMD Millipore, Billerica, Massachusetts, USA). Transcription was blocked by actinomycin D (Sigma-Aldrich). Q-VD-Oph (R&D Systems) was used to inhibit caspase activity.
JNK-dependent downregulation of FoxO1 is required to promote RA FLS survival

**Analysis of mRNA expression**
RNA extraction from peripheral blood and large-scale expression profiling by cDNA microarrays were as described previously.\(^2\) RNA was isolated from FLS and synovial tissue biopsy samples as described previously,\(^27\) and mRNA expression levels were determined by quantitative (q)PCR or by RT\(^2\) Profiler PCR Arrays (SABiosciences-Qiagen, Frederick, Maryland, USA) as described in detail in the online supplementary methods.

**Protein extraction, immunoblotting and FoxO1 DNA binding**
Protein extracts prepared by lysis in Laemmli’s buffer were resolved by electrophoresis on 4–12% Bis-Tris SDS-NuPAGE gels (Invitrogen) and analyzed by immunoblotting as described in the online supplementary methods. Nuclear fractions were prepared as described previously,\(^28\) and FoxO1 DNA-binding activity was determined using a TransAM ELISA (Active Motif, Carlsbad, California, USA).

**Overexpression and knockdown of FoxO1**
Control adenoviruses encoding green fluorescent protein (GFP; provided by Dr S W Tas, our institute)\(^29\) and adenoviruses encoding constitutively active FoxO1 mutant (FoxO1A-DA, provided by Dr D Accili, Columbia University, New York, USA)\(^30\) were amplified and titrated in transcomplemental HEK-293 cells and purified by caesium chloride gradient ultracentrifugation as described previously.\(^31\) To silence FoxO1 expression, FLS were transfected with control or FoxO1-specific small interfering (si)RNAs (Dharmacon, Schwerte, Germany) using the DharmaFECT-1 transfection reagent (Dharmacon).

**Measurements of cell viability and apoptosis**
Cell viability was determined by MTT reduction assay as described previously.\(^28\) Induction of apoptosis was assessed using the Cell Death Detection ELISA (Roche Diagnostics, Mannheim, Germany).

**Statistical analysis**
Data are presented as mean±SEM unless otherwise indicated. Spearman’s correlation coefficient was used for correlation analyses. An overall Kruskall–Wallis test and Dunns’ post hoc test were used for analyzing sets of data requiring multiple comparisons. The Mann–Whitney U test was used for all other comparisons. \(p\leq0.05\) was considered significant.
RESULTS

FoxO1 expression in RA synovial tissue correlates negatively with clinical parameters and IL-6 expression.

A study demonstrating selective reduction of FoxO1 levels in RA PBMCs suggested that alterations in FoxO1 expression might be involved in RA pathology. Here, we compared peripheral blood FoxO1 and FoxO3a mRNA expression in 10 methotrexate-naive RA patients and 15 healthy donors (HDs) previously subjected to global gene expression profiling. While no differences in FoxO3a expression were observed between HDs and RA patients, FoxO1 levels were significantly reduced in patients compared with HDs (figure 1A,B). Analysis of mRNA obtained from RA patient synovial biopsy samples revealed significant negative correlations between synovial FoxO1 mRNA levels and patient serum C-reactive protein (CRP) levels (figure 1C; R=−0.771, p=0.0008), erythrocyte sedimentation rate (ESR) (figure 1D; R=−0.739, p=0.0003), and 28-joint disease activity score (DAS28) (figure 1E, R= −0.575, p=0.01). A strong negative relationship was also noted between synovial FoxO1 expression and local expression of IL6 (figure 1F: R=−0.628, p=0.004).

Figure 1. FoxO1 mRNA levels are reduced in rheumatoid arthritis (RA) peripheral blood, and FoxO1 expression in RA synovial tissue correlates negatively with disease activity.

Total RNA was extracted from peripheral blood (PB) from 15 healthy donors (HD) and 10 methotrexate-naive RA patients, and expression levels of (A) FoxO1 and (B) FoxO3a were retrieved from the results of gene array analysis. Data are presented as boxplots where the boxes represent the 25th and 75th centiles, the line within the box denotes the median value, and the lines outside of the box mark the 10th and 90th centiles. **p<0.01, Mann–Whitney U test. (C–F) mRNA was isolated from synovial tissue specimens from 19 RA patients, cDNA was synthesized, and expression of FoxO1 and interleukin (IL)-6 determined by quantitative PCR. Correlations of relative FoxO1 expression with (C) C-reactive protein (CRP) levels, (D) erythrocyte sedimentation rate (ESR), (E) 28-joint disease activity score (DAS28) and (F) IL-6 levels were calculated using Spearman’s rank correlation coefficient. Circles indicate individual patient values, and Spearman R and p values are indicated in each graph.
Figure 2. Inflammatory stimulation reduces FoxO1 expression and activity in rheumatoid arthritis (RA) fibroblast-like synoviocytes (FLS).

RA FLS (n=4) were either left unstimulated in medium (med) or were stimulated with 1 ng/mL interleukin (IL)-1β, 10 ng/mL tumour necrosis factor (TNF) or 1 μg/mL lipopolysaccharide (LPS) for 24 h, total RNA was extracted, reverse transcribed and changes in (A) FoxO1, (B) FoxO3a or (C) FoxO4 mRNA accumulation were monitored by quantitative PCR. Data are presented as mean±SEM relative FoxO1 expression. (D) Alternatively, RA FLS (n=6) were left untreated (med) or were stimulated with IL-1β (1 ng/mL) for the indicated time (h), and FoxO1 mRNA expression analyzed as above. The graph represents mean±SEM relative FoxO1 expression. (E) RA FLS (n=3) were left untreated (med) or were stimulated with IL-1β (1 ng/mL) for 24 h; protein extracts were prepared and analyzed by immunoblotting with FoxO1 and actin antibodies. Signal intensity was then quantified and the results of densitometric analysis are shown as mean±SEM FoxO1 expression relative to actin (left panel). In addition, one experiment representative of three independent experiments is shown (right panel). (F) RA FLS were stimulated as in (D), nuclear fractions were extracted, and levels of active FoxO1 were determined using a TransAM transcription factor DNA-binding assay. Data represent the mean±SEM fold change in FoxO1 DNA binding of four to six independent experiments. *p<0.05, **p<0.01, Kruskal–Wallis test followed by Dunns’ multiple comparison analysis.
Inflammatory stimulation rapidly downregulates FoxO1 expression in RA FLS.

These initial data raised the possibility that during inflammation in RA, FoxO proteins may be regulated not only by PKB-dependent phosphorylation, but also through changes in FoxO expression. To test this possibility, we stimulated RA FLS, which express high levels of FoxO1 compared with other cell types present in the synovial tissue,\textsuperscript{17} with IL-1β, TNF and LPS for 24 h and analyzed expression of FoxO family members. Both IL-1β and TNF caused an 80% downregulation of FoxO1 mRNA expression (p<0.01), while LPS treatment led to only a modest reduction (figure 2A). Inflammatory stimulation failed to modulate FoxO3a expression, while FoxO4 levels, expressed at lower levels in FLS than synovial macrophages,\textsuperscript{17} were significantly affected only by TNF (figure 2B,C). FoxO1 transcript levels were reduced by 25% following 2 h stimulation with IL-1β, and decreased further over time (figure 2D). Consistent with changes at the mRNA level, exposure to IL-1β caused a downregulation of FoxO1 protein levels (figure 2E), as well as a reduction in the amount of active FoxO1. A ~50% inhibition of FoxO1 DNA binding was observed within 4 h of FLS exposure to IL-1β (p<0.05), and this effect was maintained at later time points (figure 2F). These results indicate that FoxO1 expression and functional activity are rapidly reduced in IL-1β stimulated RA FLS.

PKB-independent, JNK-mediated acceleration of FoxO1 mRNA degradation is responsible for the downregulation of FoxO1 expression by cytokines in RA FLS.

We next analyzed the potential involvement of signalling and regulatory pathways typically activated by cytokines in RA FLS to identify the molecular mechanism(s) underlying FoxO1 downregulation by IL-1β. Because FoxO transcription factors are capable of regulating their own expression,\textsuperscript{10} we reasoned that the observed reduction of FoxO1 levels might be secondary to PKB-dependent FoxO1 inactivation. RA FLS were stimulated with either IL-1β or PDGF in the absence or presence of a PKB inhibitor (PKBi). Compared with PDGF, IL-1β caused only a minor induction of PKB phosphorylation, consistent with other studies (figure 3A),\textsuperscript{32} and PKBi did not rescue FoxO1 expression in the presence of IL-1β (figure 3B). Strikingly, while PDGF stimulation also reduced FoxO1 expression (p<0.05) (figure 3B), inhibition of PKB activity failed to prevent this effect, despite blocking PKB phosphorylation (figure 3A) and inactivation of FoxO1 DNA-binding activity (figure 3C).

Together, these results indicated that IL-1β mediates FoxO1 downregulation at the mRNA level independently of PI3K–PKB signalling. We next tested whether cytokine-mediated FoxO1 downregulation is dependent on MAPK signalling pathways. While inhibition of p38 or extracellular signal-regulated kinase (ERK) activity failed to significantly affect FoxO1 expression levels in IL-1β-stimulated FLS, JNK inhibition almost completely prevented IL-1β-dependent reduction of FoxO1 mRNA levels (p<0.05) (figure 3D). Similarly, JNKi rescued RA FLS FoxO1 DNA-binding activity and protein expression after IL-1β stimulation (figure 3E,F). The dose-dependent and specific inhibition of c-Jun phosphorylation by JNKi was confirmed by immunoblotting (figure 3F,G). Use of a second pan-JNKi, but not a JNK2/3-selective inhibitor, also rescued FoxO1 mRNA expression, indirectly suggesting that JNK1 may have a predominant role in regulation of FoxO1 (see online supplementary figure 1).

Analysis of 3’ untranslated regions revealed the presence of AU-rich elements (AREs)
JNK-dependent downregulation of FoxO1 is required to promote RA FLS survival.

Figure 3. c-Jun N-terminal kinase (JNK) mediates downregulation of FoxO1 expression by interleukin (IL)-1β independently of phosphatidylinositol 3-kinase (PI3K)–protein kinase B (PKB) signalling.

(A) Rheumatoid arthritis (RA) fibroblast-like synoviocytes (FLS) were left untreated (med) or were treated with 10 μM PKB inhibitor VIII (PKBi) for 30 min before stimulation with IL-1β (1 ng/mL), tumour necrosis factor (TNF) (10 ng/mL) or PDGF (10 ng/mL) for 30 min. Protein extracts were prepared and analyzed by immunoblotting with antibodies recognizing phospho-(p)PKB, PKB and tubulin (Tub). (B) RA FLS (n=7) were left unstimulated (med) or were incubated with 10 μM PKBi for 30 min followed by stimulation with 1 ng/mL IL-1β or 10 ng/mL platelet-derived growth factor (PDGF) for 4 h. RNA was extracted, cDNA was synthesized, and changes in FoxO1 mRNA levels were assessed by quantitative (q)PCR. Data are presented as the mean±SEM relative FoxO1 expression. (C) RA FLS were left untreated (med) or were stimulated with 1 ng/mL IL-1β with or without PKBi (10 mM) for 2 h, nuclear fractions were extracted, and levels of active FoxO1 were determined using an ELISA-based DNA-binding assay. Results are presented as the mean±SEM fold change in FoxO1 DNA binding from three independent experiments. (D) RA FLS (n=6) were left unstimulated (med) or were treated with SB203580 (p38 inhibitor (p38i), 10 μM), U0126 (extracellular signal-regulated kinase (ERK) inhibitor (ERKi), 10 μM) or SP600125 (JNK inhibitor (JNKi), 20 μM) for 30 min and then stimulated with 1 ng/mL IL-1β for 4 h. FoxO1 expression was analyzed by qPCR and presented as the mean±SEM relative FoxO1 expression. (E) RA FLS were left untreated (med) or were treated with SP600125 (JNKi, 20 μM) or PKBi (10 μM) for 30 min before stimulation with IL-1β (1 ng/mL) for 8 h, nuclear extracts were collected, and FoxO1 DNA-binding activity was measured as in (C). Data represent the mean±SEM fold change in FoxO1 DNA binding from four independent experiments. (F) RA FLS were treated with IL-1β (1 ng/mL) in the absence or presence of SP600125 (JNKi, 20 μM) for 8 h. Protein extracts were prepared, and protein expression of FoxO1 and actin was analyzed by immunoblotting. Alternatively, RA FLS were incubated with (G) increasing concentrations of JNKi (2–20 mM) or (H) p38i (10 μM), ERKi (10 μM) or JNKi (20 μM) for 30 min before stimulation with 1 ng/mL IL-1β for 15 min. Cells were then lysed, and protein extracts were subjected to analysis by immunoblotting with anti-phospho-(p)c-Jun or anti-histone (H)3 antibodies. *p<0.05, **p<0.01, Kruskal–Wallis test followed by Dunns’ multiple comparison analysis. All immunoblotting analyses show a representative of at least three independent experiments.
in the transcripts of FoxO family members. To determine if inflammatory cytokines modulated FoxO1 mRNA stability, we analyzed degradation of FoxO transcripts after blocking transcription with actinomycin D. A comparison of mRNA stability of FoxO transcription factors in the presence of IL-1β revealed that the FoxO1 transcript was comparatively unstable, with a half-life of ~2 h. In contrast, half-lives of FoxO3a and FoxO4 were longer than 4 h (figure 4A). In addition, FoxO1 mRNA degradation was accelerated when RA FLS were exposed to IL-1β, compared with unstimulated cells (figure 4B). Treatment of RA FLS with JNKi completely prevented IL-1β-induced acceleration of FoxO1 mRNA degradation (figure 4C), indicating that IL-1β-mediated FoxO1 downregulation is regulated by the JNK pathway through modulation of FoxO1 mRNA stability.

**FoxO1 activation modulates expression of pro- and anti-apoptotic genes and induces apoptosis in RA FLS.**

To examine the effect of rescuing FoxO1 expression and function on RA FLS activation and survival, we transduced RA FLS with an adenoviral vector encoding constitutively active FoxO1 (FoxO1ADA). Overexpression and biological activity of FoxO1ADA were confirmed by immunoblotting and FoxO1 DNA-binding assay, respectively (figure 5A,B). FoxO1ADA reduced FLS viability by ~50% (p<0.05) (figure 5C), associated with elevated levels of cytosolic histone complexed DNA fragments, a hallmark of apoptotic cell death.
JNK-dependent downregulation of FoxO1 is required to promote RA FLS survival

Figure 5. Constitutive activation of FoxO1 modulates expression of pro- and anti-apoptotic genes in rheumatoid arthritis (RA) fibroblast-like synoviocytes (FLS) and induces cell death.

FLS were transduced with the control green fluorescent protein (GFP)-encoding or the FoxO1ADA-encoding adenoviral vector at a multiplicity of infection of 500 for 48 h and serum-starved in medium containing 1% fetal bovine serum (FBS) for another 24 h before further processing. (A) Transduced cells were lysed, and protein extracts were analyzed by western blotting with antibodies specific for FoxO1 and tubulin (Tub). (B) Nuclear fractions were extracted, and transcriptional activity of FoxO1 was assessed using an ELISA-based DNA-binding assay. The data are shown as the mean±SEM fold change in FoxO1 DNA binding from three independent experiments. Viability and induction of apoptosis in RA FLS transduced with GFP- or FoxO1ADA-expressing vectors were determined using an MTT reduction assay (C) and Cell Death ELISA (D), respectively. Apoptosis was assessed in the absence or presence of the pan-caspase inhibitor, Q-VD-OPh (20 μM). Data are presented as mean±SEM absorbance (optical density (OD)) at 590 nm (C) and 415 nm (D) and are results of five and three independent experiments, respectively. *p<0.05, Mann–Whitney U test. (E) Transduced FLS (n=4) were cultured for 24 h in medium containing 1% FBS, and then were either left unstimulated (med) or stimulated with 1 ng/mL interleukin (IL)-1β for 4 h. Total RNA was then extracted, reverse transcribed, and relative mRNA expression levels of BIM, Bcl-XL, p27Kip1, A1/Bfl-1, FLIP and MnSOD were determined by quantitative PCR using a customized RT² Profiler PCR Array. Data are shown as mean±SEM relative mRNA expression compared with unstimulated cells transduced with the control GFP-expressing vector. (F) Protein extracts from unstimulated RA FLS transduced with GFP or FoxO1ADA vectors were analyzed by immunoblotting with antibodies specific for Bcl-XL, p27Kip1, BIM, FLIP, MnSOD and tubulin (Tub). (G) RA FLS were left untreated (med) or were transfected with control non-targeting small interfering (si)RNA (scrb, 20 nM) or siRNA specific for FoxO1 (siFoxO1, 20 nM). After 48 h, cells were lysed, and expression levels of FoxO1, Bcl-XL and actin were analyzed by immunoblotting. All immunoblotting analyses show a representative of at least three independent experiments.
(figure 5D). FoxO1-induced apoptosis was fully dependent on caspase activation (figure 5D). In FoxO1ADA-transduced cells, we observed altered expression of genes involved in regulation of apoptosis and cell cycle progression, many of which are known FoxO targets. Namely, FoxO1ADA reduced the mRNA levels of the Bcl-2-like protein, Bcl-XL, and enhanced expression of BIM and the cell cycle inhibitor, p27Kip1, compared with control GFP-transduced cells (figure 5E). FoxO1ADA also moderately increased mRNA expression of MnSOD in unstimulated FLS and prevented the induction of another Bcl-2 family member, Bfl-1/A1, while leaving FLIP expression unaffected (figure 5E). Changes in Bcl-XL, p27Kip1 and BIM expression following FoxO1ADA overexpression were confirmed at the protein level (figure 5F), and, reciprocally, Bcl-XL protein levels were increased in FLS after siRNA-mediated FoxO1 knockdown (figure 5G). However, FoxO1 silencing failed to influence effects of JNKi on Bcl-XL expression, indicating that FoxO1 inactivation is required, but not sufficient, to regulate Bcl-XL under inflammatory conditions (see online supplementary figure 2). Together, these data indicate that inflammatory cytokines promote the survival of RA FLS in part by reducing the expression and activity of FoxO1.

**DISCUSSION**

FoxO transcription factors integrate growth, inflammatory and stress signals to control cell fate decisions. In the absence of growth factors, cytokines and antigen receptor signalling, all of which activate the PI3K–PKB pathway, FoxO family members localise to the nucleus to modulate expression of genes regulating cell cycle progression, proliferation and survival. Genes involved in cellular inflammatory responses are also direct FoxO targets, and studies in animals lacking FoxO proteins have provided evidence for essential roles of FoxO transcriptional activity in the immune system. In RA, reduced expression and PKB-mediated inactivation of FoxO family members has been reported, and SLE FoxO1 expression in PBMCs correlates negatively with disease severity, indicating FoxO involvement in protection against autoimmune processes. Here, we show that both peripheral and synovial FoxO1 expression are strongly associated with pathology in RA, as synovial FoxO1 expression correlates negatively with clinical parameters of disease activity and local IL-6 expression. FoxO1 expression is rapidly reduced after inflammatory stimulation of RA FLS, and we identify JNK-mediated acceleration of FoxO1 mRNA degradation as a novel mechanism regulating FoxO1 expression.

Initial observations in murine B cells, which downregulate FoxO1 mRNA expression in response to B-cell receptor activation, indicated that the PI3K–PKB pathway is at least partly responsible for this effect. The promoter region of FoxO1 contains a conserved FoxO-binding motif that is indispensable for FoxO1 transcriptional induction by itself and by FoxO3a. Growth factor-mediated activation of PI3K–PKB signalling and FoxO nuclear exclusion repress the expression of FoxO1 in human fibroblasts, demonstrating that FoxO activity is required for maintaining FoxO1 expression in some cell populations. However, we find that PKB inhibition fails to influence FoxO1 expression in IL-1β- or PDGF-stimulated RA FLS. Moreover, overexpression of FoxO1ADA did not induce endogenous
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FoxO1 expression (data not shown), arguing against a positive feedback loop mode of FoxO regulation in RA FLS. Instead, we show that the FoxO1 transcript is relatively unstable compared with other FoxO family members and that degradation of FoxO1 mRNA is significantly accelerated on IL-1β stimulation in a JNK-dependent fashion. Although JNK signalling is generally associated with mRNA stabilization, as observed in the case of several cytokines and growth factors,34 35 a destabilizing role for JNK has also been reported for a small number of transcripts.36 37 The latter effects have been associated with JNK-dependent induction of ARE-binding proteins, such as TTP and AUF1. Although we observed rapid, JNK-dependent increases in TTP protein levels in IL-1β-stimulated RA FLS, silencing of TTP expression with siRNA failed to prevent IL-1β-induced downregulation of FoxO1 mRNA levels (data not shown). Interestingly, JNK can also directly regulate one member of the FoxO family, as FoxO4 phosphorylation by JNK results in nuclear translocation and transcriptional activation in cells subjected to oxidative stress.38 While endoplasmic reticulum stress has also been reported to induce FoxO1 activation via a JNK-dependent pathway,39 FoxO1 is not subject to phosphorylation by JNK, suggesting that JNK affects FoxO signaling through multiple direct and indirect mechanisms in a context dependent manner.40

Notably, JNK signalling in RA synovial tissue is activated early in the course of disease.27 JNK activation in several cell types, including FLS, contributes to pathology in part through cellular production of inflammatory cytokines and matrix metalloproteinases.41 The strong negative association between FoxO1 expression and disease severity observed in our study supports a model in which the inflammatory processes in RA synovial tissue induce JNK-dependent FoxO1 downregulation in FLS, promoting FLS accumulation and persistence. In line with this possibility, we find that overexpression of FoxO1ADA induces apoptosis of RA FLS associated with altered expression profiles of two Bcl-2 family members known to be directly or indirectly regulated by FoxO proteins, pro-apoptotic BIM and antiapoptotic Bcl-XL.8 Of these, Bcl-XL is known to be elevated in the intimal lining layer in RA synovial tissue compared with osteoarthritis.42 Reduced FoxO1 expression might also promote FLS proliferation through downregulation of the cell cycle inhibitor, p27Kip1, as well as sensitize FLS to oxidative stress and prevent DNA repair processes through downregulation of MnSOD.8 RA FLS express lower levels of MnSOD mRNA compared with osteoarthritis FLS,43 which may contribute to the accumulation of oxidative stress-induced mutations in tumor suppressor genes, such as p53, detected in RA FLS and synovial tissue.44 It is unlikely, however, that FoxO1 downregulation is sufficient by itself to promote RA FLS survival and proliferation. For example, the Bcl-XL gene locus is also sensitive to the JNK-dependent AP-1 transcription factor.45 46

In preliminary experiments, we were unable to enhance RA FLS proliferation or survival in the presence of JNKi by first silencing FoxO1 (data not shown), consistent with the convergence of other JNK-dependent pathways, as well as functional redundancies among FoxO family members.47

Collectively, the data presented here identify a novel mechanism regulating FoxO-dependent transcriptional programmes, modulation of FoxO1 mRNA stability, which represents an additional layer in a complex network responsible for fine-tuning of FoxO activity. Recent studies in animal arthritis models have suggested that therapeutic
targeting of PI3K signalling might be beneficial in RA.\textsuperscript{48–50} Although the contributions of FoxO activity to the antiarthritic effects of PI3K inhibitors have not been characterized, at least some effects of PI3K blockade can be attributed to cellular processes regulated by FoxO proteins.\textsuperscript{5,32} Our findings raise the possibility that targeting PI3Ks in RA might be partly hampered by JNK signalling and the subsequent lack of FoxO1 expression in cells exposed to inflammatory cytokines, at least in regard to PI3K inhibitor effects on cell proliferation and survival. Instead, therapeutic strategies directed against JNK, or upstream MAPK kinases (MKK)-4 and -7, may be more beneficial, as they would not only suppress inflammatory activation of immune and stromal cells,\textsuperscript{41} but also restore the capacity of FoxO1 to limit RA FLS proliferation and survival.

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JNK-dependent downregulation of FoxO1 is required to promote RA FLS survival


Supplementary figure 1. IL-1β-induced down-regulation of FoxO1 in RA FLS is predominantly mediated by JNK1.

(A) RA FLS were incubated with pan-JNK inhibitors SP600125 (20 μM), AS601245 (50 μM), or with a JNK2/3-selective inhibitor (N-(3-Cyano-4,5,6,7-tetrahydro-1-benzothien-2-yl)-1-naphthamide, JNKi IX, 1 or 5 μM) for 30 min before stimulation with 1 ng/ml IL-1β for 15 min. Cells were then lysed and protein extracts were subjected to analysis by immunoblotting with anti-phospho(p) c-Jun or anti-histone (H)3 antibodies. A representative of 2 independent experiments is shown. Selective JNK2/3 inhibition partly prevented IL-1β-induced c-Jun phosphorylation, indicating that in RA FLS both JNK1 and JNK2 are activated by IL-1β. (B) RA FLS (n = 4) were incubated with the same concentrations of JNK inhibitors as in (A) followed by stimulation with IL-1β (1 ng/ml) for 4 h. RNA was extracted and changes in FoxO1 mRNA expression were analyzed by qPCR. The graph represents mean±SEM fold change in FoxO1 mRNA expression. While both pan-JNK inhibitors prevented FoxO1 downregulation by IL-1β, the JNK2/3-selective JNKi IX had no effect on FoxO1 mRNA levels, indicating that IL-1β reduces FoxO1 expression predominantly through the JNK1 pathway.

Supplementary figure 2. Silencing of FoxO1 expression in RA FLS upregulates Bcl-XL, but fails to influence the effects of JNKi on Bcl-XL expression.

RA FLS were transfected with control non-targeting siRNA (scrβ, 20 nM) or siRNA specific for FoxO1 (siFoxO1, 20 nM). After 48 h cells were stimulated for 8 h with 1 ng/ml IL-1β in the absence or presence of SP600125 (JNKi), 20 μM). Protein extracts were prepared and analyzed by immunoblotting with antibodies recognizing FoxO1, Bxl-XL and actin. A representative of 4 independent experiments is shown.
SUPPLEMENTARY MATERIALS AND METHODS

mRNA expression analysis by qPCR
Equivalent amounts of RNA extracted from RA FLS or synovial tissue biopsies were reverse transcribed using a First-Strand cDNA synthesis kit (Fermentas, Vilnius, Lithuania), and relative expression of FoxO1, FoxO3a, FoxO4, and IL-6 was determined using Fast SybrGreen PCR Master Mix (Applied Biosystems, Foster City, CA). qPCR reactions were performed on a StepOne Plus Real-Time PCR System (Applied Biosystems,) in duplicate, and specific amplification of PCR products was confirmed by analysis of dissociation curve for each pair of primers. Relative gene expression was calculated using StepOne Software v2.1 (Applied Biosystems) and expressed as the ratio between the gene of interest and expression of 18S and/or GAPDH. Sequences of the primers used in the study are listed in the table below:

<table>
<thead>
<tr>
<th>Gene</th>
<th>forw</th>
<th>rev</th>
</tr>
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<tbody>
<tr>
<td>FoxO1</td>
<td>TCTTCCCTCTGAGTCTGGGTAATT</td>
<td>CAGAGGCTACAAAGGATTCTAGA</td>
</tr>
<tr>
<td>FoxO3a</td>
<td>TCTACCGAGTGTGAGTGTTGCTGT</td>
<td>CGACTATGCGATGCAGGTTGGA</td>
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<tr>
<td>FoxO4</td>
<td>TGGAGAACCCTGGAGTGTTGACA</td>
<td>AAGCCTCCAGGCATGACTCAG</td>
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<tr>
<td>IL-6</td>
<td>GACAGCCACTCACCTCTCA</td>
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</tr>
<tr>
<td>18S</td>
<td>CGGCTACCACTCAAGGAA</td>
<td>GCTGGAATTACCCCGCCT</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GCCAGCCGAGCCACATC</td>
<td>TGACCAGGGCGCCCAATAC</td>
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</table>

mRNA expression profiling by qPCR arrays
After 4 h stimulation with IL-1β total RNA was extracted from RA FLS transduced with control GFP-encoding adenovirus or adenovirus encoding FoxO1ADA using an RNeasy mini kit 2 (Qiagen, Venlo, The Netherlands). cDNA was synthesized from 1 µg of RNA with an RT² First Strand Kit (SABiosciences-Qiagen, Frederick, MD) and expression of 84 genes involved in the regulation of inflammation and cell survival was analyzed using a customized RT² Profiler™ PCR Array set (SABiosciences-Qiagen) according to the manufacturer’s instructions. After PCR amplification, threshold values were equalized for all samples and the threshold cycle (Ct) determined for each analyzed gene. Relative expression of each gene was calculated using StepOne Software v2.1 (Applied Biosystems) and corrected for the mean expression of three housekeeping genes (B2M, HPRT1 and RPL13A).

Immunoblotting
After electrophoresis proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA) using a wet transfer apparatus (Invitrogen). Membranes were then blocked in TBS containing 2% milk and 0.05% Tween-20 (both from Bio-Rad Laboratories) and probed overnight at 4°C with antibodies recognizing FoxO1, tubulin (both from Sigma-Aldrich), phospho-(p)PKB, PKB, p-cJun, histone 3 (all
from Cell Signaling Technology, Beverly, MA), p27Kip1, Bcl-XL, MnSOD (all from BD Transduction Laboratories), BIM and FLIP (both from Enzo Life Sciences, Farmingdale, NY). After extensive washing membranes were incubated with HRP-conjugated anti-mouse or anti-rabbit Ig antibodies (Dako, Glostrup, Denmark), and proteins visualized using a Lumi Light detection kit (Roche Diagnostics, Mannheim, Germany) or a SuperSignal West Femto Chemiluminescent Substrate (Pierce, Rockford, IL).