Regulation of inflammation by histone deacetylases in rheumatoid arthritis: beyond epigenetics
Grabiec, A.M.

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CHAPTER 8

HDAC INHIBITORS PREVENT INFLAMMATION-MEDIATED INACTIVATION OF THE FORKHEAD BOX CLASS O TRANSCRIPTION FACTOR FoxO1 IN RHEUMATOID ARTHRITIS

Aleksander M. Grabiec\textsuperscript{1,2}, Linda M. Hartkamp\textsuperscript{1,2}, Lisa G.M. van Baarsen\textsuperscript{1,2}, Chiara Angiolilli\textsuperscript{1,2}, Olexandr Korchynskyi\textsuperscript{1,2}, Paul P. Tak\textsuperscript{2,3}, Kris A. Reedquist\textsuperscript{1,2}

\textsuperscript{1}Department of Experimental Immunology, \textsuperscript{2}Division of Clinical Immunology and Rheumatology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands. \textsuperscript{3}GlaxoSmithKline, Stevenage, U.K.
ABSTRACT

Reversible acetylation is an important post-translational mechanism regulating activity of many transcription factors, including forkhead box O (FoxO) proteins. FoxOs integrate environmental signals to modulate the expression of genes regulating inflammatory responses, cell cycle and apoptosis. Phosphatidylinositol 3-kinase dependent inactivation of FoxOs has been reported to be enhanced in the synovial tissue of patients with rheumatoid arthritis (RA) compared to disease controls. The goals of this study were to examine the relationship between inflammation and FoxO expression and activity in RA, determine the effects of histone deacetylase inhibitors (HDACi) on FoxO expression and activity in RA fibroblast-like synoviocytes (FLS), and identify gene products relevant to RA regulated by FoxO proteins in RA FLS. In RA synovial tissue, we found that mRNA expression of FoxO1 negatively correlated with clinical parameters of disease activity, as well as synovial IL-6 mRNA levels. In vitro, FLS derived from RA patients expressed lower levels of FoxO1, but not FoxO3a or FoxO4, compared to osteoarthritis FLS. FLS stimulation with IL-1β caused rapid inactivation of FoxO1 DNA binding activity followed by down-regulation of FoxO1 mRNA expression. Exposure of FLS to the HDACi prior to IL-1β stimulation rescued FoxO1 DNA binding activity and expression. Over-expression of a constitutively active FoxO1 mutant altered the balance of expression of pro- and antiapoptotic genes in RA FLS, inducing BIM while suppressing Bcl-XL. Additionally, active FoxO1 induced expression of the cell cycle inhibitor p27Kip1. FoxO1 activation had dual effects on IL-1β-mediated induction of inflammatory mediators: while expression of CCL2, CXCL6, PDGF and ICAM1 was reduced, mRNA of CXCL3, IL-8 and BMP-2 were elevated in FLS transduced with a vector expressing active FoxO1. Our findings suggest that suppressed FoxO1 expression might contribute to perpetuation of inflammation in RA by promoting FLS survival and proliferation, as well as selectively modulating their inflammatory activation. The anti-inflammatory effects of HDACi in RA synovial cells might be at least partly attributed to HDACi-mediated restoration of FoxO1 expression and activity.
INTRODUCTION

Persistent activation of intracellular signalling pathways regulated by cytokines, chemokines and cell-cell contacts is critically involved in driving inflammation in chronic immune-mediated inflammatory diseases (IMIDs), such as rheumatoid arthritis (RA).\(^1,2\) In RA, the accumulation and retention of activated immune cells in affected joints, as well as their interactions with stromal fibroblast-like synoviocytes (FLS), stimulate production of inflammatory mediators and matrix-degrading enzymes which promote irreversible degradation of bone and cartilage.\(^3\) Amongst altered signalling pathways observed in RA, the contributions of nuclear factor-κB (NF-κB), mitogen-activated protein kinases, spleen tyrosine kinase (Syk) and the Janus tyrosine kinase (JAK)/signal transducers and activators of transcription (STAT) pathway to RA pathobiology have been well-documented and attempts to target these pathways therapeutically have led to initial successes in the clinic.\(^4-6\) In contrast, the role of phosphoinositide 3-kinase (PI3K) signalling and its downstream targets, such as forkhead box class O (FoxO) transcription factors, remains poorly understood in RA.\(^7\)

FoxO transcriptional activity is tightly regulated by a complex interplay between phosphorylation, acetylation, and ubiquitilation. Phosphorylation of FoxO family members FoxO1, FoxO3a and FoxO4 by the c-Jun N-terminal kinase (JNK) promotes their nuclear import and enhances transcriptional activity to induce expression of genes regulating cellular stress responses, cell cycle progression and/or apoptosis.\(^8\) In the nucleus, active FoxO proteins interact with histone acetyl transferases (HATs), such as p300, cyclic-AMP responsive element binding (CREB)-binding protein (CBP) and CBP-associated factor (P/CAF) which leads to FoxO acetylation on conserved lysine residues. Although initial studies demonstrated that acetylation reduces FoxO transcriptional activity,\(^9,10\) the picture emerging from more recent reports is more complex and indicates that sequential acetylation and deacetylation steps not only modulate FoxO DNA-binding affinity, but also act in a context-dependent manner to alter FoxO gene target specificity.\(^11\) While acetylated FoxO proteins can induce expression of pro-apoptotic genes, including Bim and Fas ligand, subsequent FoxO deacetylation, mediated by the classical class I/II zinc-dependent histone deacetylases (HDAC) or the class III HDAC Sirt1, targets FoxO to transcribe genes which promote cell cycle arrest and survival in response to environmental stress signals.\(^12-14\) Alternatively, FoxO can be monoubiquitylated on the same lysine residues, which strongly stimulates its transcriptional activity and prevents subsequent acetylation.\(^15\) FoxO transcriptional inactivation is mediated by activation of PI3Ks, which results in the generation of phosphorylated lipid metabolites. These in turn activate pleckstrin homology domain-containing proteins, such as protein kinase B (PKB). PKB-mediated phosphorylation of FoxO transcription factors disrupts their interaction with target DNA sequences and facilitates FoxO interaction with 14-3-3 proteins, ultimately leading to FoxO inactivation and nuclear export.\(^16\) Phosphorylation of FoxO by PKB can lead to its polyubiquitylation, leading to proteosomal degradation.\(^17\)

While the roles of FoxO family members in the regulation of cellular stress responses, proliferation and survival are well-characterized, a complex picture emerges from studies analyzing involvement of FoxO transcriptional activity in modulating inflammatory activation of immune cells. In macrophages, FoxO1 potentiates Toll-like receptor-4 (TLR4) signalling and directly induces expression of IL-1β,\(^18,19\) possibly contributing to obesity-related chronic inflammation and insulin resistance. FoxO1 also promotes the production of inflammatory cytokines and chemokines in adipocytes through induction of the CCAAT/enhancer binding
protein-β (C/EBPβ). On the other hand, increased activity of FoxO1 suppresses NF-κB signalling in the context of cholesterol-induced endoplasmatic reticulum stress to promote macrophage apoptosis while dampening inflammatory activation. A similar phenomenon is observed in FoxO4-deficient mice, which develop more severe mucosal inflammation compared to wild-type mice in a colitis model, associated with increased NF-κB activity in the colonic mucosa. Collectively, these studies indicate that depending on the cellular and disease context, FoxO transcription factors may either promote production of inflammatory mediators, or suppress inflammation by interference with NF-κB signalling.

Previous studies have suggested that persistent activation of the PI3K-PKB axis in the inflamed synovium, observed both in animal arthritis models and in RA synovial tissue, might contribute to pathobiology at least partly through the inactivation of FoxO transcription factors. Indeed, PKB-inactivated FoxO proteins are readily detected in RA synovial tissue, and synovial macrophages express elevated levels of inactive FoxO4 compared with disease controls. Supporting a model in which cell exposure to inflammatory stimuli leads to FoxO inactivation, stimulation with TNFα or IL-1β causes rapid PKB-mediated phosphorylation of FoxO4 and FoxO1 in macrophages and RA FLS, respectively. Notably, alterations in FoxO signalling in RA are not restricted to the site of inflammation, as reduced levels of FoxO1 protein are found in peripheral blood mononuclear cells (PBMCs) of RA patients compared to healthy individuals. Similar changes in FoxO expression have also been reported in other inflammatory conditions: colonic epithelial cells of patients with inflammatory bowel disease express significantly lower levels of FoxO4 compared with healthy controls, indicating that both inactivation as well as reduced expression of FoxO family members might contribute to perpetuation of chronic inflammation in IMIDs.

The present study was undertaken to characterize inflammation-mediated regulation of FoxO1 in RA. We find strong negative associations between FoxO1 expression in RA synovial tissue and clinical parameters of disease activity, as well as local expression of IL-6. Altered FoxO1 expression in RA is also maintained ex vivo, as we observe reduced FoxO1 mRNA levels in RA FLS compared to osteoarthritis (OA) FLS. FoxO1 DNA binding activity and expression is further suppressed by inflammatory stimulation, processes reversed by exposure of RA FLS to HDAC inhibitors (HDACi). Forced expression of constitutively active FoxO1 in RA FLS regulated the expression of a subset of IL-1β-inducible genes previously identified as sensitive to HDACi, suggesting that HDACi mediate their anti-inflammatory effects in RA in part due to restoration of FoxO1 activity and expression.

**MATERIALS AND METHODS**

**Patients and synovial tissue samples.** Synovial biopsy specimens were obtained by arthroscopy from an actively inflamed joint of 20 patients with RA as previously described, embedded in TissueTek OCT (Miles Diagnostics, Elkhart, IN), snap-frozen by immersion in methylbutane (-80°C), and stored in liquid nitrogen until further processing. All RA patients fulfilled the 1987 revised criteria of the American College of Rheumatology. The study was approved by the Medical Ethics Committee of the Academic Medical Center, University of Amsterdam, The Netherlands, and all patients provided written informed consent before participation in the study. Clinical characteristics are detailed in Table 1.
Cell culture. FLS were isolated from synovial biopsies of RA patients, cultured as previously described, and used for experiments between passages 4 and 9. 24 h prior to each experiment culture medium was replaced with medium containing 1% foetal bovine serum (FBS) (Invitrogen). FLS were then treated with medium alone or medium containing 1 μM trichostatin A (TSA) (Sigma-Aldrich, St Louis, MO) or 250 nM ITF2357 (Italfarmaco, Cinisello Balsamo, Italy), followed by stimulation with IL-1β (R&D Systems, Minneapolis, MN), TNFα (Biosource International, Camarillo, CA) or LPS (Sigma-Aldrich) for 1-24 hours.

RNA extraction and quantitative PCR. Total RNA was isolated from FLS and synovial tissue biopsies as described before, and equivalent amounts of RNA were reverse transcribed using a First-Strand cDNA synthesis kit (Fermentas, Vilnius, Lithuania). Gene expression levels were determined by quantitative (q)PCR reaction performed on a StepOne Plus Real-Time PCR System (Applied Biosystems, Foster City, CA) using Power SybrGreen PCR Master Mix (Applied Biosystems). Sequences of the primers used in the study are listed in Table 2. All qPCR reactions were performed in duplicate and specific amplification of PCR products was confirmed by analysis of dissociation curve for each primer pair. Relative gene expression was calculated using StepOne Software v2.1 (Applied Biosystems) and expressed as the ratio between the gene of interest and average expression of 18S and GAPDH.

Measurement of FoxO1 DNA binding activity. FLS were left untreated or were treated with 1 μM TSA, followed by stimulation with IL-1β (10 ng/ml) for 4 or 8 h, and nuclear fractions were extracted using a Nuclear Extraction Kit (Active Motif, Carlsbad, CA). The protein content was assessed by a BCA Protein Assay Kit (Pierce, Rockford, IL) and DNA binding activity of FoxO1 in the nuclear extracts was determined using a TransAM transcription factor ELISA (Active Motif) according to the manufacturer’s instructions.

Overexpression of a constitutively active FoxO1 mutant and gene expression profiling. Control adenoviruses encoding GFP (provided by Dr SW Tas, AMC, Amsterdam, The Netherlands) and adenoviruses encoding constitutively active FoxO1 mutant (FoxO1ADA, provided by Dr. D. Accili, Columbia University, New York) were amplified and titrated in transcomplemental HEK-293 cells and purified with cesium chloride gradient ultracentrifugation as described.
previously. FLs were transduced at a multiplicity of infection (MOI) of 500 and 48 h after transduction were serum starved for 24 h in DMEM containing 1% FBS. Cells were then either left unstimulated or stimulated for 4 h with 1 ng/ml IL-1β in the presence or absence of 250 nM ITF2357. After stimulation total RNA was extracted using an RNeasy mini kit (Qiagen, Venlo, The Netherlands). cDNA was synthesized from 1 μg of RNA with an RT2 First Strand Kit (SABiosciences) 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) according to the manufacturer’s instructions. Briefly, cDNA was diluted in RT2 SybrGreen Rox qPCR Master Mix (SABiosciences) and 25 μl of the experimental cocktail was added to each well of the PCR array. After PCR amplification, threshold values were manually 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 Microsoft Excel spreadsheet software (Microsoft, Redmond, WA) and corrected for the mean expression of three housekeeping genes (B2M, HPRT1 and RPL13A).

**Table 2. Sequences of primers used for qPCR analysis.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
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<td>CAGAGGCTACCAAGGATCATGA</td>
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<tr>
<td>FoxO3a</td>
<td>TCTACGAGTGATGCGCTTT</td>
<td>CGACTATGCAAGCTGTTG</td>
</tr>
<tr>
<td>FoxO4</td>
<td>TGGAGAAGCTGGAGGTGACA</td>
<td>AAGCCTCCAGGATCATCAG</td>
</tr>
<tr>
<td>TNFα</td>
<td>CCCATGGTGCAAACCCCT</td>
<td>TGAAGTCAAGGCTCCTGAT</td>
</tr>
<tr>
<td>MMP1</td>
<td>AGTGAACGGAAAACCAGATGCTGA</td>
<td>GCTCTTGGCAAATCTGCGGT</td>
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<td>IL-6</td>
<td>GACAGCCACTCTACCTTTCA</td>
<td>CTCCTTGGCTGTTACAC</td>
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<tr>
<td>18S</td>
<td>CGGCTACCACATCCAAGGAA</td>
<td>GCTGGAATTACCGCGGCT</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GCCAGCCGAGCCACATC</td>
<td>TGACCAGGCCCAAATAC</td>
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</table>

Measurement of cell viability. RA FLS viability was assessed 96 hours after transduction by incubating cells with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich) (1 mg/ml) for 2 hours, followed by solubilization of cells in acidified isopropanol solution containing 0.1% Igepal CA-630 (Sigma-Aldrich) and measurement of optical density at 590 nm.

Western blotting. Transduced FLS were lysed in 1x Laemmli’s buffer as described before, and equivalent amounts of protein extract were resolved by electrophoresis on 4-12% Bis-Tris SDS NuPAGE gels (Invitrogen). Proteins were then transferred to polyvinylidene difluoride
membranes (Bio-Rad Laboratories, Hercules, CA), membranes were blocked in Tris-buffered saline (pH 8.0) containing 0.05% Tween-20 (Bio-Rad) and 2% milk (Bio-Rad), washed and probed overnight at 4°C with antibodies against FoxO1, tubulin (both from Sigma-Aldrich) and p27Kip1 (Becton Dickinson, San Jose, CA). After extensive washing membranes were incubated with IRDye-800-labelled anti-mouse immunoglobulin secondary antibody (LI-COR Biosciences, Bad Homburg, Germany), and proteins visualized using an Odyssey infrared imaging system (LI-COR Biosciences).

**Statistical analyses.** Data are presented as mean +/- SEM unless otherwise indicated. Correlations of FoxO1 expression with clinical parameters of disease activity and expression of inflammatory mediators were analyzed using the Spearman’s rank correlation coefficient. The Mann-Whitney U test was used for all other comparisons. P values < 0.05 were considered statistically significant.

**RESULTS**

**FoxO1 expression in RA synovial tissue correlates with clinical parameters and IL-6 expression.** Aberrant expression and/or activity of FoxO transcription factors has been reported in a number of chronic inflammatory diseases, including inflammatory bowel disease and RA.22,25 A recent study demonstrating selective reduction of FoxO1 mRNA and protein levels in RA peripheral blood mononuclear cells suggested that alterations in FoxO1 expression might be involved in RA pathobiology.26 To determine if local FoxO1 expression at the site of inflammation is associated with disease activity, we extracted RNA from synovial biopsies of 20 RA patients and analyzed mRNA expression of FoxO1 by qPCR. Strong negative correlations were observed between FoxO1 mRNA expression in RA synovial tissue and the levels of serum C-reactive protein (CRP) (Fig. 1A; \( R = -0.771, P = 0.0008 \)) and the erythrocyte sedimentation rate (ESR) (Fig. 1B; \( R = -0.739, P = 0.0003 \)). Synovial FoxO1 expression also negatively correlated with the 28-joint disease activity score (DAS28) (Fig. 1C, \( R = -0.575, P = 0.01 \)). To get more insight into the relationship between FoxO1 expression and local production of inflammatory mediators, the transcript levels of TNF\( \alpha \), MMP-1 and IL-6 in RA synovial tissue were determined by qPCR. While FoxO1 was not associated with TNF\( \alpha \) or MMP-1 expression (data not shown), we found a strong negative correlation between synovial FoxO1 and IL-6 mRNA levels (Fig. 1D: \( R = -0.628, P = 0.004 \)).

**RA FLS express reduced levels of FoxO1 compared to FLS from OA patients.** We next tested whether potential alterations in FoxO1 mRNA levels are maintained in synovial cells ex vivo. Since FoxO1 is predominantly expressed by FLS in the synovial tissue,25 expression of FoxO family members was analyzed in FLS derived from RA and OA patients by qPCR. FoxO1, FoxO3a and FoxO4 mRNA were each detected in both RA and OA FLS (Fig. 2). While no differences were observed in FoxO3a and FoxO4 expression between RA and OA FLS, the levels of FoxO1 mRNA were significantly reduced in FLS derived from RA patients compared to OA FLS (\( p < 0.05 \)).

**Inflammatory stimulation inactivates and downregulates expression of FoxO1 in RA FLS.** Previous reports indicated that inflammatory stimulation with cytokines or TLR ligands induced PKB-dependent phosphorylation and nuclear exclusion of FoxO transcription factors.18,25 Consistent with these observations, stimulation of RA FLS with IL-1\( \beta \) causes a rapid inactivation...
Figure 1. FoxO1 expression in RA synovial tissue negative correlates with disease activity and local IL-6 mRNA levels. Total RNA was extracted from synovial tissue specimens from 20 RA patients, reverse transcribed and expression of FoxO1 and IL-6 determined by quantitative PCR. Correlations of relative FoxO1 expression with (A) the C-reactive protein (CRP) levels, (B) erythrocyte sedimentation rate (ESR), (C) 28-joint disease activity score (DAS28) and (D) IL-6 expression were calculated using Spearman’s correlation coefficient. Circles indicate individual patient values, and Spearman R values and P values are indicated in each graph.

Figure 2. Expression of FoxO1 but not other FoxO family members is reduced in RA FLS compared to OA FLS. RA FLS (n = 6) and OA FLS (n = 5) were cultured for 24 h in medium containing 1% FBS, total RNA was extracted, cDNA synthesized and relative expression of FoxO1, FoxO3a and FoxO4 analyzed by quantitative PCR. Circles indicate values for individual FLS lines and dashes indicate the means. *p < 0.05, Mann-Whitney U test.
of FoxO1: 50% inhibition of FoxO1 DNA binding was detectable within 1 h after FLS exposure to IL-1β, and this effect was maintained at later time points (Fig. 3A). Stimulation with IL-1β not only reduced FoxO1 activity in RA FLS, but also downregulated FoxO1 mRNA expression in a time-dependent manner, reaching 70% suppression of FoxO1 mRNA levels after 8 h exposure to IL-1β (Fig. 3B). At the same time stimulation with IL-1β failed to modulate expression of FoxO3a and FoxO4 (Fig. 3C and D), identifying FoxO1 as the only FoxO family member susceptible to regulation by this inflammatory stimulus in FLS. Reduction of FoxO1 mRNA expression was not restricted to IL-1 receptor triggering, and was long-lasting, as reductions in FoxO1 transcript levels were found after 24 h of FLS stimulation with IL-1β, TNFα or LPS (Fig. 3E).

**HDACi prevent cytokine mediated inactivation and downregulation of FoxO1 in RA FLS.** Since the activity of FoxO transcription factors can be regulated by reversible acetylation, we next tested whether HDACi could modulate the suppressive effects of inflammatory stimulation on FoxO1 DNA binding and expression. RA FLS were either left untreated or treated with 1 μM TSA for 30 min prior to stimulation with IL-1β for 8 h. Exposure to TSA efficiently prevented

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**Figure 3. Stimulation with IL-1α reduces FoxO1 transcriptional activity and FoxO1 mRNA expression in RA FLS.** (A) RA FLS were either left unstimulated in medium (med) or were stimulated with 10 ng/ml IL-1β for 1 – 8 h, nuclear fractions were extracted and levels of active FoxO1 were determined using an ELISA-based DNA-binding assay. Data represent the mean ± SEM optical density at 450 nm of two to four independent experiments. (B-D) FLS were left untreated (med) or were stimulated with IL-1β (1 ng/ml) for the indicated time (h), total RNA was extracted, cDNA was synthesized and temporal changes in FoxO1 (B), FoxO3a (C), or FoxO4 (D) mRNA accumulation were monitored by quantitative PCR. (E) Alternatively, FLS were left unstimulated or were treated with IL-1β (1 ng/ml), TNFα (10 ng/ml) or LPS (1 mg/ml) for 24 h and FoxO1 mRNA expression analyzed as above. Data are presented as mean ± SEM relative FoxO1 expression of two (B-D) or three (E) independent experiments.
IL-1β-mediated reduction of FoxO1 DNA binding, inducing it 40% above basal levels ($p < 0.01$) (Fig. 4A). TSA not only restored FoxO1 activity in the presence of inflammatory signalling, but also overcame IL-1β-induced downregulation of FoxO1 mRNA expression (Fig. 4B). Induction of FoxO1 mRNA was noted 1 h after TSA treatment and was maintained at later time points. Similar effects were observed after FLS exposure to 250 nM ITF2357 in the presence of IL-1β stimulation (data not shown). The effects of HDACi on FoxO1 mRNA expression in RA FLS were specific, as TSA treatment had no effect on FoxO4 and FoxO3a mRNA expression (Fig. 4C and data not shown).

**Figure 4.** Histone deacetylase inhibitors (HDACi) prevent IL-1β-mediated downregulation of FoxO1 transcriptional activity and expression in RA FLS. (A) RA FLS were either left unstimulated (med) or were stimulated with 10 ng/ml IL-1β with or without 1 μM trichostatin A (TSA) 8 h, and FoxO1 DNA-binding activity was assessed in nuclear extracts using an ELISA-based DNA-binding assay. Data represent the mean +/- SEM optical density at 450 nm of five independent experiments. **$p < 0.01$, Mann-Whitney $U$ test. Alternatively, FLS were left untreated (med) or were treated with 1 μM TSA prior to stimulation with IL-1β (10 ng/ml) for the indicated time (h), RNA was isolated, reverse transcribed and relative expression of (B) FoxO1 and (C) FoxO4 was determined by quantitative PCR. Data represent the mean +/- SEM of four independent experiment. *$p < 0.05$, Mann-Whitney $U$ test.

**Overexpression of constitutively active FoxO1 modulates RA FLS inflammatory activation and viability.** To gain more insight into biological consequences of altered FoxO1 expression in RA FLS we transduced the cells with an adenovirus encoding constitutively active FoxO1 (FoxO1ADA). Overexpression and biological activity of the construct was confirmed by Western blotting and FoxO1 DNA-binding assay, respectively (Fig. 5A and B). In line with previous studies reporting induction of apoptosis after forced expression of active FoxO transcription factors, overexpression of FoxO1 reduced the viability of RA FLS by approximately 60% (Fig. 5C). We next used low density qPCR array system to analyze effects of FoxO1ADA on expression profiles of known FoxO1 transcriptional targets as well as genes previously identified to be regulated by HDACi in RA FLS (chapter 7). We observed altered expression of a number of genes involved in regulation of apoptosis and cell cycle progression: FoxO1ADA reduced the mRNA levels of the Bcl-2-like protein Bcl-XL, while expression of BIM and the cell cycle inhibitor p27Kip1 was elevated in FLS expressing FoxO1ADA compared to cells transduced with the control GFP-encoding vector (Fig. 5D). FoxO1-mediated induction of p27Kip1 was also observed at the protein level as detected by Western blotting of FLS protein extracts (Fig. 5A).
Forced expression of active FoxO1 not only influenced expression of genes regulating cell survival, but also modulated inflammatory activation of RA FLS in a complex fashion. IL-1β-mediated mRNA induction of the chemokines CXC chemokine ligand-6 (CXCL6) and CC chemokine ligand-2 (CCL2), platelet-derived growth factor (PDGF) as well as intracellular adhesion molecule-1 (ICAM1) was inhibited in FoxO1ADA-expressing FLS compared to FLS overexpressing GFP (Fig. 6A-D). On the other hand, constitutive FoxO1 activation induced mRNA accumulation of IL-8, CXCL3 and bone morphogenic protein-2 (BMP-2) in the presence of IL-1β stimulation (Fig. 6E-G), while leaving IL-1β mRNA levels unaffected (Fig. 6H). All genes assessed which were modulated more than 2-fold in FoxO1ADA-expressing FLS compared to FLS expressing GFP after 4 hour cell exposure to IL-1β are listed in Table 3. Collectively, these results indicate that persistent activation and resistance to apoptosis of RA FLS might be at least partly attributed to reduced expression of FoxO1, and suggest that restoration of FoxO1 expression and activity by HDACi might be one of the mechanisms contributing to therapeutic effects of HDAC inhibition in RA.

**DISCUSSION**

FoxO transcription factors play a central role in integrating extracellular signals to modulate cell fate choices. In the absence of growth factor, cytokine and antigen receptor signalling, all of which activate the PI3K/PKB pathway, FoxO family members localize to the nucleus to...
Table 3. List of genes identified as differentially regulated in IL-1β-stimulated FLS expressing FoxO1ADA. Results are presented as fold induction of mRNA expression in FLS transduced with FoxO1ADA compared to cells expressing control GFP vector after 4 h stimulation with 1 ng/ml IL-1β. Data represent mean of two independent experiments and standard deviation.

<table>
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<th>Gene</th>
<th>Fold induction (FoxO1ADA vs GFP)</th>
<th>Standard deviation</th>
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<tr>
<td>ADORA2A</td>
<td>0.18</td>
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<td>ANGPT2</td>
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<td>BCL2A1</td>
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<td>BCL2L11</td>
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<td>BMP2</td>
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<td>CCL2</td>
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<td>CD44</td>
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<td>CDKN1B</td>
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modulate expression of genes regulating cell cycle progression, proliferation and survival. In recent years it has become apparent that several genes involved in cellular inflammatory responses are direct FoxO targets, and studies in animals lacking FoxO proteins in the immune cells provided evidence for essential roles for FoxO transcriptional activity in the immune system both under homeostatic conditions and in chronic inflammation. In RA, PKB-mediated inactivation as well as reduced expression of FoxO family members have been reported, both of which might contribute to pathology. In systemic lupus erythematosus (SLE), another chronic IMID, FoxO1 expression in PBMCs negatively correlates with disease severity, indicating involvement of FoxOs in the protection against autoimmune processes. In this study, we show that FoxO1 mRNA expression in RA synovial tissue negatively correlates with clinical parameters of disease activity and local IL-6 expression, that FoxO1 activity and expression are rapidly reduced after inflammatory stimulation of RA FLS in an HDACi-sensitive manner, and that restoration of FoxO1 activity and expression modulates a subset of gene products regulating cellular survival, proliferation, and activation in RA FLS.

Importantly, we observed reduced mRNA levels of FoxO1, but not other FoxO family members, in RA FLS compared to OA FLS, suggesting that alterations in FoxO expression in synovial cells are maintained ex vivo even in the absence of persistent exposure to inflammatory stimulation. FoxO1 expression in FLS was also selectively downregulated by IL-1β in a time-dependent manner. Reduced expression and/or inactivation of FoxO1 in RA FLS...
FLS might contribute to their invasive properties and relative resistance to apoptosis. In line with this possibility, we observed altered expression profiles of two Bcl-2 family members, which are directly or indirectly regulated by FoxO proteins: proapoptotic BIM was induced, while expression of the antiapoptotic protein Bcl-XL was downregulated in FLS expressing constitutively active FoxO1, supporting the model in which FoxO1 activation might promote apoptosis. Consistently, overexpression of FoxO1ADA reduced FLS viability as determined by an MTT assay. Additional experiments are still needed to verify if reduced MTT signal in FoxO1ADA-expressing FLS results from induction of apoptosis, suppression of proliferation or effects on cell adhesion, all of which can affect viability measurements using this method. Curiously, Bcl-XL is elevated in the intimal lining layer in RA synovial tissue compared to OA, raising the possibility that reduced expression of FoxO1 might promote FLS survival through Bcl-XL upregulation. It remains to be tested if alterations in Bcl-XL levels are maintained in FLS in vitro and are associated with FoxO1 expression and/or activity. Reduced levels of FoxO1 expression might also sensitize FLS to oxidative stress and prevent DNA repair processes through downregulation of manganese-dependent superoxide dismutase (MnSOD) and growth arrest and DNA-damage-inducible protein 45 (GADD45), both of which are FoxO transcriptional targets. Indeed, RA FLS express lower levels of MnSOD mRNA compared to OA FLS. Downregulation of reactive oxygen species scavengers might in turn promote oxidative stress-induced accumulation of mutations in tumour suppressor genes, such as p53, further contributing to FLS persistence and proliferation in RA synovial tissue.

Inflammatory stimulation of RA FLS not only induced rapid PKB-mediated inactivation of FoxO1, as observed previously, but also caused rapid alterations in FoxO1 mRNA expression. While further studies are needed to identify the mechanism(s) underlying IL-1β-mediated reduction of FoxO mRNA accumulation, initial observations in murine B cells, which downregulate FoxO1 expression in response to B cell receptor activation, indicate that the PI3K-PKB pathway is at least partly responsible for this effect. Furthermore, the promoter region of FoxO1 contains a conserved FoxO-binding motif, and this site is indispensable for FoxO1 transcriptional induction by itself and by FoxO3a. Consistently, treatment of human fibroblasts with growth factors, leading to activation of PI3K signalling and FoxO nuclear exclusion, represses the expression of FoxO1 and, to a lesser extent, other FoxO family members, demonstrating that FoxO activity is required for maintaining FoxO1 expression. However, in this study we observed that overexpression of an active FoxO1 mutant in RA FLS failed to modulate endogenous FoxO1 expression (data not shown), arguing against the positive feedback loop mode of FoxO regulation in this cellular model.

We identify HDACi as potent regulators of FoxO1 activity and expression in RA FLS. FLS treatment with TSA not only prevented IL-1β-mediated inactivation of FoxO1, but actually induced FoxO1 DNA binding activity above the levels observed in resting cells. FoxO1 mRNA expression in the presence of IL-1β was regulated by HDACi in a similar manner. HDAC activity plays a central role in modulating FoxO transcriptional activity in response to environmental stimuli by modulating FoxO gene target specificity. Although studies have identified the class III HDAC Sirt1 as a prominent regulator of FoxO acetylation status, observations that TSA, an inhibitor of class I/II HDACs, induces FoxO acetylation indicate that zinc-dependent HDACs also deacetylate FoxO proteins. Notably, HDACi suppress production of inflammatory cytokines by immune cells derived from both healthy individuals and RA patients. HDACi are also potent therapeutics in animal arthritis models, and protective effects of HDACi in...
autoantibody- and adjuvant-induced arthritis in rodents are associated with induction of the cell cycle inhibitor p21\(^\text{WAF1/Cip1}\), a transcriptional target of FoxOs.\(^{54,55}\) Similarly, HDACi upregulate p21\(^\text{WAF1/Cip1}\) and reduce proliferation of RA FLS in vitro.\(^{53,56,57}\) These observations suggest that at least some of the anti-arthritic effects of HDACi might be attributable to acetylation-mediated modulation of FoxO1 activity by HDACi.\(^7\) However, since direct interaction between FoxO proteins and class I/II HDACs has never been demonstrated experimentally, it is possible that restoration of FoxO1 activity in IL-1\(\beta\)–stimulated FLS results solely from transcriptional induction of the FoxO1 gene. Additional experiments are needed to test if treatment with HDACi maintains FoxO1 in the acetylated state, and to verify whether FoxO1 transcriptional induction by HDACi is mediated by epigenetic mechanisms related to relaxation of chromatin structure at the FoxO1 promoter, or result from HDACi regulation of signalling pathways and/or activity of transcription factors that regulate FoxO1 expression.

Importantly, we identify subsets of cytokines and other mediators of inflammation that are either suppressed or induced by constitutive FoxO1 activation in cytokine-stimulated FLS. This finding is in line with recent reports demonstrating complex roles for FoxO transcription factors in modulation of cellular inflammatory responses. While a relative loss of FoxO activity associates with certain autoimmune phenomena,\(^{37}\) chronic FoxO activation has been shown to maintain inflammation in the context of insulin resistance in obesity and diabetes,\(^{58,59}\) suggesting that FoxO proteins might interpret contextual signals in a cell- and tissue-specific manner to either suppress or promote inflammatory processes. Notably, FoxO1ADA had hardly any effect on basal expression of these mediators compared to the degree of induction of their mRNAs by IL-1\(\beta\). This indicates that either transcriptional coactivation of these genes by FoxO1 is minor compared to other transcription factors, that further biochemical modification of FoxO1 or activation of co-factors is required, or that these loci are not direct FoxO1 targets.

For example, FoxO1 might modulate expression of these gene products through cross-talk with other signalling pathways, such as NF-\(\kappa\)B. FoxO4 directly interacts with the Rel-homology domain of NF-\(\kappa\)B p50 which might inhibit its DNA binding activity,\(^{22}\) and in vivo, elevated NF-\(\kappa\)B activity renders mice lacking FoxO4 more susceptible to trinitrobenzene sulfonic acid-induced colitis.\(^{22}\) In macrophages, FoxO1 drives the expression of inhibitor of \(\kappa\)B \(\varepsilon\) (\(I\kappa B\varepsilon\)) which attenuates NF-\(\kappa\)B activation during endoplasmatic reticulum stress.\(^{21}\) A similar mode of NF-\(\kappa\)B regulation has been reported in FoxO3a-deficient mice which develop spontaneous autoimmunity due to persistent T cell proliferation and survival. In this model, the lack of FoxO3a leads to reduction of \(\kappa\)B\(\varepsilon\) expression and the resulting increases in NF-\(\kappa\)B activity protect T cells against apoptosis,\(^{60}\) suggesting that at the systemic level, FoxO activity has anti-rather than proinflammatory functions and protects against systemic autoimmunity. Acetylation of FoxO proteins might also be involved in the FoxO-NF-\(\kappa\)B cross-talk as murine macrophages homozygous for an allele encoding FoxO1 in its deacetylated form are characterized by impaired nuclear translocation of the p65 subunit of NF-\(\kappa\)B after free cholesterol loading, possibly through attenuation of p105 proteolysis.\(^{61}\) Collectively, these observations show that FoxO proteins modulate NF-\(\kappa\)B signalling at multiple levels, implying that FoxOs may fine-tune immune and inflammatory responses through interplay with other transcription factors.\(^{52}\) The mechanisms underlying modulation of cytokine and chemokine expression in RA by FoxOs remain unknown and studies of FLS overexpressing acetylation-deficient and acetylation-mimicking FoxO1 mutants, possibly in combination with silencing of endogenous FoxO1 expression may be helpful in determining the exact role of FoxO1 inflammatory activation of FLS.
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