Ras family GTPase signaling contributions to inflammation and joint destruction in rheumatoid arthritis

de Launay, D.

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chapter 6
Inhibition of FoxO Family Member Transcription Factors in Rheumatoid Synovial Tissue
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Inhibition of FoxO Family Member Transcription Factors in Rheumatoid Synovial Tissue

J Ludikhuize, D de Launay, D Groot, TJM Smeets, M Vinkenoog, ME Sanders, SW Tas MD PhD, PP Tak MD PhD, and KA Reedquist PhD

Division of Clinical Immunology and Rheumatology, Academic Medical Center, University of Amsterdam, The Netherlands

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Abstract

Objectives: Phosphatidylinositol 3-kinase (PI3K)-dependent activation of protein kinase B (PKB) has been observed in rheumatoid arthritis (RA) synovial tissue, and interfering with PI3K-dependent PKB activation is protective in animal arthritis models. PKB can regulate cellular survival and proliferation via phosphorylation-dependent inactivation of FoxO family transcription factors. We examined if FoxO transcription factors are differentially inactivated in RA synovial tissue and if this inactivation correlates with disease parameters.

Methods: We assessed FoxO family expression and phosphorylation in synovial biopsies from 12 RA and 9 inflammatory osteoarthritis (OA) patients by immunohistochemistry and quantitative computer-assisted image analysis. IL-1β and TNF-α-induced phosphorylation of FoxO1 and FoxO4 in cultured fibroblast-like synoviocytes (FLS) and macrophages, respectively, was detected by immunoblotting.

Results: FoxO1, FoxO3a, and FoxO4 were expressed and phosphorylated in both RA and OA synovial tissue. Phosphorylation of FoxO1 was observed in both RA FLS and synovial macrophages. FoxO3a and FoxO4 were phosphorylated in T lymphocytes and macrophages, respectively. FoxO1 and FoxO4 were phosphorylated in RA and OA FLS, and macrophages, respectively, following IL-1β and TNF-α stimulation. Inactivation of FoxO4 was significantly enhanced in RA synovial sublining compared to inflammatory OA. Strong negative correlations were observed between inactivation of FoxO4 in RA synovial tissue and CRP and ESR serum levels.

Conclusion: Although all three FoxO family members are phosphorylated in both RA and OA synovial tissue, inactivation of FoxO4 in synovial macrophages is significantly enhanced in RA. Cell-specific inactivation of FoxO family members may differentially regulate cell survival and proliferation in the RA synovium.
Introduction

Inflammation and bone destruction in the affected joints of rheumatoid arthritis (RA) patients results from the improper recruitment, activation, proliferation, survival and retention of white blood cells and stromal cells [1]. While the mechanisms leading to the induction of RA are poorly understood, defects in cellular apoptosis contribute to the accumulation and persistence of inflammatory neutrophils, macrophages, lymphocytes and fibroblast-like synoviocytes (FLS) in RA synovial tissue [2,3]. Intracellular signaling pathways, including NF-κB, p53, and phosphatidylinositol 3-kinase (PI3K) pathways, regulate apoptosis and cell survival in the RA synovium through modulation of expression and activity of proteins involved in apoptosis, and a better understanding of these signaling pathways may provide new therapeutic tools in the treatment of RA (2-5). PI3Ks have recently emerged as potential key mediators of cell recruitment to, and persistence in, the RA synovial joint [5;6]. PI3Ks are heterodimeric enzymes which can be activated by recruitment of the regulatory subunit p85 to activated growth factor and cytokine receptors, or direct binding of the catalytic subunit to activated G-protein coupled receptors, including chemokine receptors, or Ras family GTPases. The resultant generation of phosphorylated lipid metabolites recruits and activates pleckstrin homology (PH) domain-containing proteins, such as the protooncogene product protein kinase B (PKB, also known as Akt). PI3K signaling is attenuated by the tumor suppressor phosphoinositide phosphatase, phosphatase and tensin homolog deleted on chromosome 10 (PTEN). Involvement of PI3K in RA was suggested by the observation that PTEN mRNA expression is decreased in the intimal lining layer compared to the synovial sublining, and in invasive RA FLS in the severe combined immunodeficiency (SCID) mouse model of RA [7]. This would be predicted to result in activation of PI3K signaling, a notion substantiated by the finding that PI3K-dependent activation of PKB is enhanced in RA synovial tissue and FLS, compared to tissue and FLS obtained from OA patients [8]. Many of the effects of PI3K on cellular proliferation and survival are mediated by PKB-dependent phosphorylation and subsequent inactivation of three FoxO family transcription factors, FoxO1, FoxO3a, and FoxO4 [9]. Activated PKB phosphorylates FoxO family members on conserved residues, directly interfering with FoxO binding to target DNA sequences [10;11], and/or promoting FoxO association with 14-3-3 proteins mediating FoxO nuclear export and exclusion [11]. Unphosphorylated active FoxO family proteins promote transcription of genes which regulate cell cycle progression and survival, including FasL, p27KIP1 [12;13], cyclin D proteins [14], Bim [15], Bcl-6 [16], and manganese-dependent superoxide dismutase [17]. In overexpression studies, constitutively active FoxO proteins can stimulate cell-cycle arrest or apoptosis, in a protein- and cell-specific manner. A potential role for FoxO family members in autoimmune disease has been suggested by analysis of FoxO3a-deficient mice, which display an age-dependent T cell lymphoproliferation, associated with multi-organ inflammation [18]. As these studies suggested a potential role for PKB-dependent inactivation of FoxO family members in human chronic inflammatory disease, we examined the expression and PKB-dependent phosphorylation status of FoxO family members in RA synovial tissue.
Activatie van een ontstekings pathway hoeft niet per se bij te dragen aan de ontsteking
Patients and Methods

Patient samples
Synovial biopsy samples were obtained from clinically active joints of twelve patients with active RA and 9 patients with inflammatory osteoarthritis (OA) undergoing joint needle arthroscopy as previously described [19]. RA patients fulfilled the American College of Rheumatology (formerly American Rheumatism Association) revised criteria for the diagnosis of RA [20]. OA patients fulfilled established criteria [21] and had a joint effusion in the absence of rheumatologic disease other than OA. The patients were followed at least one year to allow confirmation of the diagnosis. Patient characteristics are shown in Table 1. A synovial biopsy was obtained from the wrist of one RA patient and all other RA and OA biopsies were obtained from knee joints. Synovial biopsy specimens were immediately embedded in Tissue Tek OCT (Miles Diagnostics, Elkhart, IN), snap-frozen by immersion in methylbutane (-80°C) and stored in liquid nitrogen until further processing. All patients provided informed consent prior to the start of this study. This study was approved by the Medical Ethics Committee of the Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands.

Table 1 / Characteristics of study patients

<table>
<thead>
<tr>
<th>Disease diagnosis</th>
<th>RA</th>
<th>OA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>56.2 (41–72)</td>
<td>54.5 (43–76)</td>
</tr>
<tr>
<td>No. men/no. women</td>
<td>6/5</td>
<td>4/4</td>
</tr>
<tr>
<td>% receiving DMARDs</td>
<td>40</td>
<td>0</td>
</tr>
<tr>
<td>% rheumatoid factor positive</td>
<td>80</td>
<td>12</td>
</tr>
<tr>
<td>ESR, mm/hour</td>
<td>65.8 (21–108)</td>
<td>21.5 (13–63)</td>
</tr>
<tr>
<td>CRP, mg/liter</td>
<td>28.7 (8–53)</td>
<td>10.5 (7–55)</td>
</tr>
<tr>
<td>Disease duration, median months (range)</td>
<td>75.1 (2–300)</td>
<td>35 (2–240)</td>
</tr>
<tr>
<td>Morning stiffness, median minutes (range)</td>
<td>36.2 (0–80)</td>
<td>NA</td>
</tr>
<tr>
<td>Assesment of pain, mm (0–100 mm VAS)</td>
<td>56.8 (40–90)</td>
<td>10.0 (0–90)</td>
</tr>
<tr>
<td>Swollen joint count</td>
<td>7.1 (3–14)</td>
<td>1.0 (1–1)</td>
</tr>
</tbody>
</table>

Except when indicated otherwise, values represent the median (range). RA = rheumatoid arthritis; OA = osteoarthritis; DMARDs = disease-modifying antirheumatic drugs; ESR = erythrocyte sedimentation rate; CRP = C-reactive protein; VAS = Visual analog scale; NA = not applicable.
Immunohistochemical analysis
Serial sections of six different biopsy samples were cut with a cryostat (5 μm), fixed with acetone, and endogenous hydrogen peroxide activity was quenched with 0.3% hydrogen peroxide. Sections were stained overnight at 4°C with rabbit polyclonal antibodies against phospho-PKB (Thr308), FoxO1, phospho-FoxO1(Ser256) (all from New England BioLabs, Beverly, MA), FoxO3a, phospho-FoxO3a (Ser253) (both from Upstate Biotechnology, Lake Placid, NY), goat polyclonal antibodies against FoxO4 and phospho–FoxO4 (Ser193) (both from Santa Cruz Biotechnology, Santa Cruz, CA), or PTEN monoclonal antibody (clone 6H2.1, Cedarlane Laboratories, Hornby, Ontario, Canada). Equivalent concentrations of irrelevant control polyclonal rabbit and goat, or monoclonal mouse antibodies were used as negative controls. Bound antibody was detected according to a 3-step immunoperoxidase method using a biotinylated tyramine amplification method as previously described [22]. Slides were incubated with horseradish peroxidase (HRP)-conjugated swine anti-rabbit, swine anti-goat, or goat anti-mouse immunoglobulin (Dako, Glostrup, Denmark), followed by biotinylated tyramide (Dako), streptavidin-HRP (Dako) and developed with amino-ethylcarbazole (AEC, Vector Laboratories, Buringame, CA). Slides were counterstained with Mayer’s hematoxylin (Merck, Darmstadt, Germany) and mounted in Kaiser’s glycerol gelatin (Merck). Cell-specific expression or phosphorylation of proteins in synovial tissue was assessed by inclusion of antibodies against T lymphocytes (anti-CD3, Becton Dickinson, San Jose, CA), FLS (anti–CD55, mAb67, Serotec, Oxford, UK), and macrophages (anti–CD68, clone DK25, and anti-CD163, clone Ber-MAC3, both from Dako). Staining with anti-phospho-FoxO was developed with AEC as above. Sections were then labeled with antibodies against cell markers, followed by goat antimouse alkaline phosphatase-conjugated antibody (Dako), and development with FastBlue (Vector). Alternatively, sections were labeled with primary antibodies against phospho–FoxO proteins overnight at 4°C, followed by incubation with Alexa-488-conjugated goat anti-rabbit or rabbit anti-goat antibodies (Molecular Probes Europe, Leiden, The Netherlands). Sections were then incubated with mouse monoclonal antibodies (see above) against CD3, CD55, CD68, or CD163 for one hour at room temperature, followed by labeling with Alexa-596-conjugated goat anti-mouse antibody (Molecular Probes Europe). After labeling, slides were mounted in Vectashield (H-1000, Vector) and analysed using a fluorescence microscope (Leica DMRA, Wetzlar, Germany) coupled to a CCD camera and Image-Pro Plus software (Media Cybernetics, Dutch Vision Components, Breda, The Netherlands).

Digital image analysis
For analysis of FoxO expression and phosphorylation in RA and OA synovial biopsies, 18 high-power fields were analyzed by computer-assisted digital imaging analysis using the Qwin analysis system (Leica, Cambridge, UK) as previously described [23;24]. Briefly, all sections were coded and analyzed in a random order by and independent observer blinded to the clinical diagnosis (MV). The number of positive cells per mm² tissue was calculated. Additionally, to assess the degree of phosphorylation in positive cells, we also calculated the integrated optical density (IOD) per cell (expressed as median
(IOD)/nucleus/mm²). Values were calculated for both intimal lining layer and synovial sublining, and normalized for tissue cellularity. For double stainings, slides were analyzed qualitatively by two independent observers (JL and TJMS).

Cell culture and stimulation
RA and OA FLS were isolated from patient synovial biopsy samples by enzymatic digestion and culture in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum (FCS). Experiments were conducted using fourth to ninth passage FLS. FLS were plated in 12-well tissue culture dishes and cultured in medium containing 0.5% FCS for 24 hours prior to stimulation. Monocytes were obtained from healthy donor peripheral blood by sequential density gradient centrifugation on Lymphoprep (Nycomed, Torshov, Norway) and Percoll (Pharmacia Biotech, Uppsala, Sweden) as previously described [25], and differentiated into macrophages by culture for 7–10 days in medium containing 500 units/ml recombinant GM-CSF (R&D Systems Europe Ltd., Abingdon, UK). Differentiation into CD68+ macrophages was confirmed by flow cytometric analysis. FLS or macrophages were left unstimulated or stimulated over timecourses ranging from 5–60 minutes with 125 pg/ml recombinant IL-1β or 10 ng/ml recombinant TNF-α (both compounds from R&D Systems Europe Ltd.). Stimulations were quenched by washing cells in cold phosphate-buffered saline (PBS) and lysis in 1x Laemlli’s sample buffer.

Immunoblotting analysis
Proteins from FLS and macrophage lysates were resolved on 9% sodium dodecyl sulphate (SDS)-polyacrylamide gels and transferred to polyvinylidene difluoride membrane (PVDF) (Bio-Rad, Hercules, CA). Membranes were blocked for 1 hour at room temperature in Tris-buffered saline (10 mM Tris, pH 8.0; 150 mM NaCl)/0.05% Tween–20 (Bio-Rad) (TBS/T) containing 2% milk protein (Bio-Rad). Membranes were then incubated overnight at 4°C in primary antibodies diluted 1:1000 in TBS/T. Primary rabbit antibodies used for immunoblotting were: anti-FoxO1 (FKHR), anti-phospho-FoxO1 (FKHR), anti-FoxO4 (AFX), and anti-phospho-FoxO4 (AFX) (all from Cell Signalling). Blots were then washed extensively, incubated for 1 hour at room temperature with HRP-conjugated anti-rabbit immunoglobulin (Bio-Rad), washed again, and developed using an enhanced chemoluminescence detection kit (Amersham, Little Chalfont, U. K.).

Statistical analysis
Wilcoxon’s nonparametric signed ranks test was used to compare protein expression and/or phosphorylation between intimal lining layer and synovial sublining within diagnosis groups. The Mann-Whitney U test was used to compare differences between OA and RA expression and/or phosphorylation of FoxO transcription factors. Correlations between patient clinical parameters and FoxO expression or phosphorylation were assessed using Spearman’s rank correlation coefficient. P values less than 0.05 were considered statistically significant. Given the exploratory nature of the study, statistical significance was determined without adjustment for multiple comparisons.
Results

Expression and phosphorylation of FoxO family members in RA synovial tissue.

In accordance with previous reports [8], phosphorylated active PKB was readily detected in RA synovial tissue, and observed throughout the synovial sublining and intimal lining layer (Figure 1). We next examined the expression and phosphorylation status of FoxO family members in RA synovial tissue. Although FoxO1 was ubiquitously expressed throughout the intimal lining layer and synovial sublining, phosphorylated inactive FoxO1 was predominantly detected in the intimal lining layer. Both FoxO3a and phospho-FoxO3a were observed primarily in the synovial sublining. FoxO4 and phospho-FoxO4 were also most readily detected in the synovial sublining, although staining could also be observed in the intimal lining layer. No detectable staining was observed with irrelevant control polyclonal rabbit or goat antibodies.

Figure 1 / FoxO family members are expressed and phosphorylated in RA synovial tissue. RA synovial tissue sections were stained with control irrelevant rabbit or goat IgG, or antibodies against phospho (p) PKB, pFoxO1, FoxO1, pFoxO3a, FoxO3a, pFoxO4, and FoxO4 as indicated. Stainings were developed with biotin tyramide enhancement horseradish peroxidase and AEC, followed by Mayer’s hematoxyline counterstaining.
Cell-specific inactivation of FoxO family proteins in RA synovial tissue.

As FoxO family members are known to have cell-specific functions in the regulation of cell proliferation and survival, we sought to identify cells in which FoxO family members had been inactivated by phosphorylation (Figure 2A). Phospho-FoxO1 was observed in CD55-positive FLS and, less frequently, in both CD68 and CD163 positive macrophages, primarily in the intimal lining layer (indicated by arrows). Phosphorylated FoxO3a was observed primarily in CD3-positive T lymphocytes. Not all T cells displayed inactivation of FoxO3a, but rather, phosphorylated FoxO3a was found almost exclusively in T cell aggregates. Phosphorylated FoxO4 was detected only in CD68 and CD163-positive macrophages. These results were confirmed by an independent methodology, immunofluorescent double-labelling of RA synovial biopsy tissue (Figure 2B). Here, FoxO1 phosphorylation was observed only in CD55-positive FLS, and FoxO4 phosphorylation only in CD68 and CD163-positive synovial macrophages. FoxO3a phosphorylation was again observed in CD3-positive T lymphocytes. We were unable to detect phosphorylation of any of the FoxO proteins in synovial B lymphocytes (data not shown).

Figure 2 / Phosphorylation of FoxO family members in specific cell-types in RA synovial tissue.
(a) Representative double-staining of RA synovial tissue with phospho-specific anti-FoxO family antibodies and cell-specific markers. Synovial tissue sections were stained overnight at 4°C with anti-phospho (p)FoxO antibodies, followed by antibodies to detect CD3-positive T lymphocytes, CD55-positive FLS, and CD68 and CD163-positive macrophages. After biotin thiyramide enhancement, staining was developed with AEC (red, pFoxO protein) and fast blue (blue, cell-specific markers). Arrowheads show representative double-stained cells, except for pFoxO3a staining in which colocalization with CD3-positive T cells is readily evident. (b) Representative merged micrographs of immunofluorescent double-staining of RA synovial tissue with phosphospecific anti-FoxO antibodies (green) and CD markers (red).
Comparison of FoxO protein expression and phosphorylation status in RA and OA patients.

We next sought to determine if FoxO family members were differentially phosphorylated in RA and inflammatory OA synovial tissue. Therefore, we performed quantitative computer-assisted image analysis of FoxO expression and phosphorylation on synovial biopsy samples. Twelve RA and nine inflammatory OA patients were included in this study (see Table 1 for patient characteristics). The median age for the RA patients was 60 years (range 41-72), and median disease duration was 11 months (range 2-300). Three RA patients were using methotrexate (12.5, 15, and 25 mg/week) at the time of biopsy, and two were being treated with prednisolone (5 and 20 mg once daily). The median age for OA patients was 54 years (range 43-76), with a disease duration of 35 months (range 2-240). Four OA patients were being treated with NSAIDs at the time of biopsy. No statistically significant differences in either expression or number of expressing cells were observed for FoxO1, FoxO3a or FoxO4 between RA and inflammatory OA synovial tissue, using antibodies recognizing total (both phosphorylated and non-phosphorylated) FoxO proteins, either in the intimal lining layer or the synovial sublining (data not shown). Phosphorylation of FoxO1 was enhanced, but insignificantly so, in both RA intimal lining layer (median IOD and range 13,891 [range, 0-40,047]) and synovial sublining (15,641 [range, 19-43,362]) compared to OA synovial tissue (intimal lining layer 3032 [range, 19-24,228]; synovial sublining 3660 [range, 23-32,752]) (Figure 3). A similar but insignificant trend was observed in the number of cells staining positive for phospho-FoxO1 (RA intimal lining layer 755 positive cells/mm² [range, 0-1,573]; OA intimal lining layer 262 [range, 2-1,042]; RA synovial sublining 1028 [range, 3-2,065]; OA synovial sublining 370 [range, 6-1,505]). For FoxO3a, although the intensity of protein phospho-
ylation was statistically insignificant between RA and OA intimal lining layer and synovial sublining, the number of phospho-FoxO3a positive cells in RA intimal lining layer (441 [range, 154–1,245]) was decreased compared to OA (988 [range, 274–1,697]) (p< 0.005). Of interest, significant differences in FoxO4 phosphorylation were observed between RA and OA. The intensity of phospho-FoxO4 staining was significantly higher in intimal lining layer (RA 2,561 [range, 0–14,569]; OA 136 [range, 0–1,650]; p< 0.05) and synovial sublining (RA 16,404 [range, 640–117,572]; OA 1,171 [range, 299–6,896]; p< 0.01) in RA compared to OA. Similarly, the number of phospho-FoxO4 positive cells was significantly elevated in RA intimal lining layer (RA 89 [range, 0–556]; OA 18 [range, 0–89]; p< 0.05) and synovial sublining (RA 904 [range, 53–4,221]; OA 80 [range, 30–422]; p< 0.05).

To measure the degree of inactivation of each FoxO family member in synovial tissue, we calculated the ratio of phospho-specific staining intensity to the number of cells staining positive for phospho-specific antibodies (Figure 3, right panels). Again, no statistically significant differences were observed in the degree of phosphorylation of FoxO1 or FoxO3a between RA and OA synovial tissue, either in the intimal lining layer or synovial sublining. In contrast, in RA synovial sublining, but not the intimal lining layer, the degree of FoxO4 phosphorylation was significantly higher than in OA (p< 0.05). Thus, although there is no difference in FoxO1, FoxO3a or FoxO4 protein expression between RA and OA, FoxO4 phosphorylation is significantly enhanced in RA synovial tissue.

**Figure 3 / Comparison of FoxO family phosphorylation in RA and OA synovial lining and sublining.**

Synovial biopsy sections of 12 RA and 9 OA patients were stained with antibodies against (a) phosphorylated (p) FoxO1, (b) pFoxO3a and (c) pFoxO4. Integrated optical density (IOD, left panels), number of positive cells (middle panels), and specific inactivation (ratio of IOD phosphorylated FoxO protein to number of positive cells) (right panels) were calculated by computer-assisted digital image analysis. Each box plot indicates the 25th to 75th percentiles and the bar within each box indicates median values. Lines outside the box plots indicate the 10th and 90th percentiles. * indicates statistically significant differences for values p < 0.05 and ** for p < 0.005 between RA and OA or intimal lining layer (lin) and synovial sublining (sub).
Inflammation stimuli promote PI3K-dependent phosphorylation of FoxO proteins in FLS and macrophages.

Pap and colleagues have previously reported that mRNA expression of PTEN, a suppressor of PI3K signaling, is depressed in RA synovial tissue, particularly in the invasive intimal lining layer [7]. As this might provide a mechanism for regulating FoxO phosphorylation status in synovial tissue, we examined if FoxO phosphorylation levels were associated with differential expression of PTEN. RA synovial biopsy sections were stained with anti-PTEN monoclonal antibody, and expression levels assessed in RA synovial intimal lining and sublining layers quantified by digital imaging analysis. Surprisingly, we found that at the protein level no significant differences could be observed in PTEN expression levels between RA intimal lining and sublining layers, nor between RA and OA synovial tissue (Figure 4A). Additionally, no significant correlation was observed between PTEN expression and FoxO4 phosphorylation levels in either the RA intimal lining (R= -0.31, p= 0.402) or sublining layer (R= 0.44, p= 0.224) (Figure 4B). Similarly we failed to find any significant correlation between PTEN expression and phosphorylation levels of FoxO1 or FoxO3a (data not shown). These results suggest that PI3K-dependent FoxO phosphorylation in RA synovial tissue is regulated independently of changes in PTEN expression. To determine if inflammatory mediators present in RA synovium could modulate PI3K-dependent FoxO phosphorylation, cultured RA FLS were stimulated for various times with IL-1β (Figure 4C, left panels) or TNF-α (Figure 4C, right panels), cells lysed, and phosphorylation and expression of
FoxO1 monitored by immunoblotting. Both stimuli resulted in the time-dependent phosphorylation of FoxO1 without altering total protein expression. Similar results were also obtained following CD40 ligation (data not shown), and were representative of experiments conducted in 3 independent RA FLS lines. FoxO1 was phosphorylated in response to these stimuli in 2 OA FLS lines examined, with similar magnitudes of response and kinetics observed in RA FLS (data not shown). Treatment of human macrophages derived from healthy donor peripheral blood with IL-1β (Figure 4D, left panels), TNF-α (Figure 4D, right panels) enhanced phosphorylation of FoxO4. Soluble CD154 also stimulated PI3K-dependent FoxO phosphorylation in both FLS and macrophages (data not shown).

Figure 4 / FoxO phosphorylation levels are regulated not by PTEN expression, but by extracellular signals.
(a) Comparison of PTEN protein expression in RA and OA synovial tissue intimal lining (lin) and sublining (sub) layers. (b) Lack of correlation between inactivation of FoxO4 and PTEN protein expression in RA synovial tissue intimal lining and sublining layers. Pearson R values (R) and P values are indicated. (c) Phosphorylation of FoxO1 in RA FLS following stimulation with IL-1β and TNF-α. RA FLS were stimulated for the indicated times in minutes (min) with IL-1β (125 pg/ml) or TNF-α (10 ng/ml) prior to lysis and detection of phosphorylated FoxO1 (pFoxO1) and total FoxO1 protein by immunoblotting. (d) Phosphorylation of FoxO4 in human macrophages following stimulation with IL-1β and TNF-α. In vitro –differentiated human macrophages were stimulated for indicated times with IL-1β and TNF-α as in (c) and phosphorylated FoxO4 (pFoxO4) and total FoxO4 detected by immunoblotting of whole cell lysates.
Correlation of FoxO phosphorylation with clinical parameters.
We next examined if PI3K-dependent phosphorylation of FoxO family members correlated with laboratory and clinical parameters of disease activity in RA (Figure 5). In both RA intimal lining layer and synovial sublining, significant negative correlations were observed between the phosphorylation status of FoxO4 with serum C-reactive protein (CRP) levels (intimal lining layer $R = -0.82$, $p = 0.049$; synovial sublining $R = -0.41$, $p = 0.024$) and erythrocyte sedimentation rate (ESR) (intimal lining layer $R = -0.74$, $p = 0.034$; synovial sublining $R = -0.77$, $p = 0.034$). In contrast, FoxO4 phosphorylation status did not correlate with serum rheumatoid factor (RF) levels (intimal lining layer $R = -0.15$, $p = 0.726$; synovial sublining $R = -0.09$, $p = 0.823$) (data not shown). Statistically significant negative correlations of synovial sublining FoxO1 phosphorylation and intimal lining layer FoxO3a phosphorylation with CRP and ESR, but not RF, were also observed (data not shown). No significant correlations were observed between phosphorylation status of any of the FoxO proteins and patient age, sex, disease duration, morning stiffness, VAS or swollen joint count (data not shown).
**Figure 5** / Correlation of FoxO4 phosphorylation levels (IOD/positive cell) in RA synovial lining (upper panels) and sublining (lower panels) with patient CRP (mg/ml) (left panels) and ESR (mm/hrs) (right panels). Pearson R values (R) and P values are indicated.
Discussion

Our data provide the first direct evidence that FoxO family members, targets of the PI3-kinase/PKB signaling pathway, are modulated in human chronic inflammatory disease. Although we find that each of the three family members, FoxO1, FoxO3a, and FoxO4 are expressed and phosphorylated on PKB-dependent residues in both RA and OA synovial tissue, phosphorylation of FoxO4 in synovial macrophages, particularly those in the synovial sublining, is significantly enhanced in RA. RA patients with high phosphorylation levels of FoxO family members appear to represent a distinct subset of patients displaying low disease activity, as assessed by ESR and CRP. Interestingly, we have previously noted a similar correlation between disease activity and mRNA expression of insulin-like growth factor 1 (IGF-1), which targets PI3K signaling pathways [26;27]. It is currently unknown which mechanisms might regulate PI3K signaling pathways in RA, as growth factors, inflammatory cytokines, TNF receptor family members and chemokines can all activate specific PI3K isoforms. We observed no significant correlation between phosphorylation of different FoxO family proteins within patients (data not shown). Additionally, we were unable to detect a relationship between FoxO phosphorylation levels and expression of PTEN protein. However, we did find that inflammatory extracellular stimuli present in synovial tissue, such as IL-1β and TNF-α, were able to promote FoxO phosphorylation in FLS and macrophages in vitro. Together, this may suggest that FoxO protein phosphorylation is regulated by heterogeneous cytokine and cell-cell contacts in RA synovial tissue. In the present study, we observed PKB-dependent phosphorylation of FoxO1 in synovial FLS. In vitro, PI3K/PKB signaling has been shown to protect RA FLS against TNF-α and Fas-induced cell death [8;28]. Inactivation of FoxO1 in RA FLS may also provide a mechanism for the accumulation of reactive oxygen species-induced mutations in p53 and other tumor suppressor genes, as in quiescent fibroblasts, active FoxO protects cells from oxidative stress through transcriptional regulation of manganese-dependent superoxide dismutase (MnSOD) [17] and promotes DNA repair via upregulation of Gadd45 [29]. Consistent with this, MnSOD mRNA levels are depressed in RA FLS compared to OA FLS in vitro [30]. Therefore, inactivation of FoxO1 in RA FLS may make these cells prone to the accumulation of oxidative stress-induced mutations in tumor suppressor genes and allow these cells to persist and proliferate in the presence of elevated inflammatory cytokine levels observed in RA [31]. We found that phosphorylation of FoxO3a in RA synovial tissue was restricted to a subset of T lymphocytes aggregated in lymphoid follicles. T lymphocytes in these structures are thought to either represent oligoclonal expansions of T cells responding to antigenic stimulation, or, alternatively, apoptosis-defective aggregates of T cells with a restricted T cell receptor repertoire. FoxO protein activation status can influence both interleukin-2–dependent T cell survival, as well as cell cycle progression [32], and mice lacking FoxO3a display age-dependent T lymphoproliferation disease associated with multi-organ inflammation [18]. It will therefore be of interest to explore whether FoxO3a activity is differentially regulated in specific T cell subpopulations in RA. Although PKB-dependent phosphorylation of transfected FoxO4 has been
observed *in vitro*, synovial tissue macrophages represent one of the first cell types in which regulation of endogenous FoxO4 has been reported. RANKL-dependent phosphorylation of FoxO4 was recently reported in murine osteoclasts [33]. Thus, FoxO4 may play a specific role in cells of the myeloid lineage. FoxO4-deficient mice do not have overt developmental defects [34], but analysis of myeloid lineage development and function in these mice has not been reported. Activation of PKB promotes survival in human blood-derived macrophages by maintaining transcription of the anti-apoptotic Bcl-2 family protein Mcl-1 [35], and PKB activity in bone marrow-derived murine macrophages is stimulated by both growth factors and inflammatory CD40 signaling [36;37]. Although direct regulation of Mcl-1 transcription by FoxO family members has not been reported, FoxO4 may provide a link between PKB and regulation of anti-apoptotic gene products in macrophages. Consistent with this, we have observed phosphorylation of FoxO4 in human blood-derived macrophages *in vitro* following IL-1β, TNF-α and CD40 stimulation. Because synovial sublining macrophage numbers are a highly sensitive biomarker of patient responses to treatment [38;39], further analysis of the activation status of intracellular signaling pathways regulating FoxO transcriptional activity in RA synovial tissue, in conjunction with analysis of gene target expression profiles in RA patients may identify molecular networks that differentially support inflammation and joint destruction, or predict therapeutic responses, in distinct RA patient subpopulations.
Acknowledgements

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