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Resistin and Insulin/Insulin-like Growth Factor Signaling in Rheumatoid Arthritis

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Objective. **Human resistin has proinflammatory properties that activate NF-κB–dependent pathways, whereas its murine counterpart is associated with insulin resistance. The aim of this study was to examine potential cross-talk between resistin and insulin/insulin-like growth factor (IGF) signaling in rheumatoid arthritis (RA).**

Methods. **Levels of IGF-1, IGF binding protein 3, and resistin were measured in the blood and synovial fluid of 60 patients with RA and 39 healthy control subjects. Human RA synovium was implanted subcutaneously into SCID mice, and the mice were treated with resistin-targeting small interfering RNA. Primary synovial fibroblasts from patients with RA, as well as those from patients with osteoarthritis, and the human fibroblast cell line MRC-5 were stimulated with resistin. Changes in the IGF-1 receptor (IGF-1R) signaling pathway were evaluated using histologic analysis, immunohistochemistry, and reverse transcription–polymerase chain reaction.**

Results. **Resistin and IGF-1R showed different expression profiles in RA synovia. Low levels of IGF-1 in RA synovial fluid were associated with systemic inflammation and inversely related to the levels of resistin. Stimulation of synovial fibroblasts with resistin induced phosphorylation of IGF-1R to a degree similar to that with insulin, and also induced phosphorylation of transcription factor Akt. This was followed by gene expression of *GLUT1*, *IRS1*, *GSK3B*, and the Akt inhibitors PTPN and PTEN. Abrogation of resistin expression in vivo reduced the expression of IGF-1R, the phosphorylation of Akt, and the expression of PTPN and PTEN messenger RNA in RA synovium implanted into SCID mice.**

Conclusion. **Resistin utilizes the IGF-1R pathway in RA synovia. Abrogation of resistin synthesis in the RA synovium in vivo leads to reductions in the expression of IGF-1R and level of phosphorylation of Akt.**

Rheumatoid arthritis (RA) is an inflammatory joint disease that is morphologically characterized by leukocyte infiltration of synovial tissue, followed by hyperplasia and pannus formation. The pannus subsequently invades the articular cartilage and subchondral bone. Observations of the excessive growth of synovial tissue have focused interest on the role played by different growth factors in the pathogenesis of RA. Many growth factors are present within the human synovia, making them potential targets for antirheumatic treatments (1).

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Insulin and insulin-like growth factors (IGFs) are polypeptide hormones with high sequence similarity. Insulin has extensive metabolic and mitogenic effects that regulate utilization of carbohydrates, while IGF-1 is an important growth factor involved in cell growth, differentiation, and cell survival. It is one of the most potent activators of Akt phosphorylation (2). Cellular effects of insulin and IGF-1 are mediated through their hybrid receptors, which comprise either 2 insulin receptor (IR) isoforms or 1 of these isoforms plus the homologous IGF-1 receptor (IGF-1R) (3). The affinity of the hybrid receptor for its ligand modulates tissue metabolism, growth, and survival.

Activation of the IR/IGF-1R is followed by mobilization of the IR substrate and phosphatidylinositol 3-kinase (PI3K), resulting in the phosphorylation and activation of Akt (protein kinase B). This activation leads to inhibition of apoptosis and increased cell survival, mediated through inhibition of downstream substrates, such as glycogene synthase kinase 3 (GSK-3). GSK-3 inactivates glycogen synthase, the rate-limiting enzyme in glycogen synthesis, and also attenuates IR/IGF-1R signaling by inhibiting the IR substrate (3–5). The tumor suppressor PTEN is also a potent inhibitor of Akt activity (6). Several proinflammatory cytokines increase the synthesis of suppressor of cytokine signaling proteins, resulting in ubiquitination and degradation of the IR substrate, which thus modulates the activation of Akt.

In addition to the canonical IR/IGF-1R signaling pathways, ligation of IGF-1R may lead to cross-talk and induce signaling via receptors belonging to the cytokine receptor family. IGF-1 promotes survival and delays apoptosis of human neutrophils and T lymphocytes (2,7), both of which contribute to the pathologic development of inflammatory diseases such as RA (8). Therefore, the IR/IGF-1R signaling pathway may play an important role in the progression of the disease. IGF-1 contributes to the proliferation of fibroblasts and to the synthesis of cartilage and bone extracellular matrix proteins within RA synovium (9,10). Previous studies have shown that high expression of IGFs is associated with low-grade inflammation, and that production of IGF-1 is suppressed in patients with RA (11,12).

Resistin is an adipokine that affects insulin signaling in rodents. Previous research on the effects of resistin on the insulin/IGF-1 signaling pathway has mainly been performed in insulin-sensitive tissues (e.g., muscle, fat, and liver). In human hepatocytes, resistin down-regulates IR substrate 2 (IRS-2), up-regulates GSK-3β), and suppresses Akt phosphorylation (13,14). In a humanized mouse model, macrophage-derived human resistin increases white adipose tissue inflammation via serine phosphorylation of IRS-1 (15). In muscle tissue, resistin induces an accumulation of triglycerides, which results in protein kinase Cθ activity, and increases phosphorylation of IRS-1. Resistin also activates the PI3K–Akt signaling pathway in human aortic smooth muscle cells (16). Thus, the molecular mechanisms by which resistin affects signaling through insulin/IGF-1R in humans may be tissue specific.

In humans, resistin has a proinflammatory function, activating the NF-κB signaling pathway and inducing production of cytokines such as tumor necrosis factor α (TNFα), interleukin-1β (IL-1β), and IL-6 (17,18). Previously, we and others have shown that resistin levels are increased in RA patients and that it accumulates in inflamed joints (17,19,20). However, evidence of a clear role for resistin in human RA is lacking, because of the functional differences between human resistin and its murine counterpart. In this study, synovial tissue from RA patients was transplanted into mice with severe combined immunodeficiency (the SCID mouse model), to yield a humanized mouse model of rheumatoid synovitis. This model allows the effects of various molecules on rheumatoid synovium to be studied directly.

We examined the role of resistin in IR/IGF-1R signaling within RA synovium, and showed that resistin utilizes the IGF-1R pathway, possibly competing with other ligands for these receptors. Long-term abrogation of resistin synthesis in RA synovium in vivo reduced the cellularity of the synovium, the expression of IGF-1R, and the level of phosphorylated Akt.

MATERIALS AND METHODS

Materials. Recombinant human resistin was purchased from PeproTech, and recombinant insulin was from Novo Nordisk. TNFα, fetal calf serum (FCS), gentamycin, Dulbecco's modified Eagle's medium (DMEM)–Glutamax, and antibodies to resistin were from Sigma-Aldrich. RPMI 1640 without L-glutamine culture medium was from PAA Laboratories. Normocin was from InvivoGen. Antibodies directed against phosphorylated Akt (Ser-473) and the IGF-1R used for immunohistochemistry were from Cell Signaling Technology, and antibodies directed against phosphorylated IGF-1R (Tyr-1161) and the IGF-1R used for immunofluorescence were purchased from Abcam. Collagenase/Dispase was from Roche. The RNeasy kit was purchased from Qiagen. The High-Capacity complementary DNA (cDNA) Archive Kit along with primers and probes were purchased from Applied Biosystems (sequences are available from the corresponding author upon request).

Protected small interfering RNA (siRNA; siSTABLE) targeting the RETN gene, as well as nontargeting control

Table 1. Demographic and clinical characteristics of the patients with rheumatoid arthritis stratified by the presence or absence of systemic inflammation*

	Inflammation (n = 33)	No inflammation (n = 27)	*P*
Sex, no. (%) female	19 (58)	17 (63)	NS
Age, mean (range) years	60 (25–78)	55 (26–87)	NS
Disease duration, mean (range) years	7 (1–35)	7 (0.8–21)	NS
Smokers, no. (%)	10 (30)	9 (33)	NS
DMARD treatment, no. (%)	20 (61)	11 (41)	NS
Methotrexate	13	7	
Other†	7	4	
No DMARD	13	16	
Erosions on radiograph, no. (%)	20 (61)	15 (56)	NS
RF positive, no. (%)	19 (58)	7 (25)	0.007
Anti-CCP			
No. (%) positive	15 (47)	5 (19)	0.02
Median (min.–max.) IU/ml	42 (4–600)	25 (10–261)	0.02
CRP, median (min.–max.) ng/ml	45 (24–220)	7 (5–17)	0.0001
WBCs, median (min.–max.) $\times 10^9$/liter			
Blood	8.2 (5.5–16.8)	6.2 (3.9–9.0)	NS
Synovial fluid	19.5 (6.5–125)	5.6 (0.1–19.1)	0.0001
IL-6 in synovial fluid, median (min.–max.) ng/ml	2.05 (0.30–2.77)	2.01 (0.03–2.77)	NS

* Systemic inflammation was defined as a C-reactive protein (CRP) level ≥30 ng/ml. NS = not significant; DMARD = disease-modifying antirheumatic drug; RF = rheumatoid factor; anti-CCP = anti–cyclic citrullinated peptide antibodies; min.–max. = minimum–maximum; WBCs = white blood cells; IL-6 = interleukin-6.
† The "other" category of DMARDs included sulfasalazine (n = 5), gold salts (n = 4), leflunomide (n = 1), and azathioprine (n = 1). Cyclosporin A was administered in combination with methotrexate (n = 2), azathioprine (n = 1), leflunomide (n = 1), or sulfasalazine (n = 1).

RNA, were purchased from Dharmacon RNAi Technologies. Enzyme-linked immunosorbent assay (ELISA) kits for detection of IGF-1, IGF binding protein 3 (IGFBP-3), resistin, and phosphorylated IGF-1R were from R&D Systems. Picropodophyllin (PPP), a specific IGF-1R inhibitor, was kindly provided by Prof. Magnus Axelsson (Karolinska Institute, Stockholm, Sweden). TNF receptor analog (p75-IgG fusion protein; etanercept) was purchased from Wyeth Pharmaceuticals/Pfizer.

Collection and preparation of synovial fluid and blood samples. Blood and synovial fluid samples were collected from 60 patients with RA at the Rheumatology Clinic of Sahlgrenska University Hospital (Gothenburg, Sweden). The clinical and demographic characteristics of the RA cohort, stratified by the presence or absence of inflammation, are given in Table 1. In addition, as controls, blood samples were obtained from 39 healthy individuals who were matched by age and sex to the RA patients, and synovial fluid was obtained from 19 patients with noninflammatory knee joint diseases (designated the osteoarthritis [OA] group; ages 23–88 years).

Synovial fluid was obtained from the knee joints by arthrocentesis. The synovial fluid samples were then aseptically aspirated and transmitted into tubes containing sodium citrate (0.129 moles/liter; pH 7.4). Blood samples were simultaneously obtained from the cubical vein into a vacuum tube containing sodium citrate medium. Collected blood and synovial fluid samples were centrifuged at 800*g* for 15 minutes, aliquoted, and stored frozen at −20°C until used.

The Ethics Committee of the University of Gothenburg approved the study. Informed consent was obtained from all patients enrolled in the study.

Orthotropic model of synovial tissue transplantation. SCID mice lacking functional T and B cells (all female, ages 6–7 weeks) were purchased from Charles River Laboratories. Mice were housed at the animal facility in the Department of Rheumatology and Inflammation Research of Sahlgrenska University Hospital, in pathogen-free cages that were supplied with continuous air flow. All mice were fed with autoclaved laboratory chow and water ad libitum. Under sterile conditions, mice were transplanted subcutaneously and paravertebrally with human synovial RA tissue (2.0 cm^3) obtained from patients during knee replacement surgery at the Department of Orthopedics of Sahlgrenska University Hospital. The study was approved by the Ethics Committee of Gothenburg University, and the requirements of the National Board for Laboratory Animals were followed.

Following 2 weeks of implantation, which permitted transplant survival, the synovial tissue was subjected to RNA injections. To study whether targeting of resistin with siRNA modulates RA inflammation, siRNA protected from RNase degradation (siSTABLE in vivo, an siRNA targeting RETN; target sequence 5′-GCCGGCUCCCUAAUAUUUAUU-3′) was injected into the implanted tissue every second day (2 μg/mouse) during a period of 14 consecutive days. The control group of mice were injected with an identical amount of nontargeting RNA (mock RNA) sequence. Synovial tissue obtained from 1 patient was transplanted into at least 2 mice, in order to subject tissue from each patient to an siRNA targeting resistin and a control siRNA. Following 14 days of injections, the synovial tissue transplants were excised and assessed by morphologic evaluation and gene expression analysis.

Histologic examination. Human synovial tissue was submitted to routine fixation and paraffin embedding, and cut into 4-μm–thin sections. Tissue sections were deparaffinized

and endogenous peroxidase was depleted by incubation with glucose oxidase, followed by overnight incubation with a titrated amount of polyclonal rabbit anti-human resistin, polyclonal rabbit anti–IGF-1R antibodies, or phosphorylated Akt (Ser-473) antibodies. Following washing, the sections were incubated with biotinylated sheep anti-rabbit IgG antibodies (Sigma). The color reaction was completed with a Vectastain Elite ABC Kit (Vector Laboratories) and 3-amino-9-ethylcarbazole containing H_2O_2. Sections were counterstained with hematoxylin.

Cell cultures and stimulations. Primary human synovial fibroblasts were isolated from minced synovial tissue obtained during knee replacement surgery from 4 RA patients and 4 OA patients at the Department of Orthopedics, Sahlgrenska University Hospital. Cells were obtained using collagenase/Dispase (1 mg/ml), according to previously described methods (21), and cultured at 37°C in 5% CO_2 in 10-ml culture flasks suspended in DMEM–Glutamax medium supplemented with 0.25% gentamycin, 0.2% Normocin, and 10% FCS. Homogeneous cultures of synovial fibroblasts from passages 3–5, containing >98% of CD90+ cells and <1% of CD68+ cells, were used for the experiments. The human fibroblast cell line MRC-5, originally obtained from European Collection of Cell Cultures, was cultured in DMEM–Glutamax medium (Gibco) supplemented with 50 μg/ml gentamycin and 10% FCS. Cells were cultured to confluence in 24-well Nunclon Delta plates (Fisher Scientific).

Cultures of synovial fibroblasts and MRC-5 cells were stimulated with resistin (0.5–500 ng/ml), TNFα (1 ng/ml), and/or insulin (1 IU/ml). Supernatants were collected for protein analysis at 24 and 48 hours, and cells were collected for evaluation of intracellular phosphorylation and gene expression analysis at predetermined time points (5, 10, 15, 30, and 60 minutes and 12 hours). Cells were lysed and messenger RNA (mRNA) expression of *IRS1*, *GSK3B*, PTEN, and *GLUT1* was analyzed by real-time polymerase chain reaction (PCR).

Phosphorylation of IGF-1R. MRC-5 cells were cultured to confluence in 24-well Nunclon Delta plates (Fisher Scientific). Before stimulation, cells were serum starved for 6 hours in RPMI 1640 without L-glutamine medium. Cells were stimulated with resistin (500 ng/ml) or insulin (1 IU/ml) for 5, 15, 30, or 60 minutes, after which they were lysed and phosphorylation of IGF-1R was determined by ELISA (R&D Systems). To further evaluate the effect of resistin and insulin on IGF-1R, the specific inhibitor PPP (10 n*M*) was added 6 hours before stimulation with resistin or insulin was carried out.

Immunofluorescence and confocal microscopy. Human synovial fibroblasts were cultured to confluence in 8-well chamber slides (Fisher Scientific). Before stimulation, cells were serum starved for 6 hours in DMEM–Glutamax medium. Cells were stimulated with resistin (0.5–50 ng/ml) or insulin (1 IU/ml) for 5, 15, 30, 60, or 240 minutes. After stimulation, cells were fixed with 4% cold paraformaldehyde for 10 minutes, washed with phosphate buffered saline (PBS), and permeabilized with 0.1% Triton X-100 in PBS for 15 minutes. Following blocking with 5% bovine serum albumin–glycine (0.1*M*) for at least 1 hour, cells were subjected to a double-staining procedure as described previously (21). Briefly, incubation of the cells with a primary mouse anti–IGF-1R antibody was followed by secondary goat anti-mouse IgG–Alexa Fluor 488 for 30 minutes. Rabbit anti–IGF-1R–phosphorylated Tyr-1161 was then added, followed by secondary goat anti-rabbit IgG–Alexa Fluor 555.

For phosphorylation of Akt, an anti–phosphorylated Akt (anti–Ser-473) antibody was added, followed by goat anti-mouse IgG–Alexa Fluor 555. Actin filaments were stained with phallacidin–BODIPY FL (Invitrogen). Slides were mounted with Prolong gold antifade reagent containing DAPI (Invitrogen). Normal mouse IgG1 or normal rabbit serum (Dako) was used as a negative isotype control. Images were collected using a confocal microscope (LSM700; Zeiss). The background fluorescence level was set with the negative controls, and images were analyzed using Zen image-analysis software (version 2009; Zeiss).

RNA isolation and reverse transcription–PCR (RT-PCR) assays. Total RNA was extracted from synovial tissue samples, transplanted synovia, primary human synovial fibroblasts, and MRC-5 cells using the RNeasy mini kit (Qiagen). The RNA concentration was assessed spectrophotometrically with NanoDrop at 260 nm, and the quality of the RNA was assessed using Experion (Bio-Rad). The cDNA preparation was performed from 1 μg RNA using the High-Capacity cDNA Archive Kit (Applied Biosystems). Gene expression was measured with TaqMan real-time PCR (Applied Biosystems). The RT-PCR was performed using 100 n*M* of probe, 200 n*M* of forward and reverse primers, and 10 ng of total RNA in a final volume of 20 μl. The sequences of the primers and probes used in the RT-PCR are available from Applied Biosystems upon request. All data were normalized to values for the reference gene 18S, and quantitative measurement of mRNA was performed using the $\Delta\Delta C_t$ method.

Determination of cytokine levels. The levels of IGF-1, IGF-1R, IGFBP-3, and resistin were determined in blood and synovial fluid samples by ELISA with matched antibody pairs (R&D Systems), following the recommendations of the manufacturer. Serial dilutions of the corresponding recombinant cytokines were used in all assays to determine the concentration of protein in the samples. Cytokine levels are expressed in ng/ml.

Statistical analysis. Conventional statistical methods were performed using SPSS software and GraphPad Prism version 5.0. The continuous variables are presented as the median (minimum–maximum [min.–max.]). Comparisons between groups were assessed using the Mann-Whitney U test. Correlations between parameters were determined using Spearman's rank correlation tests. *P* values less than 0.05 were considered significant.

RESULTS

Reduction in the levels of IGF-1 in human RA synovial fluid. The levels of IGF-1, IGFBP-3, and resistin were measured in paired samples of blood and synovial fluid obtained from patients with RA (n = 60), and in synovial fluid samples from patients with OA (n = 19). Clinical information on the RA patients is available in Table 1 (further details are available from the corresponding author upon request). Synovial fluid

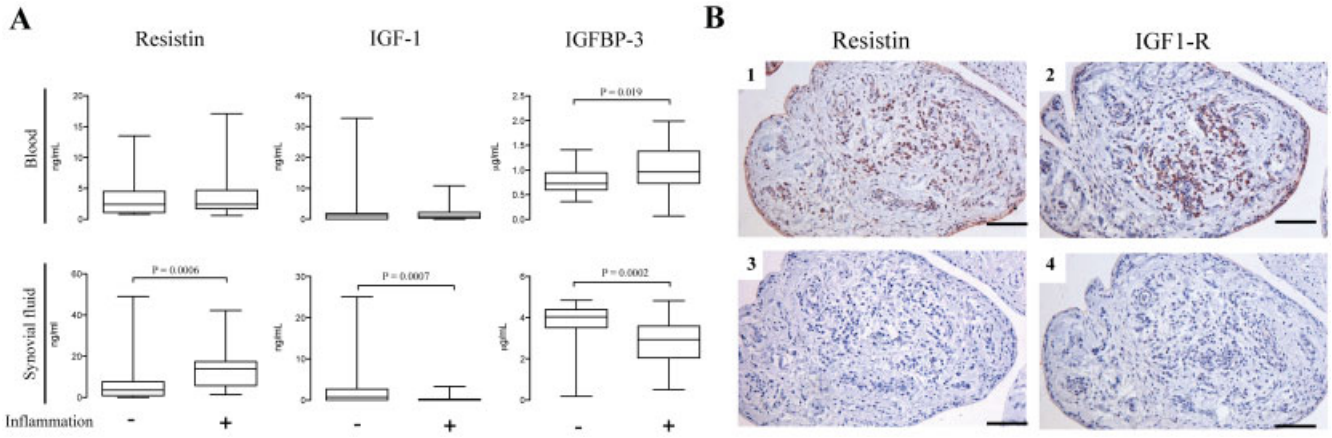

Figure 1. Reductions in insulin-like growth factor 1 (IGF-1) levels in synovial fluid from patients with rheumatoid arthritis (RA) are associated with the presence of inflammation. **A,** Levels of resistin, IGF-1, and IGF binding protein 3 (IGFBP-3) were measured in paired blood and synovial fluid samples from RA patients, stratified by the presence (C-reactive protein [CRP] level ≥30 ng/ml; n = 33) or absence (CRP level ≤29 ng/ml; n = 27) of systemic inflammation. Data are presented as box plots. Each box represents the 25th to 75th percentiles. Lines inside the boxes represent the median. Lines outside the boxes represent the minimum and maximum values. **B,** Immunohistochemical staining of human RA synovium shows differential expression of resistin (panel 1) and IGF-1 receptor (IGF-1R) (panel 2) in the apical layer of synoviocytes and in the cytoplasm of leukocytes infiltrating synovial tissue. Staining with isotype-identical Ig was used as a control for resistin (panel 3) and IGF1-R (panel 4) staining. Sections were counterstained with hematoxylin. Bars = 0.1 mm.

levels of IGF-1 were significantly lower in RA patients (median 0 ng/ml, min.–max. 0–25 ng/ml) compared with OA patients (median 12.8 ng/ml, min.–max. 4.6–25.1 ng/ml; $P < 0.0001$). In addition, IGFBP-3 levels were lower in the blood of RA patients (median 0.83 μg/ml, min.–max. 0.07–1.99 μg/ml) as compared with the blood of OA patients (median 1.69 μg/ml, min.–max. 0.17–2.92 μg/ml; $P = 0.03$). Resistin levels were significantly higher in the synovial fluid than in the blood of RA patients (median 8.00 ng/ml, min.–max. 0–49.00 ng/ml versus median 2.40 ng/ml, min.–max. 0.60–17.10 ng/ml; $P < 0.0001$). Intraarticular IGF-1 levels were inversely related to intraarticular resistin levels (Z rank −3.08, $P = 0.002$) and IL-6 levels ($r = -0.533$, $P = 0.0002$).

Stratification of RA samples according to whether systemic inflammation was present (defined as a C-reactive protein [CRP] level ≥30 mg/liter) yielded an RA inflammation group with a median CRP level of 45 ng/ml (min.–max. 24–220 ng/ml) (Table 1). Compared with samples from RA patients with no inflammation (CRP level ≤29 mg/liter), samples from RA patients with inflammation exhibited a reduction in the levels of IGF-1, in both the blood (median 1.01 ng/ml, min.–max. 0–10.82 ng/ml) and synovial fluid (median 0 ng/ml, min.–max. 0–3.31 ng/ml) (Figure 1A). IGFBP-3 levels were increased in synovial fluid samples compared with corresponding blood samples from the same RA patient (median 2.93 μg/ml, min.–max. 0.50–4.82 μg/ml versus median 0.96 μg/ml, min.–max. 0.07–1.99 μg/ml; $P < 0.001$). IGFBP-3 levels in synovial fluid from inflamed joints were significantly reduced compared with those in synovial fluid from noninflamed joints (median 2.93 μg/ml, min.–max. 0.50–4.82 μg/ml versus 4.04 μg/ml, min.–max. 0.18–4.85 μg/ml; $P = 0.0002$).

Soluble IGF-1R was not detected in blood samples from patients with RA or in blood samples from patients with OA. However, immunohistologic evaluation of human synovia showed expression of IGF-1R and resistin in both OA patients and RA patients (Figure 1B). Expression of resistin and IGF-1R was evident in the apical layer of synoviocytes and in the cytoplasm of rounded leukocytes infiltrating synovial tissue.

RT-PCR assessing the relative mRNA expression of IGF-1R and resistin in synovial tissue from patients with RA (n = 4) and patients with OA (n = 3) showed 60% higher expression of IGF-1R mRNA in OA samples compared to RA samples (mean ± SD relative quantification [RQ] 1.00 ± 0.15 versus 0.41 ± 0.11; $P = 0.0017$). RQ values for expression of resistin mRNA were high in the RA synovium (results not shown), whereas resistin mRNA could not be detected in OA samples.

Down-regulation of resistin associated with reduction in Akt activity in human RA synovium. To study the effect of long-term suppression of resistin on the IGF-1R pathway, samples of human synovial tissue

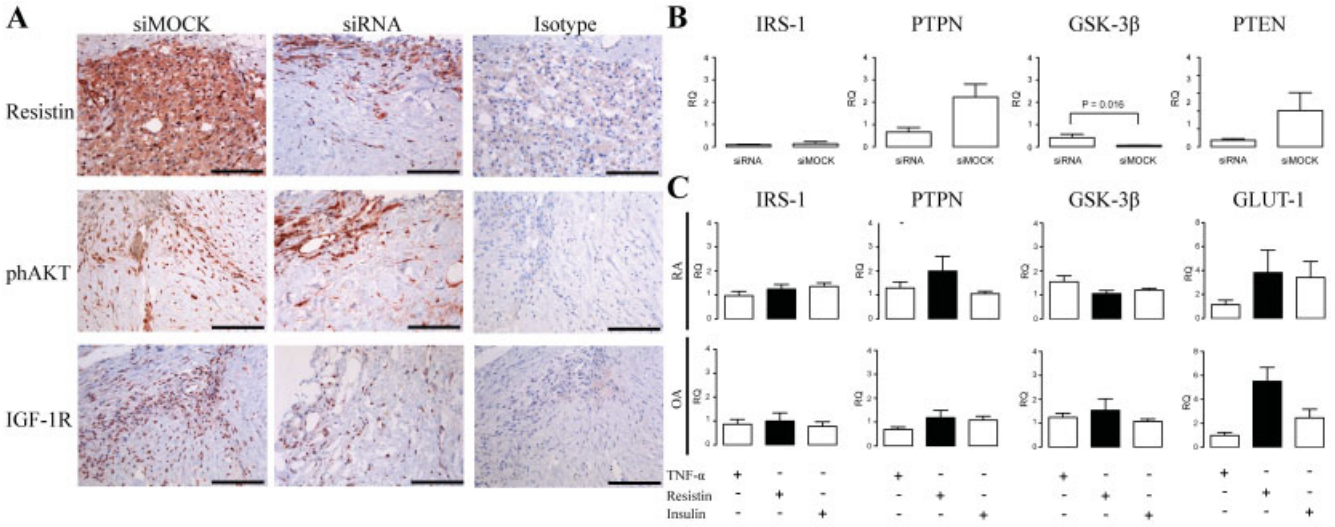

Figure 2. Depletion of resistin from human rheumatoid arthritis (RA) synovium modulates Akt-dependent pathways. **A,** Human RA synovial tissue was subcutaneously transplanted into SCID mice and treated with small interfering RNA (siRNA) targeting resistin (2 μg/mouse) or nontargeting siRNA (siMOCK) every second day for 2 weeks. Immunohistochemical staining of the treated synovial tissue showed successful depletion of resistin and a reduction in phosphorylated Akt (phAKT) and insulin-like growth factor 1 receptor (IGF-1R). Staining with isotype-identical Ig was used as a control. Sections were counterstained with hematoxylin. Bars = 0.1 mm. **B,** Analysis of mRNA expression showed that depletion of resistin with siRNA resulted in a reduction in PTPN and PTEN mRNA and an increase in *GSK3B* mRNA. *IRS1* mRNA expression was unchanged when compared with that in siMOCK-treated samples. **C,** Human synovial fibroblast cultures (from 3 patients with RA and 3 patients with osteoarthritis [OA]) were stimulated with tumor necrosis factor α (TNFα) (1 ng/ml), resistin (500 ng/ml), or insulin (1 IU/ml) for 12 hours. Analysis of mRNA in the cell lysates showed increased expression of *GLUT1* and PTPN, whereas expression of *IRS1* and *GSK3B* mRNA was unchanged. Bars show the mean ± SEM relative quantification (RQ) when compared with that in unstimulated cultures of synovial fibroblasts.

obtained from patients with RA during prosthetic surgery were transplanted subcutaneously into SCID mice (n = 6). Two weeks later (a time period permitting transplant survival), the synovial tissue was injected with either siRNA targeting resistin or a nontargeting RNA (mock RNA) sequence. After 2 weeks of treatment, the synovial tissue transplants were removed and examined for resistin expression, IGF-1R expression, phosphorylation of Akt, and gene expression.

Evaluation of the transplanted synovia revealed pronounced down-regulation of resistin synthesis in the siRNA-treated samples compared to mock-treated samples (Figure 2A). Morphologically, resistin-depleted synovium was characterized by reduced cellularity and the accumulation of fat deposits. No fat deposits were found in mock-treated synovium (Figure 2A). Down-regulation of resistin in transplanted synovium was associated with decreased phosphorylation of Akt, as compared with that in mock-treated and nontransplanted synovium from the same patient (Figure 2A). Furthermore, the down-regulation of IGF-1R was significantly more pronounced in siRNA-treated synovia.

RT-PCR analysis of Akt inhibitor genes revealed a decrease in the expression of PTPN and PTEN and a significant increase in *GSK3B* expression ($P = 0.016$) in siRNA-treated synovial tissue compared with mock-treated tissue (Figure 2B). In contrast, the expression of *IRS1* was similar in both siRNA-treated and mock-treated synovial tissue.

In addition, synovial tissue from RA patients (n = 2) was treated in vitro with daily injections of siRNA (300 ng/day) or mock RNA for 7 consecutive days. RT-PCR analysis showed that down-regulation of resistin did not affect the relative expression of IR mRNA ($RQ_{Sample\ 1} = 1.12$ and $RQ_{Sample\ 2} = 0.95$), whereas the expression of TNFα tended to increase ($RQ_{Sample\ 1} = 1.39$ and $RQ_{Sample\ 2} = 1.36$).

To further confirm the link between resistin and IGF-1R, synovial fibroblast cultures isolated from the synovial tissue of patients with RA (n = 3) and patients with OA (n = 3) were stimulated with resistin (500 ng/ml), insulin (1 IU/ml), or TNFα (1 ng/ml) for 12 hours, followed by analysis of the expression of *IRS1*, PTPN, *GSK3B*, and *GLUT1* mRNA. Resistin stimula-

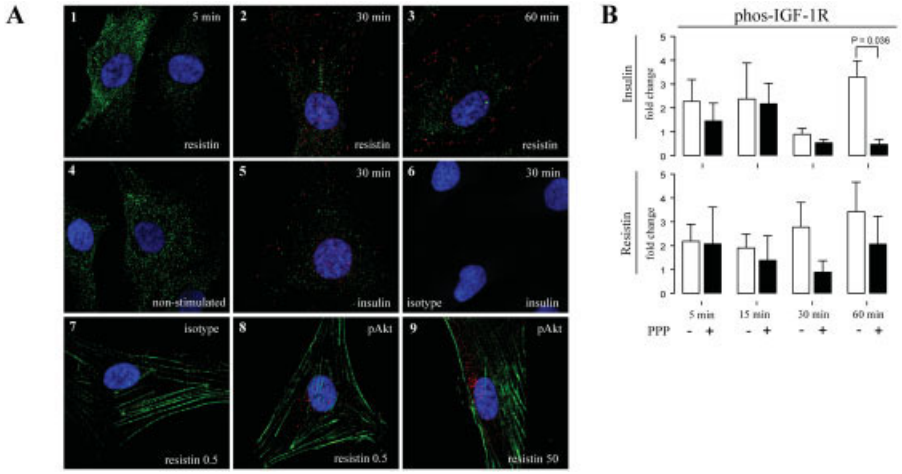

Figure 3. Stimulation with resistin induces phosphorylation of the insulin-like growth factor 1 receptor (IGF-1R) and subsequent activation of Akt. **A,** Human synovial fibroblasts were stimulated with resistin (50 ng/ml) (panels 1–3) or insulin (1 IU/ml) (panel 5) for 5, 30, or 60 minutes. Unstimulated human synovial fibroblast cultures were exposed to cell medium only (panel 4). In panels 1–6, expression of total IGF-1R in stimulated and unstimulated cultures is shown in green, while gradual accumulation of the phosphorylated IGF-1R (Tyr-1161) in resistin-stimulated and insulin-stimulated cultures is shown in red. In panels 7–9, phosphorylation of Akt (Ser-473) after 4 hours of stimulation with resistin (0.5 ng/ml [panel 8] and 50 ng/ml [panel 9]) is shown in red, while actin filaments are stained green. Staining with isotype-identical Ig was used as a control (panels 6 and 7). **B,** Stimulation of MRC-5 cells with resistin resulted in a time-dependent increase (from 5 minutes up to 60 minutes poststimulation) in IGF-1R phosphorylation (phos), similar to that observed following insulin stimulation. Addition of the IGF-1R inhibitor picropodophyllin (PPP; 10 n*M*) to the MRC-5 cell cultures reduced the levels of resistin- and insulin-induced phosphorylation. Results are presented as the mean ± SEM fold change in levels of phosphorylation compared to those in unstimulated cells from 3–5 independent experiments.

tion resulted in increased expression of PTPN and *GLUT1*. However, the expression of *IRS1* and *GSK3B* was not significantly different from that observed in unstimulated cultures (Figure 2C).

Regulation of IGF-1 production and activation of IGF-1R signaling by resistin. To further evaluate possible links between resistin and IGF-1R, cultures of synovial fibroblasts and MRC-5 cells were stimulated with resistin (0.5–500 ng/ml) or insulin (1 IU/ml) and assessed for IGF-1R phosphorylation. The phosphorylation of IGF-1R in synovial fibroblasts showed a time-dependent pattern, becoming visible in the cytoplasm following 5 minutes of stimulation with resistin, and then showing a gradual increase in phosphorylation after 30 and 60 minutes (Figure 3A, panels 1–3). Translocation of phosphorylated IGF-1R to the cell membrane was clearly seen 60 minutes after stimulation with resistin, as compared with that in unstimulated cultures (Figure 3A, panels 3 and 4). The phosphorylation of IGF-1R induced by resistin was similar to that induced by insulin (Figure 3A, panel 5, as compared with isotype control in panel 6).

Supporting results were obtained in MRC-5 cell cultures, in which resistin induced an up to 4-fold increase in IGF-1R phosphorylation compared to that in medium-stimulated MRC-5 cell cultures (Figure 3B). Furthermore, the addition of a synthetic inhibitor of IGF-1R (10 n*M* PPP) reduced both the insulin-induced ($P = 0.036$) and resistin-induced (P not significant) phosphorylation of IGF-1R in MRC-5 cells (Figure 3B). PPP specifically inhibits phosphorylation of IGF-1R without interfering with IR activity (22). These results support the notion of the direct activation of IGF-1R by resistin in the absence of IGF-1. Activation of IGF-1R was followed by activation of the transcription factor Akt, operating downstream of the IGF-1R, and by

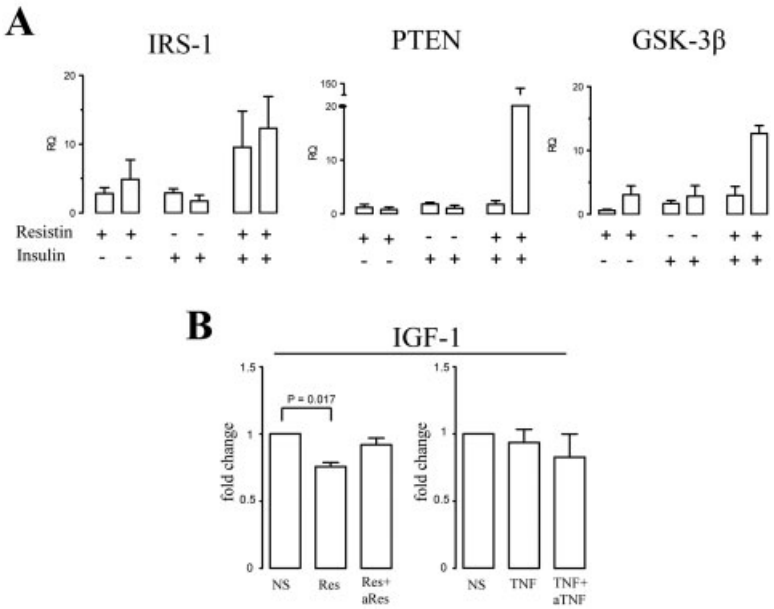

Figure 4. Resistin regulates production of insulin-like growth factor 1 (IGF-1) and expression of Akt inhibitors in the human fibroblast cell line MRC-5. **A,** MRC-5 cells were stimulated with resistin (500 ng/ml) and/or insulin (1 IU/ml), and the efforts on MRC-5 cells were assessed after 1 hour and 12 hours of stimulation, revealing increases in mRNA for *IRS1*, PTEN, and *GSK3B* following stimulation with resistin, at levels similar to those induced with insulin. **B,** Supernatants were assessed for expression of IGF-1 following 24 hours of stimulation with resistin (Res; 500 ng/ml) or tumor necrosis factor α (TNFα; 1 ng/ml), as compared with that in unstimulated (NS) cultures. Addition of antiresistin antibodies (aRes; 10 μg/ml), but not anti-TNFα antibodies (aTNF; 10 μg/ml etanercept), restored the levels of IGF-1. Bars show the mean ± SEM relative quantification (RQ) or fold change in RQ when compared with that in unstimulated cell cultures.

phosphorylation of Akt at Ser-473 (Figure 3A, panels 8 and 9, as compared with isotype control in panel 7). Perinuclear localization of phosphorylated Akt was seen in synovial fibroblasts 4 hours after stimulation with resistin.

Notably, resistin stimulation did not affect the expression of either IR mRNA ($RQ_{Sample\ 1} = 1.16$ and $RQ_{Sample\ 2} = 0.95$) or IGF-1R mRNA ($RQ_{Sample\ 1} = 0.76$ and $RQ_{Sample\ 2} = 0.60$) in MRC-5 cells. Resistin stimulation did induce an increase in *IRS1* mRNA in MRC-5 cells both 1 hour and 12 hours poststimulation, an effect also seen after insulin stimulation (Figure 4A). When MRC-5 cells were incubated with a combination of insulin and resistin, the level of *IRS1* mRNA increased even further. Resistin also induced an increase in mRNA for the Akt inhibitors PTEN and *GSK3B* in the MRC-5 cell cultures (Figure 4A). A similar increase in the production of Akt inhibitors was observed following insulin stimulation. Transcription of Akt inhibitors increased several fold in MRC-5 cells after combined stimulation with resistin and insulin. The induction of PTEN mRNA in response to resistin stimulation was consistent with the results obtained in the transplanted human RA synovial tissue, in which the down-regulation of resistin was followed by a reduction in PTEN expression (Figure 2B).

Stimulation of MRC-5 fibroblast cell cultures with resistin (500 ng/ml) led to a significant decrease in IGF-1 levels in the supernatants of resistin-stimulated cultures compared to vehicle-stimulated cultures ($P = 0.017$) (Figure 4B). Notably, neutralization of resistin using specific monoclonal antibodies (10 μg/ml, as described previously [17]) restored the production of IGF-1 by MRC-5 fibroblasts, suggesting a regulatory role for resistin in IGF-1 release. In contrast, no decrease in the levels of IGF-1 was observed following stimulation of MRC-5 cells with TNFα (1 ng/ml) or TNFα plus an anti-TNFα antibody (10 μg/ml etanercept) (Figure 4B).

DISCUSSION

Resistin has recently been shown to be an important player in the pathogenesis of RA (19,20,23), inducing an inflammatory response. However, functional differences between human and murine resistin have made studies of its role in human tissues difficult. In the present study, we evaluated the effects of resistin on IR/IGF-1R signaling in RA. Histologic evaluation of RA synovial tissue showed sufficient expression of IGF-1R, but the intraarticular levels of IGF-1 (thought to be the major ligand for IGF-1R) in RA synovia were reduced. This reduction was directly related to synovial inflammation.

Our observations support the common view regarding an association of inflammation with insulin/IGF-1 resistance and growth impairment, in which proinflammatory cytokines induce a state of resistance to several hormones, including IGF-1 (24). In the presence of certain growth factors, proinflammatory cytokines mediate antiproliferative effects by attenuating the mitogenic properties of IGF-1. IL-1β and TNFα reduce tyrosine phosphorylation of IGF-1R, thereby blocking the activation of intracellular pathways (25). Our results showed that intraarticular levels of IGF-1 are inversely related to resistin levels in patients with systemic inflammation. In addition, resistin directly inhibited IGF-1 production by synovial fibroblasts and a human fibroblast cell line in vitro. This is consistent with our previous findings, in which we showed that resistin plays a major role in the regulation of inflammation through activation of NF-κB–dependent cytokine production (17).

To further evaluate whether resistin regulates IR/IGF-1R signaling, we studied the effects of long-term inhibition of resistin in human synovial transplants in vivo. Transplanted RA synovial tissue was subjected to resistin depletion using siRNA. Resistin suppression was associated with down-regulation of IGF-1R expression and a reduction in Akt phosphorylation, subsequently leading to decreased expression of the Akt inhibitors PTEN and PTPN. *GSK3B* is important for adipogenesis, since inhibition of *GSK3B* prevents adipocyte differentiation (26–28). Histologic study of resistin-depleted synovium showed not only abrogation of Akt phosphorylation, but also reduced cellularity and accumulation of fat deposits. Gene expression analysis showed that resistin-depleted synovium had significantly higher expression of *GSK3B* compared with synovium treated with control siRNA. Abrogation of Akt phosphorylation (caused by depletion of resistin) and increased expression of *GSK3B* may explain the formation of fat deposits. Previous studies have shown that resistin induces lipolysis in human adipocytes (29).

No effect of resistin on the expression of *IRS1* or *GSK3B* in synovial fibroblasts was observed, but we did see an increase in PTPN expression and a significant increase in *GLUT1* expression. These results are consistent with those obtained from the transplanted synovia. Increased expression of *GLUT1* may be a direct result of Akt activity (30–32). A causative relationship between resistin suppression in the synovial transplants and changes in IR/IGF-1R signaling was investigated in our in vitro experiments assessing human synovial fibroblasts and the fibroblast cell line MRC-5. Phosphorylation of IGF-1R was visible in the cytoplasm of resistin-stimulated synovial fibroblasts, followed by its translocation to the cell membrane and consequent phosphorylation of transcription factor Akt. The chain of resistin-induced events, namely, the phosphorylation of IGF-1R, induction of *IRS1*, and activation of Akt inhibitors PTEN and *GSK3B*, favors the hypothesis that resistin plays a role in the regulation of IGF-1R signaling and may be considered a dynamic participant in Akt-related processes, including cell proliferation and tissue remodeling (16,29,33).

Resistin depletion in human synovium led to a reduction in IGF-1R expression. In classic insulin-sensitive tissues, such as fat deposits, hepatic tissue, and muscle cells, the down-regulation of IGF-1R has been suggested to be a potent mechanism enhancing the assembly of IR subunits and the insulin-induced phosphorylation of Akt (34). Indeed, the insulin-induced effects seen in our experiments were often similar to those observed following stimulation with resistin. Stimulation of MRC-5 cell cultures with both insulin and resistin resulted in increased production of *IRS1* and Akt inhibitors PTEN and *GSK3B*. These findings suggest that activation occurs through different receptors or independent binding sites.

IGF-1 levels are known to correlate with inflammation and CRP levels. However, resistin levels are increased by CRP and proinflammatory cytokines, such as IL-1β and TNFα (35,36). This is supported by our results obtained from the blood and synovial fluid of RA patients. Resistin levels were increased in patients with systemic inflammation, whereas IGF-1 levels were decreased. In addition, resistin stimulation of MRC-5 cells led to a decreased production of IGF-1. Although IGF-1 levels were decreased in the synovium, there was still clear expression of IGF-1R in the synovium. Abrogation of resistin in the RA synovium using siRNA led to

decreased phosphorylation of Akt, suggesting that resistin may activate Akt through IR/IGF-1R, facilitating excessive growth of synovial tissue in RA.

In conclusion, in the present study, we showed that resistin interferes with IR/IGF-1R signaling by modulating both IGF-1R expression and Akt-dependent processes in human synovial tissue. Thus, resistin represents a potential link between NF-κB–dependent inflammation and IR/IGF-1R–dependent growth. Further studies are required to elucidate the pathogenic consequences of resistin-induced local activation of Akt in inflamed rheumatoid synovium.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Mr. Svensson had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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REFERENCES

1. Paleolog EM. The vasculature in rheumatoid arthritis: cause or consequence? Int J Exp Pathol 2009;90:249–61.
2. Walsh PT, Smith LM, O'Connor R. Insulin-like growth factor-1 activates Akt and Jun N-terminal kinases (JNKs) in promoting the survival of T lymphocytes. Immunology 2002;107:461–71.
3. Rikhof B, de Jong S, Suurmeijer AJ, Meijer C, van der Graaf WT. The insulin-like growth factor system and sarcomas. J Pathol 2009;217:469–82.
4. Rickle A, Behbahani H, Ankarcrona M, Winblad B, Cowburn RF. PTEN, Akt, and GSK3β signalling in rat primary cortical neuronal cultures following tumor necrosis factor-α and trans-4-hydroxy-2-nonenal treatments. J Neurosci Res 2006;84:596–605.
5. Ciaraldi TP, Oh DK, Christiansen L, Nikoulina SE, Kong AP, Baxi S, et al. Tissue-specific expression and regulation of GSK-3 in human skeletal muscle and adipose tissue. Am J Physiol Endocrinol Metab 2006;291:E891–8.
6. Stambolic V, Suzuki A, de la Pompa JL, Brothers GM, Mirtsos C, Sasaki T, et al. Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN. Cell 1998;95:29–39.
7. Himpe E, Degaillier C, Coppens A, Kooijman R. Insulin-like growth factor-1 delays Fas-mediated apoptosis in human neutrophils through the phosphatidylinositol-3 kinase pathway. J Endocrinol 2008;199:69–80.
8. Isaacs JD. Therapeutic T-cell manipulation in rheumatoid arthritis: past, present and future. Rheumatology (Oxford) 2008;47:1461–8.
9. Keyszer GM, Heer AH, Kriegsmann J, Geiler T, Keysser C, Gay RE, et al. Detection of insulin-like growth factor I and II in synovial tissue specimens of patients with rheumatoid arthritis and osteoarthritis by in situ hybridization. J Rheumatol 1995;22:275–81.
10. Malemud CJ. The role of growth factors in cartilage metabolism. Rheum Dis Clin North Am 1993;19:569–80.
11. Lemmey A, Maddison P, Breslin A, Cassar P, Hasso N, McCann R, et al. Association between insulin-like growth factor status and physical activity levels in rheumatoid arthritis. J Rheumatol 2001;28:29–34.
12. Kasperkovitz PV, Timmer TC, Smeets TJ, Verbeet NL, Tak PP, van Baarsen LG, et al. Fibroblast-like synoviocytes derived from patients with rheumatoid arthritis show the imprint of synovial tissue heterogeneity: evidence of a link between an increased myofibroblast-like phenotype and high-inflammation synovitis. Arthritis Rheum 2005;52:430–41.
13. Luo Z, Zhang Y, Li F, He J, Ding H, Yan L, et al. Resistin induces insulin resistance by both AMPK-dependent and AMPK-independent mechanisms in HepG2 cells. Endocrine 2009;36:60–9.
14. Sheng CH, Di J, Jin Y, Zhang YC, Wu M, Sun Y, et al. Resistin is expressed in human hepatocytes and induces insulin resistance. Endocrine 2008;33:135–43.
15. Qatanani M, Szwergold NR, Greaves DR, Ahima RS, Lazar MA. Macrophage-derived human resistin exacerbates adipose tissue inflammation and insulin resistance in mice. J Clin Invest 2009;119:531–9.
16. Calabro P, Samudio I, Willerson JT, Yeh ET. Resistin promotes smooth muscle cell proliferation through activation of extracellular signal-regulated kinase 1/2 and phosphatidylinositol 3-kinase pathways. Circulation 2004;110:3335–40.
17. Bokarewa M, Nagaev I, Dahlberg L, Smith U, Tarkowski A. Resistin, an adipokine with potent proinflammatory properties. J Immunol 2005;174:5789–95.
18. Silswal N, Singh AK, Aruna B, Mukhopadhyay S, Ghosh S, Ehtesham NZ. Human resistin stimulates the pro-inflammatory cytokines TNF-α and IL-12 in macrophages by NF-κB-dependent pathway. Biochem Biophys Res Commun 2005;334:1092–101.
19. Schaffler A, Ehling A, Neumann E, Herfarth H, Tarner I, Scholmerich J, et al. Adipocytokines in synovial fluid. JAMA 2003;290:1709–10.
20. Senolt L, Housa D, Vernerova Z, Jirasek T, Svobodova R, Veigl D, et al. Resistin in rheumatoid arthritis synovial tissue, synovial fluid and serum. Ann Rheum Dis 2007;66:458–63.
21. Ekwall AK, Eisler T, Anderberg C, Jin C, Karlsson N, Brisslert M, et al. The tumour-associated glycoprotein podoplanin is expressed in fibroblast-like synoviocytes of the hyperplastic synovial lining layer in rheumatoid arthritis. Arthritis Res Ther 2011;13:R40.
22. Girnita A, Girnita L, del Prete F, Bartolazzi A, Larsson O, Axelson M. Cyclolignans as inhibitors of the insulin-like growth factor-1 receptor and malignant cell growth. Cancer Res 2004;64:236–42.
23. Migita K, Maeda Y, Miyashita T, Kimura H, Nakamura M, Ishibashi H, et al. The serum levels of resistin in rheumatoid arthritis patients. Clin Exp Rheumatol 2006;24:698–701.
24. O'Connor JC, McCusker RH, Strle K, Johnson RW, Dantzer R, Kelley KW. Regulation of IGF-I function by proinflammatory cytokines: at the interface of immunology and endocrinology. Cell Immunol 2008;252:91–110.
25. Shen WH, Zhou JH, Broussard SR, Freund GG, Dantzer R, Kelley KW. Proinflammatory cytokines block growth of breast cancer cells by impairing signals from a growth factor receptor. Cancer Res 2002;62:4746–56.
26. Ross SE, Hemati N, Longo KA, Bennett CN, Lucas PC, Erickson RL, et al. Inhibition of adipogenesis by Wnt signaling. Science 2000;289:950–3.
27. Bennett CN, Ross SE, Longo KA, Bajnok L, Hemati N, Johnson KW, et al. Regulation of Wnt signaling during adipogenesis. J Biol Chem 2002;277:30998–1004.
28. Longo KA, Wright WS, Kang S, Gerin I, Chiang SH, Lucas PC, et al. Wnt10b inhibits development of white and brown adipose tissues. J Biol Chem 2004;279:35503–9.
29. Ort T, Arjona AA, MacDougall JR, Nelson PJ, Rothenberg ME,

Wu F, et al. Recombinant human FIZZ3/resistin stimulates lipolysis in cultured human adipocytes, mouse adipose explants, and normal mice. Endocrinology 2005;146:2200–9.
30. Wieman HL, Wofford JA, Rathmell JC. Cytokine stimulation promotes glucose uptake via phosphatidylinositol-3 kinase/Akt regulation of Glut1 activity and trafficking. Mol Biol Cell 2007;18: 1437–46.
31. Jacobs SR, Herman CE, Maciver NJ, Wofford JA, Wieman HL, Hammen JJ, et al. Glucose uptake is limiting in T cell activation and requires CD28-mediated Akt-dependent and independent pathways. J Immunol 2008;180:4476–86.
32. Rathmell JC, Fox CJ, Plas DR, Hammerman PS, Cinalli RM, Thompson CB. Akt-directed glucose metabolism can prevent Bax conformation change and promote growth factor-independent survival. Mol Cell Biol 2003;23:7315–28.
33. Adams TE, Epa VC, Garrett TP, Ward CW. Structure and function of the type 1 insulin-like growth factor receptor. Cell Mol Life Sci 2000;57:1050–93.
34. Engberding N, San Martin A, Martin-Garrido A, Koga M, Pounkova L, Lyons E, et al. Insulin-like growth factor-1 receptor expression masks the antiinflammatory and glucose uptake capacity of insulin in vascular smooth muscle cells. Arterioscler Thromb Vasc Biol 2009;29:408–15.
35. Kaser S, Kaser A, Sandhofer A, Ebenbichler CF, Tilg H, Patsch JR. Resistin messenger-RNA expression is increased by proinflammatory cytokines in vitro. Biochem Biophys Res Commun 2003;309:286–90.
36. Hu WL, Qiao SB, Li JJ. Decreased C-reactive protein-induced resistin production in human monocytes by simvastatin. Cytokine 2007;40:201–6.