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ORIGINAL ARTICLE

Distinctive gene expression signatures in rheumatoid arthritis synovial tissue fibroblast cells: correlates with disease activity

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Gene expression profiling of rheumatoid arthritis (RA) and osteoarthritis (OA) joint tissue samples provides a unique insight into the gene signatures involved in disease development and progression. Fibroblast-like synovial (FLS) cells were obtained from RA, OA and control trauma joint tissues (non-RA, non-OA) and RNA was analyzed by Affymetrix microarray. Thirty-four genes specific to RA and OA FLS cells were identified ($P<0.05$). HOXD10, HOXD11, HOXD13, CCL8 and LIM homeobox 2 were highly and exclusively expressed in RA and CLU, sarcoglycan-γ, GPR64, POU3F3, peroxisome proliferative activated receptor-γ and tripartite motif-containing 2 were expressed only in OA. The data also revealed expression heterogeneity for patients with the same disease. To address disease heterogeneity in RA FLS cells, we examined the effects of clinical disease parameters (Health Assessment Questionnaire (HAQ) score, C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), rheumatoid factor (RF)) and drug therapies (methotrexate/prednisone) on RA FLS cell gene expression. Eight specific and unique correlations were identified: human leukocyte antigen (HLA)-DQA2 with HAQ score; Clec12A with RF; MAB21L2, SIAT7E, HAPLN1 and BAIAP2L1 with CRP level; RGMB and OSAP with ESR. Signature RA FLS cell gene expression profiles may provide insights into disease pathogenesis and have utility in diagnosis, prognosis and drug responsiveness.
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Keywords: *rheumatoid arthritis; osteoarthritis; synovial fibroblast; microarray*

Introduction

Rheumatoid arthritis (RA) is a common, relapsing autoimmune disease affecting approximately 1% of the population worldwide.[1] RA presents clinically with joint swelling, deformity, pain, stiffness and weakness.[2] The primary sites of tissue damage are joints, with the rheumatoid synovial environment presenting as an area of intense immunological activity,[3] but systemic involvement of the eyes, kidneys and lungs may also occur. The affected RA joint is characterized by proliferation of synovial lining cells, pannus accumulation over articular cartilage and the infiltration of inflammatory cells, including mononuclear cells and lymphocytes. Fibroblast-like synovial (FLS) cells are thought to be responsible for pannus formation and contribute to bone and cartilage destruction.

One of the hallmarks of RA is synovial hyperplasia. A critical resident cell type in affected synovial tissue (ST) is the CD68$^-$/MHCII$^-$ FLS cell.[4] FLS cells synthesize and secrete many pro-inflammatory mediators – cytokines, chemokines, growth factors, matrix metalloproteases (MMP) – that are involved in autocrine and paracrine regulation of inflammation[3] and, therefore, are critical effectors in regulating the inflammatory response in RA. FLS cells in RA are thought to transform into cells that proliferate in an anchorage-independent manner, lack contact inhibition and secrete cytokines constitutively. Many growth factors, such as platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), transforming growth factor-β (TGF-β) and activin are expressed in RA and drive fibroblast proliferation *in vitro*.[5,6]

The activated phenotype of distinct cell populations in affected RA patients determines disease severity. A clear understanding of those factors that contribute to the activated phenotype is required. In recent years microarray gene expression technology has been applied to investigate different pathogenic processes in RA, using ST[7,8] or RA peripheral blood mononuclear cells[9,10] and few studies have examined joint FLS cells.[11,12] A trend towards enhanced expression for many pro-inflammatory genes has been consistently reported, with no single emergent profile. Given the critical contributions of FLS cells to disease pathogenesis in RA, we undertook a comprehensive analysis of the gene expression profiles in RA ST FLS cells from affected joints, comparing gene expression with expression profiles of ST FLS cells from affected joints from osteoarthritis (OA) patients and joints from surgical trauma patients (non-OA/RA).

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To address RA patient heterogeneity, gene expression data were considered in the context of standard measures of disease activity, namely Health Assessment Questionnaire (HAQ) score, rheumatoid factor (RF), erythrocyte sedimentation rate (ESR) and serum C-reactive protein (CRP) and taking into account patient medications such as methotrexate (MTX) and prednisone. Viewed altogether, the data suggest signature gene expression profiles associated with RA are influenced by disease activity and medication status.

Results

Extensive in vitro *cell passaging alters gene expression profiles*
Because of limited specimen size, it was necessary to expand the ST FLS cell population *in vitro* to obtain sufficient cells for RNA extraction. At the outset, we undertook experiments to assess the effects of *in vitro* culturing of ST FLS cells on gene expression profiles. Since the cytokine-rich microenvironment in the RA joint is responsible for regulating the expression of many of the genes expressed in the ST FLS cells, prolonged *in vitro* culture may not be representative of *in situ* FLS cell gene expression. ST FLS cells were isolated from RA joints from 16 different patients and cultured *in vitro* for either 2/3 ($n=8$) or 8 ($n=8$) passages. RNA was isolated and analyzed using the Affymetrix chip U133A representing 14 500 genes. The data are expressed as the average fold change in gene expression when cells are maintained in culture, and 148 genes were identified whose expression was significantly ($P<0.05$) different between early and late passage FLS (Figure 1, Supplementary Table 1). A significant downregulation of immune regulatory genes, such as *IGHC1*, *PTPRC*, *IGJ* and *IGCL2*, was observed by passage number 8 of ST FLS cells compared to passage 2/3. Additionally, we observed an upregulation of expression for 88 genes when FLS cells were maintained in culture for eight passages. Accordingly, all subsequent experiments were performed on ST FLS cells maintained in culture for $\leqslant 3$ passages, in an attempt to obtain gene expression data representative of *in situ* RA ST FLS cells.

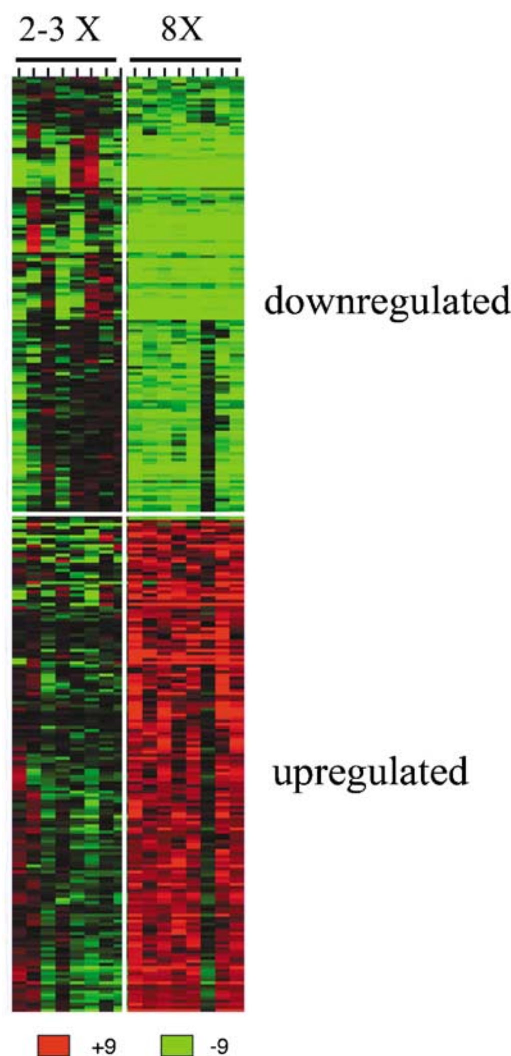

Figure 1 Hierarchical cluster diagram of differential gene expression in RA ST FLS cells maintained in culture for low- or high-passage number. Gene expression levels in the 2–3 × passage number ($n=8$) RA FLS cell samples were used as baseline and the fold change is shown relative to the average expression level measured for each gene in the 8 × passage number group ($n=8$). Data are visualized colorimetrically with heat plots, 'red' representing elevated gene expression and 'green' decreased gene expression.

Distinguishing gene expression profiles between ST FLS cells from OA and RA joints
Next, we examined the gene expression profiles of ST FLS cells from affected joints from 17 RA and 20 OA patients. Gene expression levels in ST FLS cells from non-RA or non-OA joints, collected from six trauma patients at the time of orthopedic surgery, served as the experimental baseline. RNA was prepared from harvested ST FLS cells maintained for $\leqslant 3$ passages in culture, then analyzed using the Affymetrix U133 plus 2.0 array, representing over 47 000 transcripts. For each gene, the baseline level of expression is represented as the average of the intensity signals obtained for each of the six control specimens. Hierarchical clustering was performed as a non-bias analysis of gene expression. The data in Figure 2 indicate the fold change in gene expression for RA and OA joint ST FLS cells, relative to the controls. The expression levels of genes representing 287 unique probe sets were identified as significantly ($P<0.05$) different between RA and OA ST FLS cells, taking a twofold difference as the minimal cutoff (Table 1). The majority of the products of these genes are involved in regulation of the immune response, according to the Gene Ontology biological function classification (www.geneontology.org). As anticipated, genes involved in proteolysis, signal transduction and regulation of transcription were also identified as differentially regulated in OA and RA ST FLS cells.

Genes expressed in affected joints from ST FLS cells which may be considered potential unique biomarkers of either RA or OA were defined as genes whose expression was either upregulated or downregulated $\geqslant$3-fold relative to control levels, and are distinguishable between RA and OA based on a $<$1-fold change in expression in the other distinct disease group. Using these criteria, and focusing exclusively on the 287

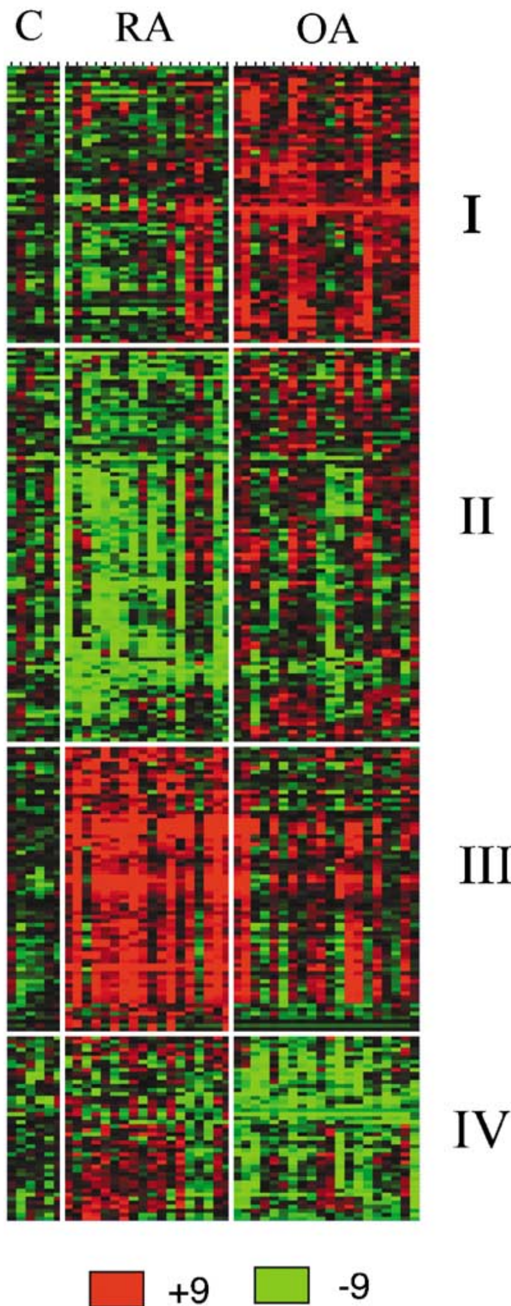

Figure 2 Hierarchical clustering of differential gene expression in RA and OA ST FLS cells. Expression levels in control (non-RA, non-OA, $n=6$) ST FLS cell samples were used as baseline and the fold change in expression is shown relative to the average control value. Data are visualized colorimetrically with heat plots, 'red' representing elevated gene expression and 'green' decreased gene expression. Gene expression levels for 287 probe sets are significantly different ($P<0.05$) between the RA and OA groups and four distinct categories are identified: (I) increased in OA, (II) decreased in RA, (III) increased in RA and (IV) decreased in OA. OA, osteoarthritis; RA, rheumatoid arthritis.

significant probe sets, several disease-specific genes were identified. In RA ST FLS cells, five genes were identified with expression levels upregulated >3-fold above control levels, with <1-fold upregulation in OA ST FLS cells, namely genes for the homeobox proteins D10, D11 and D13 (*HOXD10, HOXD11, HOXD13*), for chemokine ligand 8 (*CCL8/MCP-2*) and for LIM homeobox 2 (*LHX2*) (Figure 3, Table 1, bold). The expression levels of six genes were exclusively upregulated in OA joint ST FLS cells: clusterin (*CLU*), sarcoglycan-γ, *GPR64*, *POU3F3*, peroxisome proliferative activated receptor-γ (*PPARG*) and tripartite motif-containing 2 (*TRIM2*). The expression levels of 26 other genes were also upregulated in *both* OA and RA joint ST FLS cells, thereby not fulfilling the criterion as unique biomarkers. Of these, using a threefold difference in expression levels between OA and RA specimens, RA joint ST FLS cells expressed elevated gene expression for *ADAMDEC1*, *IGSF6*, *MMP-12*, *PLCB4* and human leukocyte antigen (*HLA*) genes, whereas OA joint ST FLS cells exhibited elevated gene expression for *CSN1S1*, *CP*, *GPR49*, *MAB21L2*, *MMP-10* and *GABA* receptors. Another defining characteristic was the downregulation of specific genes, notably *EDNRB* and *PRG4* in RA, and *CTSC*, *NRN1* and *TBX5* in OA FLS.

Validation of RA- and OA-specific gene profiles

To validate further the expression levels of RA- or OA-specific genes, *HOX10*, *HOXD11*, *LHX2*, *PPARG*, *POU3F3* and *TRIM2* gene levels were examined in a distinct set of RA and OA patient samples using real-time quantitative reverse transcrition (RT)-PCR. As illustrated in Figure 4, RA-specific genes *HOXD10*, *HOXD11* and *LHX2* are preferentially upregulated in RA-patient ST FLS cells, whereas OA-specific genes *PPARG*, *POU3F3* and *TRIM2* are preferentially upregulated in OA patient ST FLS cells compared to RA ST FLS cells. These data are in agreement with our microarray findings.

Distinguishing the effect of clinical parameters or drug therapies on RA FLS gene expression profiles

A number of studies have used microarray technology to investigate gene expression in RA, yet because of the complicated molecular processes effecting joint inflammation, the multiple pathophysiological processes ongoing at different stages of the disease, the inherent interpatient variability and the different tissues that have been analyzed, no consistent unique RA biomarkers have been identified in specific-cell populations. Although our study has focused on the RA-joint FLS cell, at end-stage disease, we next took into consideration other parameters that would contribute to FLS cell gene expression, including measures of disease activity and medication status. Gene expression levels in five RA joint FLS cell samples from patients with high (>1.8) HAQ scores were compared with gene expression levels in RA joint FLS cell samples from eight patients with low (<1.8) HAQ scores. Our analysis identified 19 genes for which gene expression levels were significantly different between the two groups. Nine of these genes exhibited a higher level of expression ($\geqslant$2-fold above control gene expression) in ST FLS cells from patients with HAQ scores >1.8 (Figure 5a, Supplementary Table 2). Surprisingly, we identified elevated gene expression ($\geqslant$2-fold above control levels) for 10 genes in ST FLS cell gene expression from patients with HAQ scores <1.8 (Figure 5a, Supplementary Table 2). Statistical analysis revealed significant negative correlation with HAQ scores and gene expression for *HLA-DQA1* ($r^2=0.55$, $P=0.0024$) (Figure 6a) and *HLA-DQB1* ($r^2=0.38$, $P=0.0193$, data not shown). Similar analysis examining

Table 1 Evaluation of gene expression differences between RA ($n=18$) and OA ($n=20$) ST FLS cells

Gene description	*Gene symbol*	*RA*	*OA*
ADAM-like, decysin 1	*ADAMDEC1*	23.83	5.24
Major histocompatibility complex, class II, DQ α1	*HLA_DQA1*	18.82	1.82
Major histocompatibility complex, class II, DR α	*HLA-DRA*	18.22	6.44
Major histocompatibility complex, class II, DR β4	*HLA-DRB4*	13.61	1.91
Leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 1///lysozyme (renal amyloidosis)	*LILRB1/LYZ*	12.97	4.84
MMP-12 (macrophage elastase)	*MMP12*	11.71	3.12
Chemokine (C-C motif) ligand 18 (pulmonary and activation-regulated)	*CCL18*	8.69	3.12
Absent in melanoma 2	*AIM2*	8.35	1.39
Collagen, type X, α1 (schmid metaphyseal chondrodysplasia)	*COL10A1*	8.07	1.23
Endothelial differentiation, sphingolipid G-protein-coupled receptor, 3	*C9orf47*	7.30	1.75
Immunoglobulin superfamily, member 6	*IGSF6*	5.57	1.53
Membrane-spanning 4-domains, subfamily A, member 6A	*MS4A6A*	4.81	1.22
Phospholipase C, β4	*PLCB4*	4.63	1.58
Homeo box D11	***HOXD11***	**4.49**	**−1.85**
Chemokine (C-C motif) ligand 18 (pulmonary and activation-regulated)	*CCL18*	4.18	1.78
GATA binding protein 6	*GATA6*	4.11	1.80
LIM homeobox 2	***LHX2***	**3.91**	**−1.32**
Fc fragment of IgG, low affinity IIa, receptor for (CD32)	*FCGR2A*	3.89	1.67
Fructose-1,6-bisphosphatase 1	*FBP1*	3.59	1.40
Chemokine (C-C motif) ligand 8	***CCL8***	**3.40**	**−1.41**
Chemokine (C-C motif) ligand 3-like, centromeric	*MGC12815*	3.30	1.56
Lymphocyte antigen 86	*LY86*	3.29	1.46
Bone marrow stromal cell antigen 1	*BST1*	3.25	1.15
Homeo box D13	***HOXD13***	**3.19**	**−1.16**
Brain expressed, X-linked 1	*BEX1*	3.17	1.37
Homeo box D10	***HOXD10***	**3.07**	**−1.04**
Cytokine receptor-like factor 1	*CRLF1*	−2.86	−1.23
Dual specificity phosphatase 4	*DUSP4*	−3.45	−1.52
Endothelin receptor type B	*EDNRB*	−3.57	1.46
SAM domain containing 1	*LOC389432*	−2.86	−1.27
Nedd4 family interacting protein 1	*NDFIP1*	−4.55	−1.41
Proteoglycan 4	*PRG4*	−4.76	2.17
Secreted frizzled-related protein 1	*SFRP1*	−2.56	−1.10
Secreted frizzled-related protein 2	*SFRP2*	−4.55	−1.67
Mab-21-like 2 (*Caenorhabditis elegans*)	*MAB21L2*	7.32	20.63
Casein αs1	*CSN1S1*	2.07	17.43
Defensin, β1	*DEFB1*	1.17	8.77
MMP-10 (stromelysin 2)	*MMP10*	2.81	7.19
Ceruloplasmin (ferroxidase)	*CP*	1.72	6.30
G-protein-coupled receptor 49	*GPR49*	1.79	6.23
Ecotropic viral integration site 1	*EVI1*	2.09	5.89
ELOVL family member 7, elongation of long chain fatty acids (yeast)	*ELOVL7*	2.08	5.36
Coagulation factor V (proaccelerin, labile factor)	*F5*	1.59	5.17
Clusterin (complement lysis inhibitor, SP-40,40, sulfated glycoprotein 2, testosterone-repressed prostate message 2, apolipoprotein J)	***CLU***	**1.02**	**5.06**
LIM domain only 2 (rhombotin-like 1)	*LMO2*	1.54	4.52
Tropomodulin 1	*TMOD1*	1.54	4.96
G-protein-coupled receptor 64	***GPR64***	**1.06**	**4.90**
POU domain, class 3, transcription factor 3	***POU3F3***	**1.01**	**4.67**
Solute carrier family 7 (cationic amino-acid transporter, y+system) member 11	*SLC7A11*	1.34	4.62
GABA A receptor, α4	*GABRA4*	1.74	4.58
Sema domain, immunoglobulin domain (Ig), short basic domain, secreted (semaphorin) 3A	*SEMA3A*	1.35	4.46
Sarcoglycan, γ (35 kDa dystrophin-associated glycoprotein)	***SGCG***	**−1.25**	**4.38**
GABA A receptor, β1	*GABRB1*	1.53	4.26
Carboxylesterase 1 (monocyte/macrophage serine esterase 1)	*CES1*	1.17	3.59
G-protein-coupled receptor 27	*GPR27*	1.26	3.47
Insulin-like growth factor binding protein 2, 36 kDa	*IGFBP2*	1.10	3.28
Sine oculis homeobox homolog 3 (*Drosophila*)	*SIX3*	1.18	3.24
Tissue inhibitor of metalloproteinase 4	*TIMP4*	1.36	3.23
Tripartite motif-containing 2	***TRIM2***	**1.09**	**3.18**
Solute carrier family 7 (cationic amino-acid transporter, y+system), member 2	*SLC7A2*	1.16	3.16
Peroxisome proliferative activated receptor, γ	***PPARG***	**1.04**	**3.01**
Leucine-rich repeat containing 15	*LRRC15*	−1.59	−3.23
Neuritin 1	*NRN1*	1.42	−2.78
Cathepsin C[a]	*CTSC*	1.71	−2.82
T-box 5	*TBX5*	−1.04	−2.63
Chemokine (C-C motif) ligand 13	*CCL13*	1.20	−2.33
Thy-1 cell surface antigen	*THY1*	−1.59	−2.78
Microfibrillar-associated protein 5	*MFAP5*	−1.37	−2.78

Abbreviations: FLS, fibroblast-like synovial; GABA, γ-aminobutyric acid; MMP, matrix metalloproteinase; OA, osteoarthritis; RA, rheumatoid arthritis; ST, synovial tissue.
Values represent the average change in gene expression (+/−) relative to control baseline samples. Values identified on a gray background reflect genes whose expression levels are significantly different ($P<0.05$) between OA and RA ST FLS cell samples and only >2.5-fold changes are shown.
Genes identified in bold, represent unique disease-specific biomarkers as identified using the criteria outlined in the Results section.
[a]Average of two probe sets.

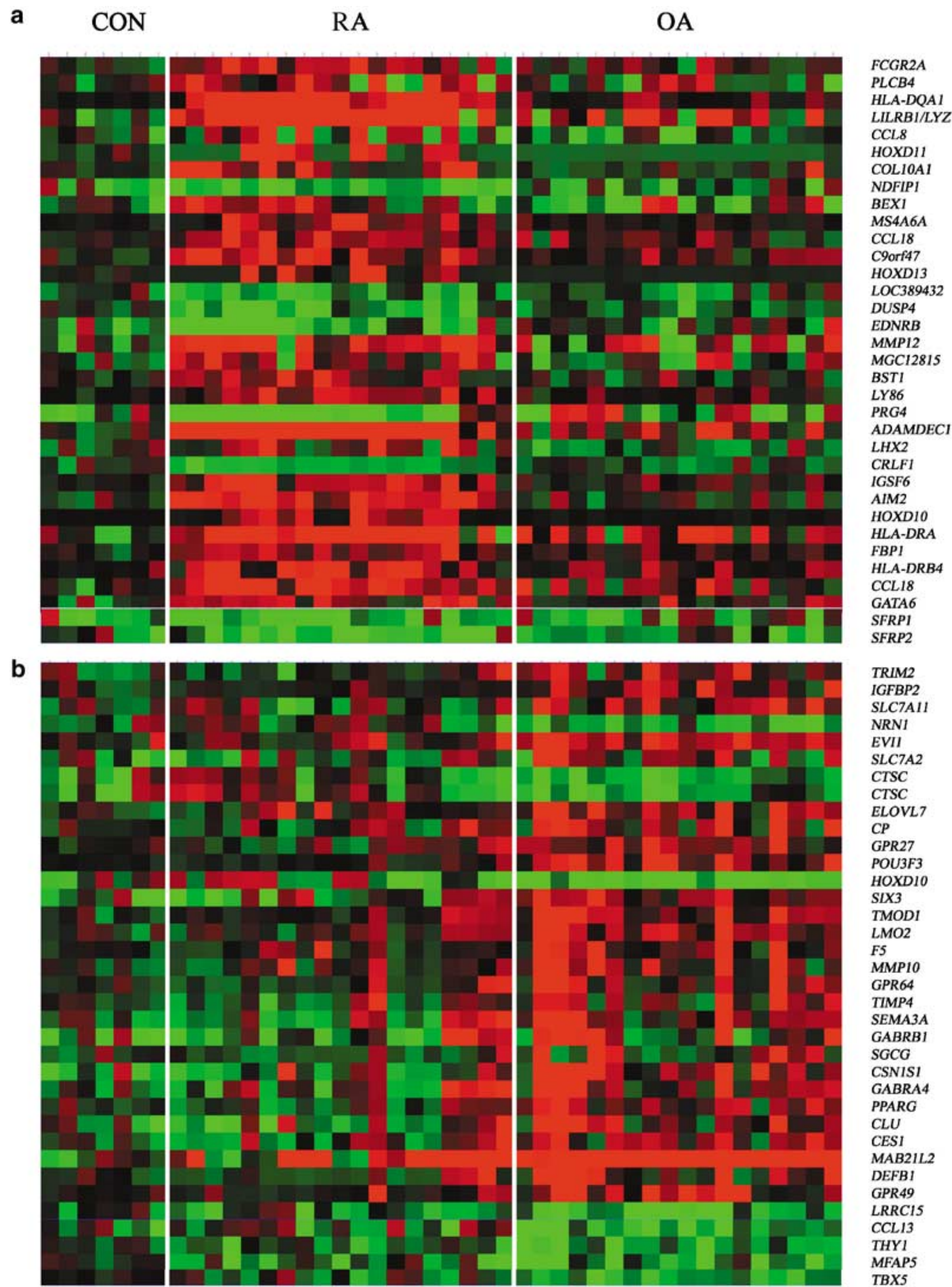

Figure 3 Cluster diagram of specific genes differentially regulated ⩾3-fold in RA versus OA ST FLS cells that may represent disease-specific biomarkers. (**a**) Genes significantly ($P<0.05$) upregulated or downregulated ⩾3-fold in RA samples; (**b**) genes significantly ($P<0.05$) upregulated or downregulated ⩾threefold in OA samples. Data are visualized colorimetrically as per Figure 2. OA, osteoarthritis; RA, rheumatoid arthritis.

gene expression levels in ST FLS cells from six RA patients with high RF values (>40 IU/ml) and five RA patients with low RF values (<40 IU/ml) identified 13 genes for which their expression was found to be significantly ($P<0.05$) distinct based on high or low RF values (Figure 5c, Supplementary Table 3). Most notably, elevated gene expression for *Clec12A*, a C-type lectin-like receptor, which may act as a negative regulator of immune cell function, correlated with RF values >40 IU/ml ($r^2=0.74$, $P=0.0007$; Figure 6c).

We next examined whether signature gene expression profiles may be defined in terms of serum CRP levels in the RA cohort. Data for five RA patients with high serum CRP (>8 mg/l) and five RA patients with low CRP (<8 mg/l) were analyzed. The expression levels of 18 genes were found to be significantly upregulated in RA

ST FLS cells from patients with CRP levels >8 mg/l, and the expression levels eight genes were elevated in ST FLS cells from patients with CRP levels <8 mg/l (Figure 5b, Supplementary Table 4). Statistical analysis for significance showed that elevated expression levels for *BAIP2L1* ($r^2 = 0.82$, $P = 0.0003$), *HAPLN1* ($r^2 = 0.71$, $P = 0.0021$), *SIAT7E* ($r^2 = 0.48$, $P = 0.0274$) and *MAB21L2* ($r^2 = 0.53$, $P = 0.0172$) correlated with serum CRP levels >8 mg/l (Figure 6b). While the expression levels of nine genes were elevated in the ST FLS cells from the RA patient cohort with serum CRP levels <8 mg/l, our analysis revealed no statistically significant correlation between their expression levels and serum CRP values <8 mg/ml.

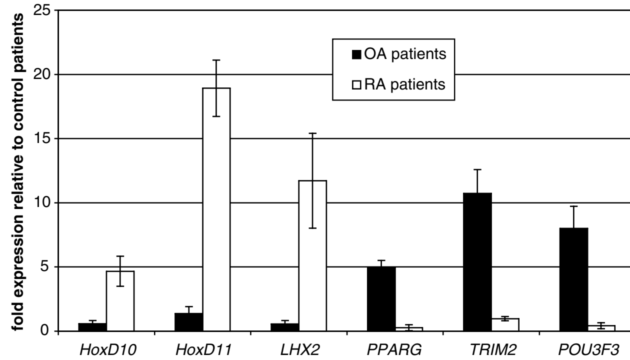

Figure 4 Quantitative real-time RT-PCR validation of potential RA and OA biomarkers. RNA was extracted from ST FLS cells from eight OA (■) and eight RA (□) patients, using the RNeasy kit. cDNA synthesis was carried out using random primers and 100 ng total RNA. A total of 500 ng of cDNA was used as a template for real time PCR. For each sample, reference (*HPRT*) and target (*HOXD10*, *HOXD11*, *LHX2*, *PPARG*, *TRIM2* and *POU3F3*) reactions were performed. The data are presented as the fold expression relative to gene expression levels in ST FLS cells from three trauma control patients. Values ± s.e. were calculated using Relative Quantification software (Roche). *LHX2*, LIM homeobox 2; OA, osteoarthritis; *PPARG*, peroxisome proliferative activated receptor-γ; RA, rheumatoid arthritis; *TRIM2*, tripartite motif-containing 2.

Further analysis of the data in the context of five RA patients with ESR of >50 mm/h and four RA patients with ESR <50 mm/h, identified 12 genes for which expression levels were elevated and five genes for which expression levels were decreased in patients with high ESR (Figure 5d, Supplementary Table 5). Statistical analysis revealed significant correlations between the extent of gene expression in RA ST FLS cells for *RGMB* ($r^2 = 0.57$, $P = 0.0188$) and *OSAP* ($r^2 = 0.65$, $P = 0.008$) (Figure 6d) and ESR in these RA patients.

Our data identify a significant correlation between CRP and ESR as objective markers of RA disease activity ($r^2 = 0.5$, $P = 0.0339$; Figure 5e). Accordingly, we examined FLS cell gene expression in a combined group of patients ($n = 9$) with high levels of CRP (>8 mg/l) and/or ESR (>50 mm/h) and compared the signatures of gene expression with those for a combined group of patients with low CRP and/or ESR ($n = 4$). We identified

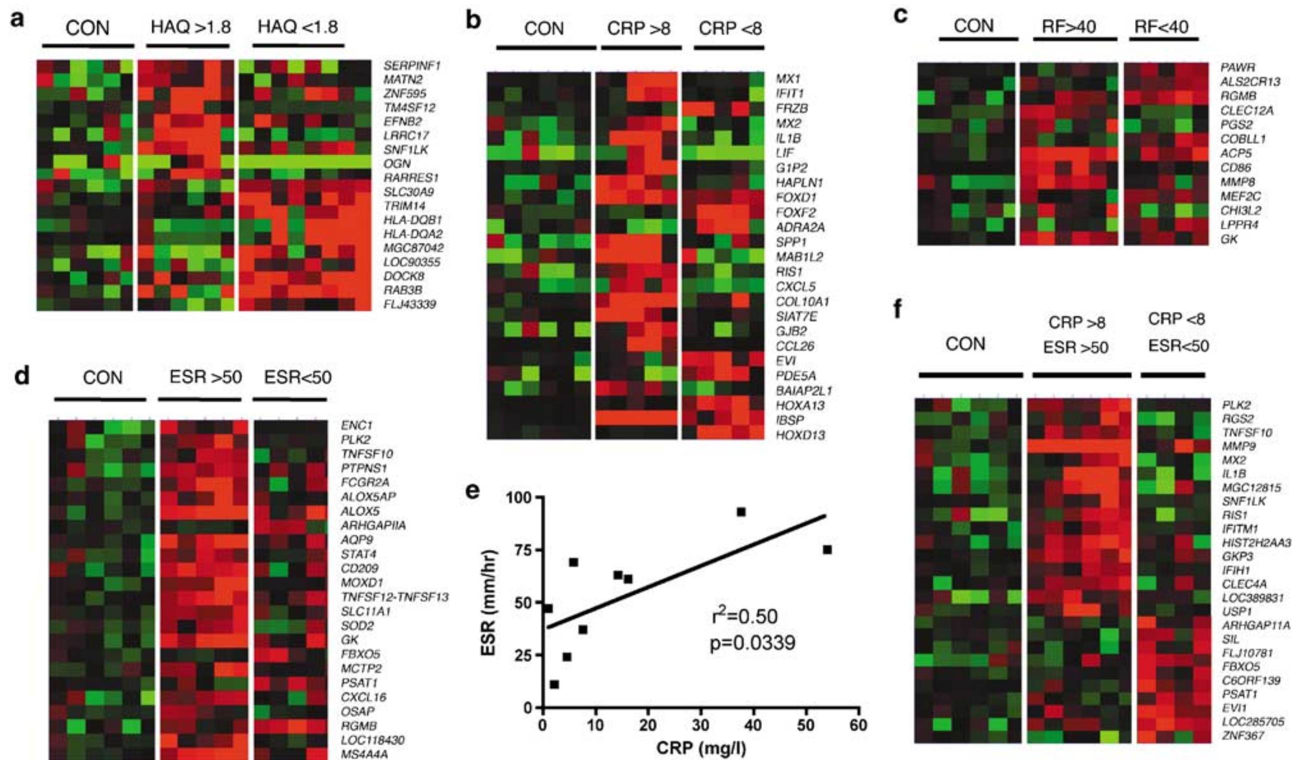

Figure 5 Cluster diagrams of genes whose expression levels correlate with clinical markers of disease activity. Genes are identified whose expression levels are significantly different ($P < 0.05$) in RA FLS cells from patients with (**a**) high HAQ scores compared to low HAQ scores; (**b**) high CRP levels compared to low CRP levels; (**c**) high RF levels compared to low RF levels; (**d**) high ESR compared to low ESR. (**e**) Regression analysis of ESR with CRP levels. (**f**) Genes are identified whose expression levels are significantly different ($P < 0.05$) in RA FLS cells from patients with high ESR and/or CRP levels compared to low ESR and/or CRP levels. Data are visualized colorimetrically as per Figure 2. CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; HAQ, Health Assessment Questionnaire; RF, rheumatoid factor.

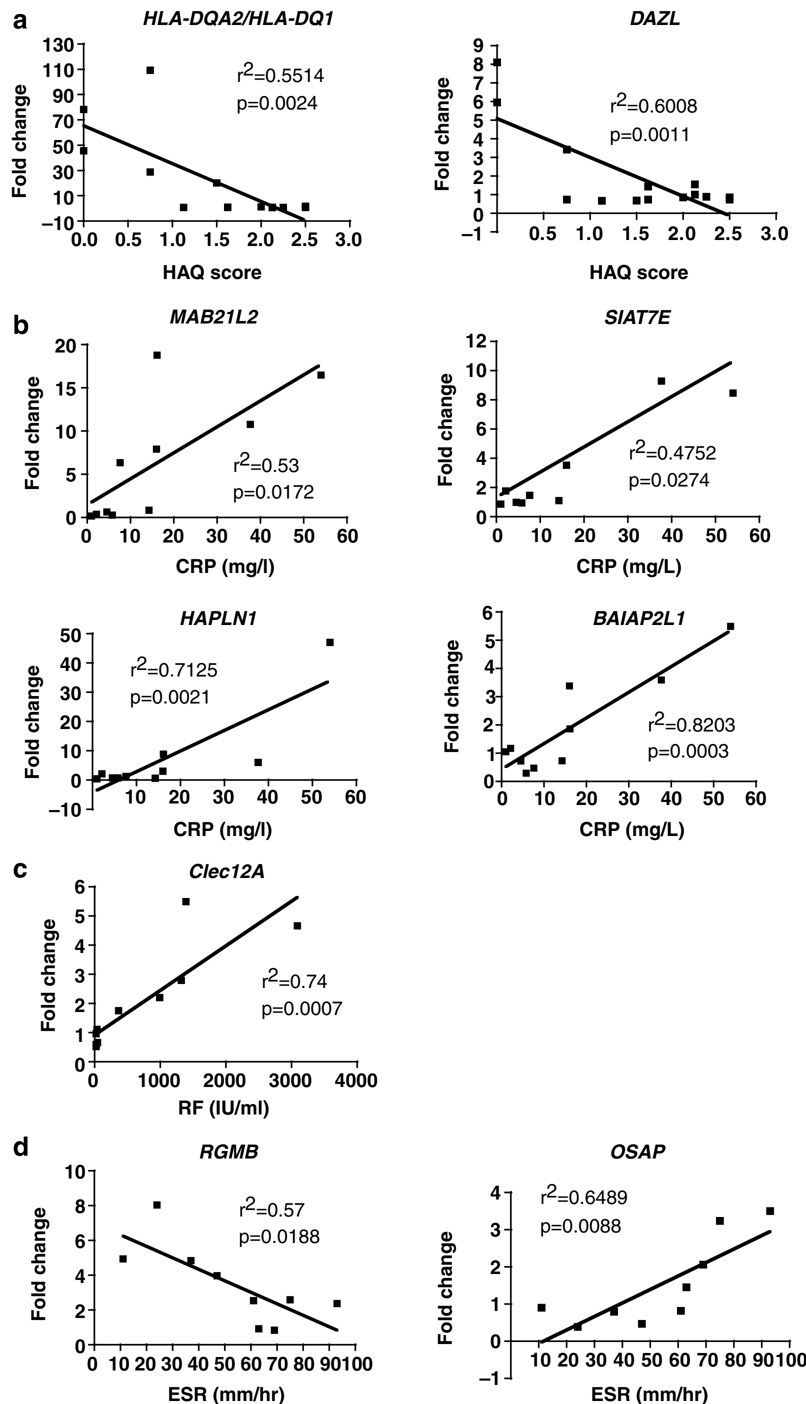

Figure 6 Expression profiles of selected genes correlate with clinical parameters. Regression plots denoting correlates of expression levels for distinct genes with specified objective markers of disease activity: (**a**) HAQ; (**b**) CRP; (**c**) RF and (**d**) ESR. CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; HAQ, Health Assessment Questionnaire; HLA, human leukocyte antigen; RF, rheumatoid factor.

22 genes that were significantly different between the two groups, setting the cutoff at ⩾3-fold differences in expression levels (Figure 5f, Supplementary Table 6). Nine of these genes, *interleukin (IL)-1B, PLK2, MX2, TNFSF10, RGMB, EVI1, PSAT1, FBXO5* and *ARHGAP11A*, had been identified as either upregulated or downregulated in the earlier CRP or ESR analyses. By combining these two markers of disease activity, an additional subset of genes was identified, implicated in acute inflammation, namely *MMP-9, CCL3, CLEC4A* and *TNFSF10*.

In a final series of analyses, the effects of medication with MTX or prednisone on ST FLS cell gene expression levels were evaluated. Gene expression levels were compared in 10 RA patients receiving MTX therapy and 8 not receiving it. The data in Figure 7a and

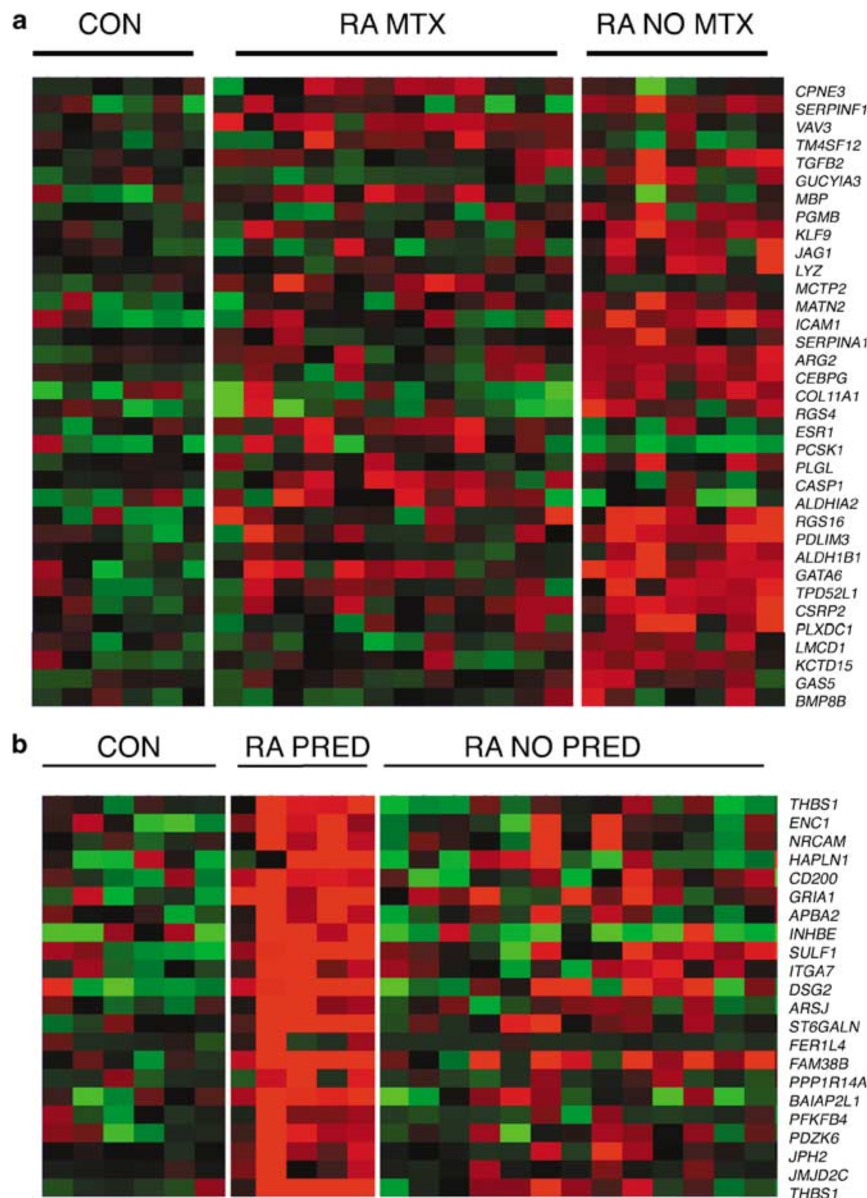

Figure 7 Specific gene expression signatures in RA ST FLS cells are associated with MTX and prednisone therapies. Hierarchical cluster diagram of genes whose expression levels correlate with MTX (**a**) or prednisone (**b**) therapy. Data are visualized colorimetrically as per Figure 2. MTX, methotrexate.

Supplementary Table 7 demonstrate that MTX therapy significantly contributed to the elevated gene expression of 11 genes, *MCTP2*, *ALDH1A2*, *CASP1*, *ESR1*, *VAV3*, *MBP*, *TM4SF12*, *CPNE3*, *PCSK1*, *SLC16A4* and *SERPINF1*, and decreased gene expression of 26 genes, including *ICAM1*, *RGS16* and *GATA6*. In the context of prednisone treatment, we compared the gene expression profiles in ST FLS cells from five RA patients receiving prednisone to 13 not receiving it. We observed upregulated expression of 23 genes in patients undergoing prednisone therapy (Figure 7b, Supplementary Table 8).

Discussion

RA is a complex, polygenic autoimmune disease that develops over time and involves many different cell types, including both recruited and resident cells. Diagnosis of RA is difficult since there is no single test or etiological agent, but rather is based on the presence of a percentage of clinical parameters, radiographic evidence of joint destruction and/or the presence of RF. Identification of specific genes or biochemical events exclusively involved in the pathogenesis and progression in RA would be invaluable for diagnosis and/or monitoring treatment responses. A number of microarray studies have analyzed RA and OA tissues in an effort to identify disease-specific genes. While these studies have provided some insights, they have not provided a consistent, comprehensive disease profile, in part due to differences in cell types examined, small sample sizes and, in some studies, lack of appropriate control tissues. Herein, we have employed Affymetrix oligonucleotide microarray gene chip analyses, repre-

senting over 47 000 transcripts, to provide a comprehensive genome-wide FLS cell profile for representative RA and OA STs from affected joints. Using this approach, unique, disease-specific genes were identified. While many genes were differentially regulated, heterogeneity of gene expression among patients with the same disease was observed. RA disease activity, as revealed by HAQ score, CRP, ESR or RF levels, was associated with specific gene expression signatures in ST FLS cells. Additionally, prednisone and MTX treatment affected gene signatures. The results presented describe a methodology to monitor joint pathology and the efficacy of drug therapies.

The synovium of affected joints is generally regarded as the primary site of pathology and the cytokine and growth factor rich environment in both RA and OA affected joints influences ST FLS cell gene expression. Activation of the rheumatoid synovium occurs through both soluble factors and cell–cell interactions. Using unsupervised hierarchical clustering, we could clearly differentiate the gene expression profile in RA FLS cells from OA FLS cells. We identified many differentially regulated genes, several of which had been previously identified in RA (*HLA-DR*,[13] *CCL18*[14] and *BST1*[15]) or OA (*CLU*[16] and *DEFB1*[17]). However, many genes for which expression were significantly upregulated in RA were also expressed at albeit lower levels in OA patient FLS cells, making these genes unlikely candidates as RA-specific biomarkers. Examination of genes exclusive to each disease revealed a small subset of genes for RA or OA FLS cells. In the case of both diseases, only a subset (30–50%) of patients' FLS cell samples showed this unique expression signature, suggesting FLS cell expression heterogeneity within patients with the same disease. Previous studies have reported RA FLS cell gene expression heterogeneity.[11,18,19] Heterogeneity of gene expression is not surprising, given that RA is a complex inflammatory process that changes over time. Additionally, the clinical presentation of RA is heterogeneous and treatment outcomes vary.[20] Although even limited passaging of the FLS cells in culture may invoke cell activation and outgrowth of a subpopulation of cells, our data would suggest that this was not the case. Close scrutiny of the cultures revealed that they were uniform in the context of morphology of cells and all cells stained positive for the fibroblast marker 5B5. Moreover, among the expressed genes we identified, were genes that had been previously identified in RA and OA FLS cells.

There are at least two distinct types of RA FLS cells, one characterized by activation of the adaptive immune response and the second, similar to OA, involved in extracellular matrix remodeling and fibrosis and likely involving fibroblast de-differentiation.[18,19] Interestingly, Kasperkovitz *et al*.[11] reported a correlation between an unique gene expression signature in a subset of RA FLS cell samples and the presence of α-smooth muscle actin (SMA) expressing myofibroblasts involved in wound healing. This population of α-SMA-positive cells was also reported in another study,[21] where these cells were identified in 50% of the RA patient tissues. The origin of myofibroblasts within the tissue is unclear, derived from either TGF-*β* stimulated fibroblasts or differentiated from stem cells – fibrocytes – recruited from peripheral blood.[22,23] Here, we identified four transcription factors (*HOXD10*, *HOXD11*, *HOXD13* and *LHX2*) involved in development, including limb outgrowth,[24] as genes whose expression was unique to RA ST FLS cells. The LIM-homeobox transcription factor, *LHX2*, has been implicated in regulating hematopoietic and hair follicle stem cells.[25,26] The Hox genes are a large family of transcriptional regulators primarily involved in development and mutations in *HOXD13* are associated with synpolydactyly in humans.[24] *HOXD13* can activate the Eph receptor tyrosine kinase, EphA7, which plays a major role in limb development in the mouse.[27] *HOXD9* has been shown to be expressed in synovial fibroblasts[28] and may contribute to enhanced synovial proliferation,[29] thereby contributing to synovial hyperplasia. In other studies, the expression of genes such as bone morphogenetic protein (BMP)[30,31] Wnt and frizzled,[32] associated with growth and development, have also been identified in RA tissues. Here, we show some of the genes involved in embryonic limb development are expressed in the RA joint, thereby supporting a hypothesis that regulatory mutations affecting multiple Hox genes are associated with limb malformations, similar to what is observed in RA.

While useful data relating to gene expression in RA FLS cells may be obtained by this approach, it is clear that RA FLS cell gene expression profiles are not uniform among patients. Many factors may influence FLS cell gene expression and analysis of gene expression in the context of variables in the patient cohort, specifically clinical parameters or current drug therapies, may help to characterize the signature gene profiles. RA is primarily a disease affecting the joints, yet there is systemic involvement[33] with specific laboratory measurements that serve to classify disease activity and disease severity. Elevated CRP and ESR levels, the presence of antibodies to RF and multiple joint involvement are indicators of poor prognosis. Elevated HAQ scores and CRP levels are associated with reductions in bone mineral density[34] and evidence for serum TNF-α, MMP-1 and MMP-3 expression is associated with elevated CRP levels and ESR in RA.[35] IL-1B polymorphisms are also associated with higher clinical disease scores, including CRP levels. Kasperkovitz *et al*.[11] were the first to report a correlation between RA FLS cell phenotype, in the context of gene expression, and the intensity of the inflammatory response in affected joints. Here, we show specific RA FLS cell gene expression signatures associated with HAQ score, CRP levels, RF levels and ESR. Specifically, we show a correlation between CRP levels and gene expression for several diverse genes, including glycosyl transferase gene (*SIAT7E*), an insulin receptor tyrosine kinase (*BAIAP2L1*), an antagonist for bone morphogenic protein 4 (*MAB21L2*),[36] and a factor involved in stabilizing the extracellular cartilage matrix and involved in chondrocyte differentiation and maturation (*HAPLN1*).[37] Interestingly, we also identified expression of a number of interferon (IFN) inducible genes including *Mx1*, *Mx2*, *G1P2* and *IFIT1* in the FLS cells in patients with high CRP levels. Increases in IFN stimulated gene expression in RA ST have been reported[38] and inhibition of cytokine signaling mediated by SOCS3 alleviates the symptoms of RA in a mouse collagen induced arthritis model[39] stressing the importance of IFN in the pathogenesis of RA.

Other potential correlates identified included ESR with gene expression for *RGMB* and *OSAP*, and elevated

serum RF levels with increased FLS gene expression for *CLEC12A*. RGMB is a glycosyl-phosphatidyl inositol (GPI)-anchored protein member of the repulsive guidance system and, during development, RGMB enhances BMP signaling by binding to BMP2 and BMP4.[40] Since BMPs are involved in bone formation, we infer that patients with elevated ESR may have decreased regenerative capacity. OSAP is believed to have a role in steroidogenesis,[41] perhaps contributing to the sex bias noted in RA. The expression of other genes was found to be elevated in patients with high ESR or RF values. RF was associated with CD86, a T-cell costimulatory marker. Notably, elevated synovial levels of CD86 have been detected in a proportion of RA patients.[42] Gene expression for a number of pro-inflammatory molecules were elevated in RA patients with high ESR values, including *TNFSF13* (APRIL), *CD209*, *CXCL16* and *FCGR2A*. TNFSF13 regulates B and T-cell survival and activation and can also negatively modulate IFN-γ, implying that TNFSF13 may exert multiple effects in RA.[43] CXCL16 may function to recruit mononuclear cells into affected joints, and elevated levels of CXCL16 have been detected in SF from RA patients.[44] CD209 is a high affinity receptor for ICAM2/3 and may promote cellular adhesion of FLS cells. FCGR2A is a low-affinity Fc receptor (CD32) operating to fix complement, previously implicated in the pathogenesis of RA.[45] While these specific genes were not identified in previous studies,[18,19] they are involved in activation of the adaptive immune response, suggesting that patients with high ESR and RF levels may define one of the previously described FLS cell subpopulations.

Increases in ESR and CRP levels are non-specific markers of disease activity and, therefore, not independent variables. Accordingly, a combined analysis of the effects of both of these clinical parameters on FLS cell gene expression was performed. While the combined analysis identified several pro-inflammatory genes that were not identified in the individual analysis, nine genes were identified in the combined analysis that were also identified in the individual analyses of ESR and CRP.

Disease modifying anti-rheumatic drugs (DMARDS) and corticosteroids reduce joint inflammation and cartilage and bone destruction.[46,47] Not surprisingly, both MTX and prednisone therapy altered gene expression in RA ST FLS cells. Notably, MTX treatment was associated with increased gene expression for the pro-apoptotic factor, *CASP1*, and reduced gene expression for the adhesion molecule, *ICAM1*. Pharmacological inhibition of ICAM-1 results in clinical improvement in some RA patients.[48] Prednisone therapy effectively limits inflammatory cell and FLS cell infiltration into arthritic joints.[49] Here, we observed that FLS cells from RA patients receiving prednisone exhibited altered gene expression levels for factors involved in cellular recruitment and inflammation, such as *THBS1* and *CD200*. Enhanced *CD200* expression suppresses osteoclast development[50] and we infer that prednisone therapy promotes bone development over erosion. Our data suggest that DMARDS, either directly or indirectly, alter FLS cell gene expression, reflecting both reduced inflammation and joint destruction. While a comprehensive analysis of all drug regimens in the RA patient cohort was beyond the scope of this study, our data highlight the importance of considering FLS cell gene expression in the context of drug therapy. A better understanding of the genes affected by specific therapeutics will provide insights, at the cellular level, of their effects on joint pathology and immune function. Viewed altogether, the data presented herein provide evidence, at the gene expression level, of the heterogeneity of disease pathology associated with affected joints in RA, prescribed by disease activity and influenced by medications. Notably, these data derive from a single cell population that influences disease pathogenesis. We anticipate that similar analyses of other contributing cell types – T cells, macrophages, B cells – would likewise provide evidence of complex, heterogeneic pathogenesis, the more so when stage of disease is also considered. These investigations are the subject of our ongoing studies.

Materials and methods

Patients

Sample collection involved confirmation of the diagnosis of RA/OA using clinical, serologic and radiological data and informed consent on all study participants. RA patients were diagnosed according to the American College of Rheumatology 1987 revised criteria.[43] The study included a total of 17 RA patients (18 RA samples, one patient was sampled from two knee joints), 20 OA patients and six control (non-RA, non-OA, surgery) patients. The average age in years of patients in this study was RA = 61 (36–79), OA = 66 (25–75), with a median disease duration of 19.5 years for RA (range 4–37) and 5.5 years for OA (range 1–12). Clinical parameters were recorded at the time of sample collection. RA/OA ST samples were collected from patients with erosive, end-stage disease at the time of joint replacement surgery. Control, non-OA/RA ST specimens were obtained from individuals at the time of orthopedic surgery following trauma. All ST specimens were immediately transferred to research personnel for processing.

Sample processing and microarray analysis

FLS cells from ST from affected RA joints, OA joints and tissue from trauma patients (non-RA, non-OA, surgery) were isolated from ST by enzymatic digestion with collagenase I, II, IV (3 mg/g tissue) and DNase (2 mg/g tissue) for 0.5–2 h at 37°C depending on the sample size. Single cell suspensions were collected after straining the digest through a 70 μm filter. FLS cells were washed two times and allowed to adhere, and cultured in Dulbecco's modified Eagle's medium (DMEM). Cells were passaged by trypsin digestion and split at a ratio of 1:3. Confirmation of >90% purity of FLS cell populations involved staining for prolyl 4 hydroxylase (5B5 antibody, Abcam, Cambridge, MA, USA) and fluorescence-activated cell sorting analysis.

Cells were washed and plated in DMEM containing 10% fetal calf serum and antibiotics and maintained in culture for 2, 3 or 8 passages, as indicated. RNA was isolated from the FLS cells using a Qiagen kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. RNA purity was confirmed by spectrophotometric absorbance readings at 260/280 nm. The preparation of cDNA from RNA derived from FLS cells, sample hybridization, and scanning of the U133A or

U133Plus 2.0 oligonucleotide microarray GeneChip Arrays (Affymetrix, Santa Clara, CA, USA) were performed at the Centre for Applied Genomics Microarray Facility (Hospital for Sick Children, Toronto, Ontario, Canada), in accordance with the procedures established by Affymetrix.

RNA extraction, cDNA synthesis and real-time PCR
RNA for real time RT-PCR was harvested from ST FLS cells from an additional eight RA patients, eight OA patients and three trauma patients (non-OA, non-RA, surgical), using the Qiagen RNeasy mini kit according to the manufacturer's protocol. cDNA was synthesized using 100 ng RNA in the presence of random primers and MMLV Reverse Transcriptase (Invitrogen, Burlington, ON, Canada). Reaction components were obtained from the LightCycler FastStart DNA Master SYBR Green^{PLUS} I Kit (Roche Canada, Mississauga, ON, Canada), and the LightCycler instrument (Roche) and corresponding software were used for all reactions. The PCR was performed in a final volume of 20 μl, 0.5 μM of each primer and 5 μl template cDNA (concentration 100 ng/μl). Primer sets were obtained for human *HOXD10, HOXD11, HOXD13, LHX2, PPARG, POU3F3* and *TRIM2* from SuperArray and run according to the manufacturers protocol. Primer sets for HPRT were as follows, forward primer 5′-ATCAGACTGAAGAGCT AT TGTAATGACCA-3′ and the reverse primer 5′-TGGC TTATATCCAACACTTCGTG-3′. Standard curves were established for each primer set and both reference and target reactions were performed for each sample.

Data analysis
Data were analyzed using ArrayAssist (Stratagene, La Jolla, CA, USA) software. GC-RMA analysis was performed which adjusts for background signal, normalizes and log transforms the data. Volcano plots were then used to identify differentially expressed genes between the RA and OA specimens or within different RA specimen groupings (clinical parameters or drug treatments) using a fold change of 2 and $P < 0.05$ with a variance stabilization unpaired *t*-test. For the U133A chip, gene expression data from the eight individual RA patient joint ST FLS cell samples at 2/3× passage were compared to gene expression data from the 8 individual RA patient ST FLS cell samples at 8× passage. For the U133Plus 2.0 chip, RNA from six control FLS cell samples from trauma patients (non-RA and non-OA) was used to define a baseline, and the fold change in expression from baseline for each gene relative to the expression level in the 18 RA ST FLS cell and 20 OA ST FLS cell samples was determined. All analyses were performed on those genes that were identified as differentially regulated >2-fold above baseline.

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