Epigenome Analysis Reveals TBX5 as a Novel Transcription Factor Involved in the Activation of Rheumatoid Arthritis Synovial Fibroblasts

Emmanuel Karouzakis, Michelle Trenkmann, Renate E. Gay, Beat A. Michel, Steffen Gay and Michel Neidhart

*J Immunol* published online 15 October 2014
http://www.jimmunol.org/content/early/2014/10/10/jimmunol.1400066
Epigenome Analysis Reveals TBX5 as a Novel Transcription Factor Involved in the Activation of Rheumatoid Arthritis Synovial Fibroblasts

Emmanuel Karouzakis, Michelle Trenkmann, Renate E. Gay, Beat A. Michel, Steffen Gay, and Michel Neidhart

In this study, we analyzed the methylation status of human promoters in rheumatoid arthritis synovial fibroblasts (RASF). Differentially methylated genes between RASF and osteoarthritis synovial fibroblasts (OASF) were identified by methylated DNA immunoprecipitation and hybridization to human promoter tiling arrays. The methylation status was confirmed by pyrosequencing. Gene and protein expression of differentially methylated genes was evaluated with real-time PCR, Western blot, and immunohistochemistry. Chromatin immunoprecipitation was used to measure the gene promoter–associated acetylation and methylation of histones. Transcription factor–specific targets were identified with microarray and luciferase assays. We found that the transcription factor T-box transcription factor 5 (TBX5) was less methylated in rheumatoid arthritis (RA) synovium and RASF than in osteoarthritis (OA) samples. Demethylation of the TBX5 promoter in RASF and RA synovium was accompanied by higher TBX5 expression than in OASF and OA synovium. In RA synovium, TBX5 expression was primarily localized to the synovial lining. In addition, the TBX5 locus was enriched in activating chromatin marks, such as histone 4 lysine 4 trimethylation and histone acetylation, in RASF. In our functional studies, we observed that 790 genes were differentially expressed by 2–6-fold after overexpression of TBX5 in OASF. Bioinformatic analysis of these genes revealed that the chemokines IL-8, CXCL12, and CCL20 were common targets of TBX5 in OASF. Taken together, our data show that TBX5 is a novel inducer of important chemokines in RASF. Thus, we conclude that RASF contribute to the inflammatory processes operating in the pathogenesis of RA via epigenetic control of TBX5.

Rheumatoid arthritis synovial fibroblasts (RASF) were shown to be important effector cells of joint destruction in rheumatoid arthritis (RA) (1, 2). No genetic mutation is known that could explain the hyperactive phenotype of synovial fibroblasts (SF). Therefore, we investigated the role of epigenetic gene regulation in the activated phenotype of RASF.

Epigenetics are related to changes in gene expression that occur without a change in the DNA sequence. The epigenome primarily consists of two main components: DNA methylation and histone modifications. DNA methylation is found in the sequence context of CpG dinucleotides. DNA methylation is an enzymatic reaction catalyzed by DNA methyltransferases (DNMT): DNMT1 is responsible for copying the methylation marks during cell replication, whereas DNMT3a and DNMT3b are de novo DNMT (3). The functional role of DNA methylation is generally to repress gene transcription.

Epigenetic alterations have been associated with a number of human diseases (4). Hypermethylation of the promoters of tumor suppressor genes was demonstrated in different tumors. For example, the promoters of p16 or the DNA repair genes MLH1 and BRCA1 are silenced by promoter hypermethylation in cancer cells (5). In autoimmunity, we and other investigators showed that RASF and T cells of systemic lupus erythematosus are globally hypomethylated (6). In the search for specific target genes, promoters, we found that CXCL12 and LINE-1 are differentially methylated in RASF versus osteoarthritis SF (OASF) (7–9). In systemic lupus erythematosus, the promoters of CD70 and perforin were reported to be hypomethylated in T cells (10). Recently, two independent groups published their DNA methylation signature studies analyzing RASF using the Illumina Human Methylation 450 chip (11, 12). IL6R, CAPN8, DPP4, and several HOX genes were identified as methylated target genes (12). In addition, hypomethylation was suggested to be connected with alterations in cellular behavior, such as cell migration, cell adhesion, and extracellular matrix interactions (11). Although these studies revealed novel differentially methylated, and, thus, deregulated, genes in RASF, their function was not evaluated on a single-gene level.

In the current study, we performed a stringent analysis using methylated DNA immunoprecipitation combined with a promoter tiling array (MeDIP-chip) to compare RASF with OASF and identified a novel differentially methylated gene known as T-box transcription factor 5 (TBX5). Furthermore, we performed microarray expression experiments to identify potential TBX5 target genes and elucidate their potential function in RASF.
Materials and Methods

Patients, primary cells, cell lines, and tissue preparation

Synovial tissue specimens were obtained, after written consent had been given, from RA and osteoarthritis (OA) patients undergoing joint replacement surgery at the Schulthess Clinic Zurich. They were homogenized and digested in 150 mg/ml Dispase II (Roche, Mannheim, Germany) at 37°C for 60 min. All RA patients fulfilled the American College of Rheumatology criteria for the classification of RA. Primary SF and the HEK293 cell line were cultured in DMEM supplemented with 10% FCS, 50 IU/ml penicillin-streptomycin, 2 mM l-glutamine, 10 mM HEPES, and 0.2% fungicide (all from Life Technologies Invitrogen, Basel, Switzerland). All experiments were performed using SF cultures from 4th to 6th passage.

MeDIP and promoter array analysis

Genomic DNA (gDNA) was obtained by phenol/chloroform extraction. Before immunoprecipitation, the gDNA was sheared to a size of 300–100 bp using the Bioruptor sonicator (Diagenode) for 10 cycles of 15 s on/15 s off. A total of 4 μg sonicated DNA was diluted in 450 μl Tris-EDTA buffer and denatured by boiling for 10 min at 100°C. Ten percent of sonicated gDNA was retained as input control. Methylated DNA was immunoprecipitated with 10 μl a mouse mAb against 5-methylcytosine (1 μg/ml) for 2 h at 4°C with overhead shaking. Next, polyclonal sheep anti-mouse magnetic beads were incubated with the DNA for 2 h at 4°C with overhead shaking, followed by three washes for 10 min each. Precipitated methylated DNA was eluted from the beads with 250 μl elution buffer, containing 7.5 μl proteinase K (10 μg/ml), for 3 h at 50°C with shaking (800 rpm). Finally, precipitated DNA was purified using the QIAquick PCR Purification Kit. For the promoter array experiment, seven MeDIP reactions were performed per sample and pooled; together with input samples they were hybridized to NimbleGen 720p promoter arrays. In addition, individual genes were analyzed by separate MeDIP assays and analyzed by SYBR Green quantitative real-time PCR (qPCR) (Table I). The results of the qPCR were calculated as the percentage of immunoprecipitated DNA to input DNA using the cycle threshold (Ct) values: % (MeDIP/input enrichment) = 2^(Ct(Input) – 3.32 – Ct(IP)) * 100%. In the equation, 2 is the primer amplification efficiency (E = 2), and 3.32 is the compensation factor [log2(10) = 3.32] needed to correct the Ct of diluted input.

Bisulfite pyrosequencing

gDNA was prepared from SF using the QIAamp DNA Blood Mini Kit (Qiagen, Hombrechtikon, Switzerland). The DNA (1 μg) was bisulfite modified using the Epitect Bisulfite Kit (Qiagen). Two rounds of hemi-nested PCR amplification of bisulfite-modified DNA (2 μl) were performed using AmpliTaq Gold polymerase (Applied Biosystems, Rotkreuz, Switzerland). The PCR program was 95°C for 4 min; 5 cycles of 95°C for 30 s, 52°C for 90 s, and 72°C for 2 min; 25 cycles of 95°C for 30 s, 52°C for 90 s, and 72°C for 90 s; and 72°C for 4 min. Primers were designed using the computer software MethPrimer (13) (Table I). Purified PCR fragments were cloned using the Qiagen PCR Cloning Kit, according to the manufacturer’s instructions. Positive clones were sequenced (Microsynth, Balgach, Switzerland), and data were analyzed using the BiSMa bioinformatics tool (14). For pyrosequencing analysis, the PyroMark CpG assay Hs_TBX5_02_PM (Qiagen) was used for PCR amplification of the TBX5 gene promoter region (Chr 12:114486488-114487117). The PCR products were sequenced using the PyroMark Q96 instrument (Qiagen).

ChIP assay

The ChIP assay was done as described previously (15). The primers used for qPCR are shown in Table I.

Western blot

Whole-cell lysates were prepared using RIPA buffer (50 mM Tris HCl [pH 8], 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS). Proteins were separated on a 10% SDS polyacrylamide gel and transferred to Protran nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). Membranes were blocked for 1 h at room temperature in 5% nonfat dry milk with 0.05% Tween-20 in TBS and were incubated overnight with polyclonal rabbit anti-human TBX5 (1:250; Prestige Antibodies; Sigma-Aldrich). Subsequently, membranes were incubated with HRP-conjugated secondary Abs (Jackson ImmunoResearch, Suffolk, U.K.). Bound Abs were visualized using ECL (Amersham Bioscience, Oelfingen, Switzerland). Equal protein loading was confirmed using mouse anti-human α-tubulin Abs (Sigma-Aldrich, Basel, Switzerland).

Real-time qPCR

Total RNA was isolated using the RNeasy Miniprep kit (Qiagen), including DNase I treatment. RNA was reverse transcribed using random hexamers and Multiscribe reverse transcriptase (both from Applied Biosystems, Rotkreuz, Switzerland). Samples without addition of reverse transcriptase served as a negative control. Relative quantification of mRNA levels by TaqMan or SYBR Green qPCR was done using eukaryotic 18S rRNA as an endogenous control (Applied Biosystems). The differences in the comparative Ct of sample and 18S cDNA were calculated (ΔCt). Relative expression levels were calculated using the formula ΔΔCt = ΔCt (sample stimulated) – ΔCt (sample unstimulated). Relative expression was calculated using the Equation 2

\[ \frac{2^{\Delta\Delta Ct}}{2} \]

Immunohistochemistry

Paraffin-embedded synovial and SCID mouse model tissue slides (16) were deparaffinized and treated at 80°C for 30 min with citrate buffer (pH 6). Endogenous peroxidase was blocked by treating the slides for 10 min with 3% H₂O₂ in H₂O. Tissue slides were washed with PBS/0.05% Tween-20 and blocked with 2% rabbit serum in PBS for 30 min. To analyze the expression of TBX5 in RA and OA, tissue slides were incubated overnight at 4°C with a 1:50 dilution of polyclonal rabbit anti-TBX5 Abs (Sigma-Aldrich); rabbit IgG1 was used as a negative isotype control. For detection of TBX5, biotinylated anti-rabbit Abs were incubated with the slides for 30 min, followed by an additional 30-min incubation with the VECTASTAIN ABC reagent for peroxidase (Vector Laboratories) and visualization with 3,3′-diaminobenzidine. Four photographs/patient tissue sample were acquired using an inverted microscope (Carl Zeiss).

Plasmid construction

The coding sequence of the TBX5 transcript variant 1 (NM_000192) was cloned into the pcDNA3.1(*) vector (Invitrogen) and used for over-expression experiments (Table I). For reporter gene assays, the genomic region (~1553 to +127) of the IL8 gene promoter was cloned into the

### Table I. Primer sequences used in the study

<table>
<thead>
<tr>
<th>Gene-Method</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBX5-MeDIP-qPCR</td>
<td>FW: 5′-ATATCCATGCTTATGCAAAG-3′&lt;br&gt;REV: 5′-GCGAGAAATGAGTTGTTGCAA-3′</td>
</tr>
<tr>
<td>TBX5-BS</td>
<td>FW: 5′-GTTTATTGTTTATTGTTTATT-3′&lt;br&gt;REV: 5′-ACTATTTATACCAAAATATCCATAC-3′</td>
</tr>
<tr>
<td>TBX5-SYBR</td>
<td>FW: 5′-GAGAACCACAGATCCACGCAA-3′&lt;br&gt;REV: 5′-CATGTACATCAGCAGCCAA-3′</td>
</tr>
<tr>
<td>CXCCL20-SYBR</td>
<td>FW: 5′-GCGAAATCAGAAGACCCGACA-3′&lt;br&gt;REV: 5′-ATTGCAGACCCTGCTGCTGAA-3′</td>
</tr>
<tr>
<td>CXCCL2-SYBR</td>
<td>FW: 5′-CCAGCCATCCGGCTGACTG-3′&lt;br&gt;REV: 5′-AGGGGCGCTCCTGCTGCG-3′</td>
</tr>
<tr>
<td>TBX5-cDNA cloning</td>
<td>FW: 5′-TGGAATCAGATGGCTAGAAGAGGAAAGGAAAGC-3′&lt;br&gt;REV: 5′-TCAGATGGCTAGAAGAGGAAAGGAAAGC-3′</td>
</tr>
<tr>
<td>IL-8 promoter cloning</td>
<td>FW: 5′-TGACAGCTGGCTGGAGACATTTTATTGCTTCACTGCTC-3′&lt;br&gt;REV: 5′-AGCTCGGCTCAAGAATTTTATTGCTTCACTGCTC-3′</td>
</tr>
</tbody>
</table>
pGL3 basic vector, and the GAPDH promoter (−1087 to −24) was cloned into pRL (Promega).

**Reporter gene assay**

HEK293 cells were transfected with 200 ng pGL3_IL8 plus 100 ng pRL_GAPDH using Lipofectamine 2000 (Invitrogen). Different concentrations (100, 250, 500, and 1000 ng) of the pcDNA3.1_TBX5 expression vector were co-transfected with the above plasmids. As a negative control, the empty vector was transfected for each condition separately.

Firefly luciferase activity was measured with the Dual Luciferase Reporter Assay System (Promega), and the results were normalized to the activity of Renilla luciferase.

**Microarray analysis**

OASF (n = 2) cultures were transfected with 2 μg pcDNA3.1(+)_TBX5 or pcDNA3.1(+) empty vector as a negative control using the Basic Nucleofector Kit for primary human fibroblasts (Amaxa/Lonza). After 48 h, RNA was extracted using the RNeasy Miniprep Kit (Qiagen). The RNA concentration was measured by NanoDrop, and RNA quality was checked using the 2100 Bioanalyzer. cDNA was generated by reverse transcription. The labeled cDNA was hybridized to the Human Genome U133 plus 2.0 gene expression array (Affymetrix) using Fluidics Station 450. Data were analyzed using the software standard microarray bioinformatics package of R. The DAVID bioinformatics program and the Kyoto Encyclopedia of Genes and Genomes (KEGG) were used to functionally annotate the genes identified from the microarray (17, 18). The microarray data have been deposited in the Gene Expression Omnibus database under accession number GSE60162 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE60162).

**Statistical analysis**

GraphPad Prism 5.0 software was used for statistical analysis, and the data were analyzed by the Mann–Whitney and Wilcoxon statistical tests. Data are presented as mean ± SE. The p values < 0.05 were considered significant.

**Results**

**Identification of novel differentially methylated genes by MeDIP promoter array analysis**

Initially, we used the MeDIP assay, in combination with the human promoter tiling arrays, to identify genes that are regulated by DNA methylation in RASF and OASF. The bioinformatics analysis identified 45 differentially methylated gene promoters in RASF. Trends in the data led us to investigate a few candidate genes confirmed by MeDIP-qPCR (Fig. 1A). We chose the differentially methylated gene TBX5, with a high enrichment score and known function, from the array. The MeDIP-chip enrichment region of TBX5 was located in a CpG island and overlaps with the histone 3 lysine 4 trimethylation (H3K4me3)-enriched area of nine cell lines of the ENCODE database (Fig. 1B). Because the arrays were done in only a few samples, we expanded our analysis to a larger cohort of patient samples. Therefore, we performed separate MeDIP-qPCR assays using six different RASF and OASF cell cultures. TBX5 was significantly hypomethylated in RASF (OASF: 17 ± 2.9%, RASF: 5 ± 3.5% MeDIP/input enrichment, n = 6, p < 0.05, Fig. 1C).

The TBX5 promoter in RASF is hypomethylated and marked with activating chromatin modifications

Because MeDIP analysis provides information about regional methylation changes, we corroborated our previous results for the TBX5

![Graph showing identification of differentially methylated genes in OASF and RASF.](http://www.jimmunol.org/Downloadedfrom/Graph.png)
promoter using bisulfite sequencing and pyrosequencing at the single-nucleotide level. Bisulfite sequencing of two representative RASF and OASF cultures showed reduced levels of methylation in RASF (Fig. 2A). Additionally, pyrosequencing quantification confirmed a significantly demethylated TBX5 promoter in RASF (OASF: 45.9 ± 6.9%, RASF: 4.4 ± 0.47% CpG methylation, n = 8, Fig. 2B).

Histone modifications are additional important epigenetic marks. Previous studies showed that H3K4me3 and histone 3 acetylation (H3Ac) are associated with actively transcribed genes and accompany hypomethylated genes (19). In addition, histone 3 lysine 27 trimethylation (H3K27me3) is often found in the methylated gene promoters. Therefore, we used ChIP to analyze the TBX5 promoter for histone modifications. We found that the TBX5 promoter is significantly more enriched in RASF than in OASF with regard to H3K4me3 and H3Ac. In contrast, H3K27me3 was significantly more enriched in OASF than in RASF (Fig. 2C).

RASF express TBX5 at the mRNA and protein levels

DNA hypomethylation and activating histone modifications are often associated with gene activation and correlate with mRNA expression. Therefore, we measured the expression of TBX5 in RASF versus OASF. We found that the expression of TBX5 mRNA is significantly higher in RASF than OASF (OASF: 0.96 ± 0.3-fold change, RASF: 2.2 ± 0.1-fold change, n = 10, p < 0.05, Fig. 3A). The increase in mRNA correlated with the increase in levels of TBX5 protein expression (in arbitrary units [AU]) in RASF as shown by Western blot (protein expression [in AU]) OASF: 0.20 ± 0.02, RASF: 0.47 ± 0.10, n = 4, Fig. 3B). Next, we evaluated the role of proinflammatory cytokines in the expression of TBX5. RASF were stimulated with TNF-α, IL-1β, and LPS for 24 h, and the expression of TBX5 was measured by qPCR. IL-1β and LPS significantly upregulated TBX5 mRNA in RASF but not in OASF (IL-1β; OASF 0.7 ± 0.08-fold change, RASF 1.8 ± 0.3-fold change, n = 5, p < 0.05; LPS: OASF 1 ± 0.1-fold change, RASF 1.9 ± 0.4-fold change, n = 7, p < 0.05, Fig. 3C). In contrast, TNF-α did not significantly change TBX5 mRNA between these cells (OASF 0.8 ± 0.16-fold change, RASF 0.7 ± 0.35-fold change, n = 5, p = 0.6, Fig. 3C).

Proinflammatory chemokines are TBX5 gene targets

Finally, we overexpressed TBX5 in OASF and performed microarray expression analysis. By this method, we identified 790 differentially expressed genes. Our analysis was focused on 211 annotated genes from the genome database. The overexpression of TBX5 in OASF upregulated the expression of 92 genes and downregulated the expression of 120 genes by a range of 2–6-fold (Fig. 5A, 5B). KEGG pathway analysis revealed that chemokine genes were upregulated by TBX5 (Fig. 5B). Trends in the data from the microarray and KEGG pathway analysis led us to investigate a few candidate genes confirmed by qPCR in five independent experiments. In particular, IL-8, CXCL2, and CCL20 were upregulated by a range of 2–3-fold (Fig. 5C). To confirm the above results, we performed reporter gene assays using a vector in which we had cloned the IL-8 promoter upstream of the luciferase gene. Cotransfection with different concentrations of the TBX5 vector induced IL-8 promoter activity in three independent experiments in HEK293 cells (Fig. 5D).

**FIGURE 2.** Validation of DNA methylation and histone modification analysis of the TBX5 promoter in OASF and RASF. Bisulfite sequencing and pyrosequencing analysis showed that the 5′ CpG island of TBX5 is hypomethylated in RASF. (A) Columns represent CpG sites, and rows represent clones of two independent OASF and RASF samples. Black squares are methylated CpG, and gray squares are unmethylated CpG. (B) OASF and RASF samples (n = 8) were analyzed using pyrosequencing, and the mean percentage of CpG methylation of the TBX5 locus is shown. (C) The TBX5 promoter was analyzed in ChIP samples from OASF (n = 4) and RASF (n = 4) for H3Ac, H3K27me3, H3K4me3, and IgG isotype control normalized to histone 3. RASF were highly enriched for H3Ac and H3K4me3, whereas OASF were enriched for H3K27me3. Results are shown as a ratio to histone 3 enrichment. *p < 0.05.

**TBX5 is expressed and its promoter is hypomethylated in RA synovium**

To show the pathophysiological role for TBX5 in RA, we stained synovial tissue slides with TBX5 Abs and analyzed the TBX5 gene methylation status in synovial biopsies by bisulfite pyrosequencing. Immunohistochemistry analysis revealed high expression of TBX5 in the RA sublining (Fig. 4A). In addition, we stained sections from RASF that invaded the cartilage from a SCID mouse model experiment and identified TBX5 at the sites of invasion (Fig. 4B). Total RNA extraction from synovial tissue from the same patients and qPCR analysis confirmed higher expression of TBX5 in RA synovium (OA: 0.9 ± 0.1-fold change, n = 8, RA: 3 ± 0.6-fold change, n = 12, Fig. 4C). In addition, we found that TBX5 was hypomethylated in genomic DNA extracted from the biopsies by bisulfite sequencing (Fig. 4D) and pyrosequencing (OA: 45 ± 3.5% CpG methylation, RA: 7 ± 0.7% CpG methylation, n = 5, Fig. 4E).
Discussion

The current study was designed to analyze the functional role of epigenetic modifications in SF of RA patients. We identified the transcription factor TBX5 as a novel differentially methylated gene in those cells. In addition, we found that TBX5 has an important function in the induction of proinflammatory chemokines in SF.

We and other investigators studied targets of DNA methylation at the single-gene level. These studies showed that DNA methylation can play an important role in various pathways, such as apoptosis and IL-6, CXCL12, and Ephrin-B1 expression in RASF (20–22). These results prompted us to perform a genome-wide DNA methylation analysis. We used the MeDIP-chip and compared SF from RA and OA patients. Our primary goal was to identify regional genomic alterations; for this reason, we chose the MeDIP-chip method. Bioinformatics analysis revealed several differentially methylated CpG islands. Although the statistical significance of the arrays is not highly accurate because we used only a few samples, we proved, with independent MeDIP and pyrosequencing assays, that the promoter of transcription factor TBX5 was hypomethylated in RASF. Its promoter also was associated with active histone marks. Therefore, we could conclude that epigenetic modifications are primarily responsible for the transcriptional regulation of this gene.

Promoter hypomethylation of TBX5 correlated highly with the increase in mRNA expression in RASF. The densitometry results of Western blots are expressed as arbitrary units (AU) and the protein expression was shown as a ratio of TBX5 to α-tubulin (AU). (C) IL-1β and LPS, but not TNF-α, induced the expression of TBX5 in RASF. *p < 0.05.

TBX5 belongs to the T-box transcription factor family, all of which possess a T-box DNA-binding domain in their sequence. It was shown to play an important role in tissue development and cancer. Genetic mutations in the TBX5 sequence have been associated with the Holt–Oram syndrome, which involves dysfunctions in the heart and limbs (23). Recently, it was characterized as a novel tumor suppressor and evaluated for its potential use as a biomarker for colon cancer (24). Interestingly, inactivation of the TBX5 gene occurred via DNA hypermethylation in colon cancer cell lines (24). In contrast, TBX5 is active and hypomethylated in patients with RA. Hypomethylation of gene promoters is often important in autoimmune diseases (6). Our previous studies revealed that hypomethylation is responsible for the activated phenotype of RASF (7). By treating normal SF with the DNA hypomethylator drug 5-azacytidine, we identified genes implicated in RASF, including IL receptors, growth factors, extracellular matrix proteins, matrix-degrading enzymes, adhesion molecules, transcription factors, and in the activation of Wnt signalling and Rho-GTPases (7). In addition, we found that promoter hypomethylation of CXCL12 is responsible for its upregulation in RASF (8). The above observations were confirmed in recent studies that investigated differentially methylated genes directly in RASF using methylation arrays (11). These studies identified that hypomethylated genes were connected with alterations in cellular behavior, such as cell migration, cell adhesion, and extracellular matrix interactions.

We also were interested in identifying a possible role for TBX5 overexpression in RA. To that end, we cloned the TBX5 cDNA into an expression vector and transiently transfected OASF. Using microarray analysis, we identified 790 differentially expressed genes, and bioinformatics pathway analysis revealed that the chemokine pathway was especially affected by TBX5 overexpression. More specifically, the proinflammatory chemokines IL-8, CCL20, and CXCL2 were upregulated. We reported previously that different TLR ligands and proinflammatory cytokines can induce IL-8 in RASF (25). IL-8 originally was identified as a neutrophil chemoattractant (26). CXCL2 is another neutrophil chemoattractant that is involved in inflammatory processes. In particular, the expression levels of CXCL2 were reported to be increased in the Ag-induced arthritis...
The CCL20 ligand and its receptor CCR6 are expressed in RA synovial lining cells. RASF stimulated with IL-1β significantly upregulate CCL20 mRNA expression. In addition, RASF can be induced to secrete COX-2 and matrix metalloproteinase 3 by stimulation with rCCL20 in vitro. Interestingly, all of the above chemokines are induced by IL-1β.

FIGURE 4. DNA methylation and protein and mRNA expression of TBX5 in OA and RA synovial tissues. (A) Immunohistochemical staining of TBX5 showed a stronger expression in RA synovium compared with OA synovium. Positive TBX5 staining is represented by the brown nuclear color in comparison with the IgG control (original magnification ×40). Semi-quantitative analysis of immunohistochemistry slides revealed significantly stronger expression of TBX5 in RA synovial lining than in OA synovial lining. (B) TBX5⁺-stained RASF were found at the site of invasion in the SCID mouse invasion model (original magnification ×40). (C) mRNA expression of TBX5 was significantly higher in RA synovium than in OA synovium, as shown by qPCR. The CpG island of TBX5 is hypermethylated in OA whole synovial tissues, as shown by bisulfite sequencing (D) and pyrosequencing (E). *p < 0.05.

FIGURE 5. Identification of TBX5 target genes in SF. (A) Overexpression of TBX5 was achieved using a pcDNA3.1-TBX5 expression vector. Western blot confirmed the TBX5 protein expression in OASF. (B) Heat map shows the top 211 upregulated genes, generated from the microarray expression data, as a log2 ratio of controls versus TBX5-transfected OASF patients (n = 2). GO terms analysis with DAVID bioinformatics tool of the upregulated genes revealed a trend toward the immune system and chemotaxis pathways. (C) IL-8, CXCL2, and CCL20 were confirmed to be upregulated after overexpression of TBX5 in five OASF cell cultures. (D) Dose-dependent increase in IL-8 luciferase using different concentrations of TBX5 expression vector in HEK293 cells. The baseline of luciferase empty vector was set up as 1 (dashed horizontal line). Three independent experiments were analyzed, and SE is shown as bars. *p < 0.05.
TLR ligands. In our experiments, we found that IL-1β and LPS stimulate the expression of TBX5, but not TNF-α, in RASF. Although TNF-α, IL-1β, and LPS have many similarities, there are known variations during cell signaling. TNFR1 contains a death domain that recruits different signaling molecules than the RIP domain of IL-1R and TLR4 (30). IL-1R/TLR4 interact with the same adaptor protein MyD88 and distinct TRAF ubiquitin ligases (30). It can be a cross-talk of NF-kB with TBX5 downstream of the IL-1β/R/TLR4–signaling pathway. MAPK, which are the downstream activators of these signaling pathways, can directly or indirectly phosphorylate the expression of TBX5.

We did not observe these upregulations in OASF as a result of TBX5 promoter methylation. Therefore, TBX5 is a transcription factor that regulates proinflammatory chemokines in response to specific stimuli in RASF.

In conclusion, we identified TBX5, a relevant RASF transcription factor, by epigenetic analysis. Both DNA methylation and histone modification marks were shown to be deregulated in this promoter between RASF and OASF. The hypomethylation of TBX5 in RASF causes the upregulation of TBX5 expression. OASF do not express high levels of TBX5 because of methylation of their promoter. Overexpression of TBX5 in OASF could induce the expression of proinflammatory chemokines. In turn, the chemokines may induce the infiltration of cells in the synovium and contribute to the altered tissue morphology and inflammatory process observed in RA synovium. Overall, TBX5 may be an important transcription factor for tissue activation.

Acknowledgments
We thank Andrea Patrignani, Dr. Hubert Rehrauer, and Dr. Michal Oconiewski of the Functional Genomics Center Zurich (Zurich, Switzerland) for technical and bioinformatics support of this work. We also thank Dr. Aria Minder from the Genetic Diversity Center (Zurich, Switzerland) for help with pyrosequencing and Maria Comazzi from the Center of Experimental Rheumatology (Zurich, Switzerland) for immunohistochemistry.

Disclosures
The authors have no financial conflicts of interest.

References