Genes and Immunity (2011) 12, 643–652

www.nature.com/gene

ORIGINAL ARTICLE

# DNA methylation regulates the expression of CXCL12 in rheumatoid arthritis synovial fibroblasts

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*In the search for specific genes regulated by DNA methylation in rheumatoid arthritis (RA), we investigated the expression of CXCL12 in synovial fibroblasts (SFs) and the methylation status of its promoter and determined its contribution to the expression of matrix metalloproteinases (MMPs). DNA was isolated from SFs and methylation was analyzed by bisulfite sequencing and McrBC assay. CXCL12 protein was quantified by enzyme-linked immunosorbent assay before and after treatment with 5-azacytidine. RASFs were transfected with CXCR7-siRNA and stimulated with CXCL12. Expression of MMPs was analyzed by real-time PCR. Basal expression of CXCL12 was higher in RASFs than osteoarthritis (OA) SFs. 5-azacytidine demethylation increased the expression of CXCL12 and reduced the methylation of CpG nucleotides. A lower percentage of CpG methylation was found in the CXCL12 promoter of RASFs compared with OASFs. Overall, we observed a significant correlation in the mRNA expression and the CXCL12 promoter DNA methylation. Stimulation of RASFs with CXCL12 increased the expression of MMPs. CXCR7 but not CXCR4 was expressed and functional in SFs. We show here that RASFs produce more CXCL12 than OASFs due to promoter methylation changes and that stimulation with CXCL12 activates MMPs via CXCR7 in SFs. Thereby we describe an endogenously activated pathway in RASFs, which promotes joint destruction.*
Genes and Immunity (2011) **12,** 643–652; doi:10.1038/gene.2011.45; published online 14 July 2011

**Keywords:** *CXCL12; rheumatoid arthritis synovial fibroblasts; epigenetics; DNA methylation*

## Introduction

A variety of studies describe an activated phenotype of synovial fibroblasts (SFs) in rheumatoid arthritis (RA), which is characterized by a changed morphology, deranged apoptotic behavior and increased invasive properties.[1] Recently, we found that epigenetic changes might contribute to these phenotypic changes in RASFs. We could show that DNA of RASFs is globally hypomethylated when compared with osteoarthritis (OA) SFs or normal SFs (NSFs) and that less DNA methyltransferase DNMT1, the enzyme responsible for DNA *de novo* methylation, is present in RASFs.[2] DNA methylation is well known to regulate gene expression. Transcriptional regulation by DNA methylation occurs in CpG rich regions of gene promoters, so called CpG islands. Methyl CpG binding proteins bind to methylated CpG islands and together with chromatin remodeling enzymes cause gene silencing.[3] In the normal genome, DNA methylation regulates gene expression to form tissue specific expression patterns.[4] Aberrant DNA methylation was found in various pathologies including cancer and autoimmune diseases.[5,6]

CXCL12, also known as stromal-derived factor-1 (SDF-1) is a key factor in the trafficking of lympho- and hematopoietic progenitor cells and in the early development and regeneration of tissues.[7] Moreover, the secretion of CXCL12 is increased after tissue damage by hypoxia, toxins or irradiation, leading to the immigration of progenitor cells expressing the CXCL12 receptor CXCR4.[8] A second receptor for CXCL12, CXCR7 has only most recently been identified and has been implicated in tumor growth and metastasis.[9–11] Elevated levels of CXCL12 were found in patients with multiple sclerosis, inflammatory myopathies, spondyloarthropathies and RA.[12–17] Levels of CXCL12 in the synovial fluid of RA patients are around 10 times higher than in healthy joints and reach a mean of 750 ng ml$^{-1}$.[16] It is understood that CXCL12 drives chronic inflammation by attraction of monocytes and lymphocytes into the joint and by stimulation of SFs to produce pro-inflammatory cytokines.[17–20] Previous studies also showed that cultured RASFs produce more CXCL12 than NSFs or OASFs.[16,21]

Based on these results, we analyzed in the current work whether expression of CXCL12 in RASFs is modified by changes in DNA methylation. Furthermore, to elucidate CXCL12 signaling pathways we looked at the expression of CXCR7 in SFs and tested whether CXCR7

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Received 20 January 2011; revised 3 May 2011; accepted 1 June 2011; published online 14 July 2011

mediates the production of matrix metalloproteinases (MMPs) after CXCL12 stimulation in RASFs.

## Results

*RASFs upregulate CXCL12 mRNA and protein expression*

To analyze the basal expression of CXCL12 expression in RASFs and in OASFs, we measured CXCL12 mRNA and protein levels in cell culture supernatants after 24 h. RASFs express significantly more CXCL12 mRNA than OASFs and NSFs (RASFs $6.9 \pm 0.3$ dCt, $n = 11$; OASFs $8.3 \pm 0.5$ dCt, $n = 8$; NSFs 7.5 dCt, $n = 1$; $P < 0.028$) (Figure 1a). Furthermore, we observed that the mRNA expression of CXCL12 in these patients was positively correlated with the C-reactive protein (CRP), a marker of inflammation ($r = -0.64$, $P < 0.01$). RASFs produced with $85 \pm 12\,\text{pg ml}^{-1}$ also significantly more CXCL12 protein than OASFs with $52 \pm 10\,\text{pg ml}^{-1}$ (Figure 1b).

*DNA methylation regulates the promoter of CXCL12*

To investigate whether changes in DNA methylation might influence expression of CXCL12 in RASFs, we first tested whether the CXCL12 promoter can be regulated by DNA methylation. Computational analysis predicted that there are CpG islands upstream of the transcription initiation site of the CXCL12 promoter (Figure 2a). We further analyzed the region that is 100 bp upstream of exon 1 (−741 to −477 bp) and the region that spans exon 1 (−244 to +272 bp) by bisulfite sequencing. Our findings showed that NSFs have a high percentage of methylated CpG nucleotides in the promoter upstream region from −741 to −477 bp, whereas in the region −244 to +272, that spans the transcriptional initiation site and exon 1, all the CpG nucleotides were unmethylated (Figure 2b). To see whether demethylation of the heavily methylated CpG-rich region would influence CXCL12 expression, we treated OASFs with different doses of the demethylating drug 5-azacytidine (5-azaC) and found dose-dependent upregulation of CXCL12 protein secretion (Figure 2c). Furthermore, bisulfite sequencing analysis showed demethylation of the CXCL12 promoter (−741 to −477 bp) with 1 μM 5-azaC (Figure 2d). In addition, the basal mRNA expression of CXCL12 correlated with the methylation levels of the CXCL12 promoter ($r = 0.60$, $P < 0.04$) as shown by McrBC digestion assay (Figure 2e).Taken together, the results of Figure 2 indicate that expression of CXCL12 is regulated by methylation of its promoter, and accordingly can be increased by demethylation.

*Demethylation of CXCL12 promoter upregulates CXCL12 expression in RASFs*

To investigate whether the methylation status of the CXCL12 promoter is altered in fibroblasts of RA patients, we compared the percentage of CpG methylation between RASFs, NSFs, OASFs and normal lung fibroblasts. First, a group of four pooled RASFs patients and a group of three pooled OASFs patients were compared (Figure 3a). The analyzed promoter region of CXCL12 had significantly less CpGs methylated in the RASFs group than in the OASFs group (RASFs 21% ± 7.2 versus OASFs 42% ± 7.8) (Figure 3b). We then analyzed whether the results from the pooled groups would be reflected in individual patient's analysis (Figure 3c). The CXCL12 promoter had similar changes as seen in the pooled samples (RASFs 27% ± 4.8 versus OASFs 46% ± 5.6). The CXCL12 promoter in NSFs cell cultures was strongly methylated (NSFs1 54% and NSFs2 60%). As expected, the CXCL12 promoter in lung fibroblasts was similarly strongly methylated as in NSFs or OASFs. The above results suggest that normally the expression of CXCL12 is repressed by methylation in fibroblasts, but that an epigenetic defect in SFs of RA patients causes an intrinsically increased expression of CXCL12.

*Overexpression of CXCL12 induces functional changes in RASFs*

Stimulation of SFs, chondrocytes and osteoclasts with CXCL12 *in vitro* was previously found to induce the expression of matrix-degrading enzymes.[16,22,23] As DNA demethylation induces CXCL12 overexpression, we measured mRNA expression of the collagenases MMP-1 and MMP-13, the gelatinases MMP-2 and MMP-9, stromelysin (MMP-3), and the matrix-bound MMP-14 after stimulation with CXCL12 for 24 h in RASFs. In addition, we looked at the mRNA expression of the tissue inhibitors of MMPs (TIMPs) −1, −2 and −3. CXCL12 levels in joints of RA patients have been measured to be on average 375 and $750\,\text{ng ml}^{-1}$.[16,24] We stimulated RASFs *in vitro* with $100\,\text{ng ml}^{-1}$. Stimulation with CXCL12 selectively and significantly increased the expression of MMP-1 by 6 ± 3 fold, of MMP-3 by 2 ± 0.2 fold and of MMP-13 by 4 ± 1 fold (Figure 4a). In contrast, CXCL12 neither influenced the expression of MMP-2, MMP-9 or MMP-14, nor did it change the expression levels of TIMPs.

To see whether endogenous production of CXCL12 by RASFs in culture would be enough to stimulate the expression of MMPs, we increased the concentration of

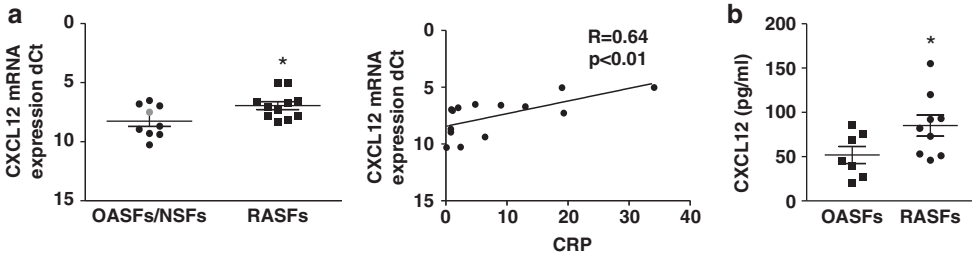


**Figure 1** RASFs express more CXCL12 mRNA and secrete more protein than OASFs. (**a**) RASFs ($n = 11$, ■) expressed significantly more CXCL12 mRNA than OASFs ($n = 8$, ●) and NSFs ($n = 1$, ●). The mRNA expression of CXCL12 correlated with the patient's C-reactive protein (CRP) levels. (**b**) Enzyme-linked immunosorbent assay was used to analyse the amount of CXCL12 release in RASFs ($n = 9$) and OASFs ($n = 7$) cell culture supernatants. RASFs released significantly more CXCL12 than OASFs. *$P < 0.05$.

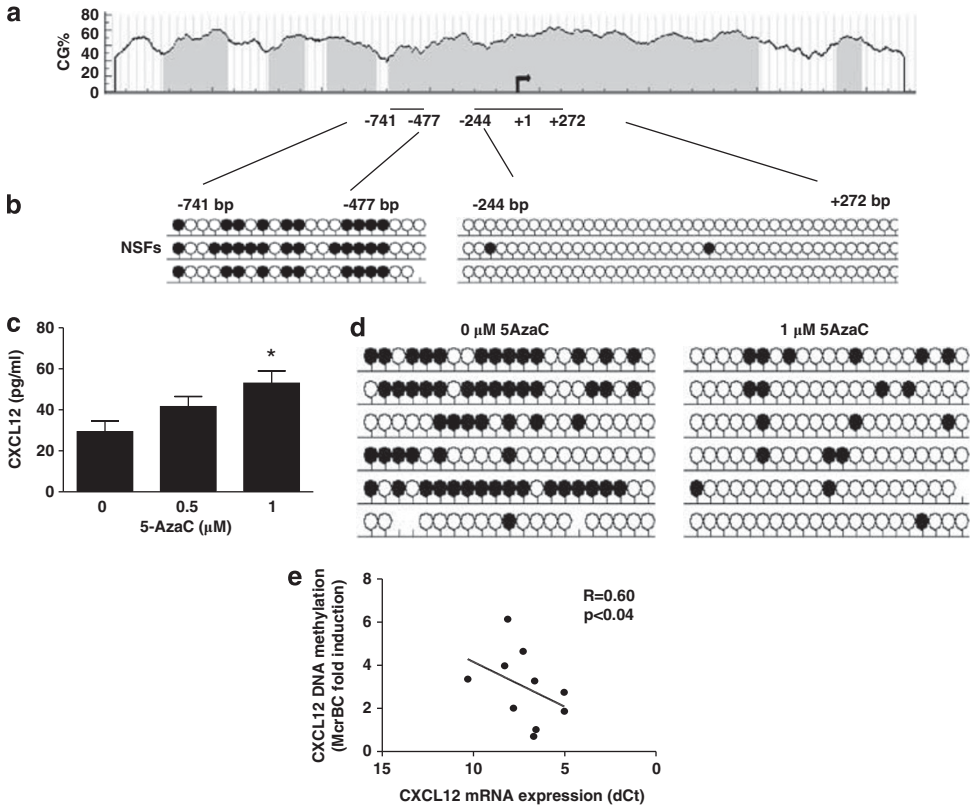


**Figure 2** Expression of CXCL12 is regulated by DNA methylation. (**a**) Computational analysis of the CXCL12 promoter region (blue-colored regions: CpG islands). The percentage of C and G nucleotides (CG %) are shown 2000 bp upstream of the transcription initiation site (arrow) and exon 1. Bisulfite-sequencing primers were designed to analyse region −741 to −477 bp and −244 to +272 bp of the CXCL12 promoter. (**b**) Methylated CpG dinucleotides were found in the region −741 to −477 bp of NSFs ($n=1$). Each circle represents a methylated (black) or unmethylated (white) CpG dinucleotide. Every row represents a different clone. (**c**) After treatment of OASFs with 0, 0.5 and 1 μM 5-azaC for 6 days, amounts of CXCL12 were determined by enzyme-linked immunosorbent assay ($n=4$). 5-azaC treatment induced dose-dependent release of CXCL12 (*$P<0.05$). (**d**) 5-azaC demethylates the analyzed promoter region as shown by bisulfite sequencing ($n=1$ OASFs, six clones were sequenced). (**e**) McrBC digestion and CXCL12 promoter (−741 to −477 bp) quantitative PCR of RASFs ($n=11$) genomic DNA. Negative correlation between CXCL12 mRNA expression and CXCL12 promoter DNA methylation was shown in RASFs. A full colour version of this figure is available at the *Genes and Immunity* journal online.

CXCL12 in the cell culture supernatants by increasing the ratio between seeded cells and medium and by prolonging incubation times and added an CXCL12-neutralizing antibody or IgG control (Figure 4b). Expression of MMP-1 mRNA was decreased 26% after 24 h, and 42% after 96 h incubation by CXCL12 blockade compared with IgG control.

We then addressed the question whether CXCL12 signals via CXCR4 or the newly found CXCL12 receptor CXCR7 in SFs. Transcription of the *CXCR4* gene produces four different mRNAs, which putatively encode four different isoforms of the protein. We analyzed the expression of these four transcripts in peripheral blood mononuclear cells, SFs, synovial tissues and HeLa cells. None of the tested SFs expressed any transcripts for CXCR4. In peripheral blood mononuclear cells all four transcripts were amplified, whereas HeLa cells expressed transcript 1, 2 and 4. In RA synovial tissues transcripts 1 and 2 were detectable, probably due to the infiltration of lymphocytes, as in OA synovial tissues none of the transcripts was found (Figure 4c). In contrast to CXCR4, CXCR7 protein was constitutively expressed in SFs (Figure 4d).

We then silenced the expression of CXCR7 with small interfering RNA (siRNA) (Figure 5a) and examined whether this would influence the induction of MMPs in RASFs after stimulation with CXCL12. After transfection of siRNA-targeting CXCR7, no increase in the mRNA expression of MMP-1, MMP-3 and MMP-13 was seen anymore after stimulation with CXCL12 (Figure 5b). From these experiments, we conclude that CXCR7 is functionally important in SFs and its activation by CXCL12 leads to expression of MMP-1, MMP-3 and MMP-13.

## Discussion

In the current paper we show that the increased expression of CXCL12 in RASFs is due to an epigenetic alteration and that high amounts of CXCL12, as found in the joints of RA patients lead to increased expression of MMPs and to joint destruction.

In cancer, both DNA hypomethylation and hypermethylation have been shown to occur.[25,26] The promoters of p16 or the DNA repair genes *MLH1* and *BRCA1* for instance have been shown to be silenced by increased DNA methylation in cancer cells.[27] Global hypomethylation was correlated with demethylation of repetitive sequences, for example, Sata, D4Z4, NBL2 and transposable elements, for example, LINE-1.[28,29] We and others have reported global hypomethylation of SFs in RA and

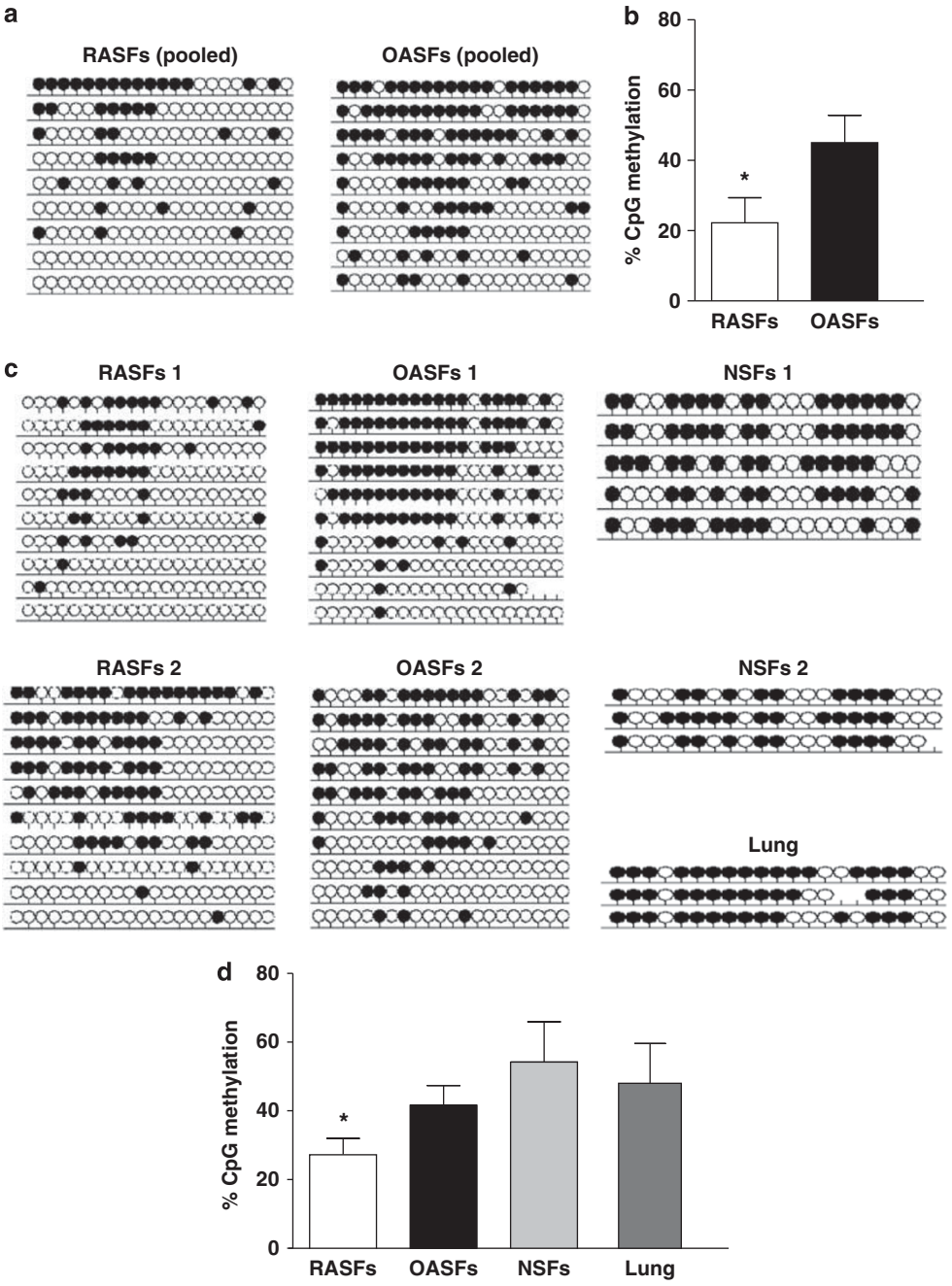


**Figure 3** RASFs have low levels of CXCL12 promoter DNA methylation. (**a**) Bisulfite sequencing of CXCL12 promoter region (−741 to −477) in RASFs and OASFs. Nine clones were sequenced from a pooled group of four RASFs and three OASFs patients. (**b**) Summary of the data as percentage of CpG methylation between pooled fibroblasts cell types shows that RASFs have significantly lower percentage of DNA methylation than OASFs. (**c**) Bisulfite sequencing of CXCL12 promoter region (−741 to −477) in SFs from two individual RA patients, two individual OA patients, two NSFs and one normal lung fibroblast culture. (**d**) Summary of the data as percentage of CpG methylation between individual fibroblasts cell types shows that RASFs have significantly lower percentage of DNA methylation than OASFs, NSFs and lung fibroblasts. The methylation percentage in **b** and **d** was calculated separately for each clone and the average of total number of clones was plotted in the histogram graph. $^*P < 0.005$.

CD4T cells in systemic lupus erythematosus.[2,30] We also showed that this global hypomethylation affects the promoter of LINE-1 transposable element in RASFs and that continuous treatment of NSFs with 5-azaC induced changes in gene expression that resemble the activated status of RASFs such as increased production of MMP-14, CD29 and cathepsins.[2] Also in systemic lupus erythematosus, hypomethylation of specific gene promoters have been shown to contribute to the pathogenesis of the disease.[30] Similar to the approach of our current study it was shown that normal T cells treated with 5-azaC upregulated *LFA1*, *CD70* and perforin and that the CpG islands in the promoters of these genes were hypomethylated in T cells of systemic lupus erythematosus patients.[31–33] DNA methylation of promoter sequences can change the binding of transcription factors.

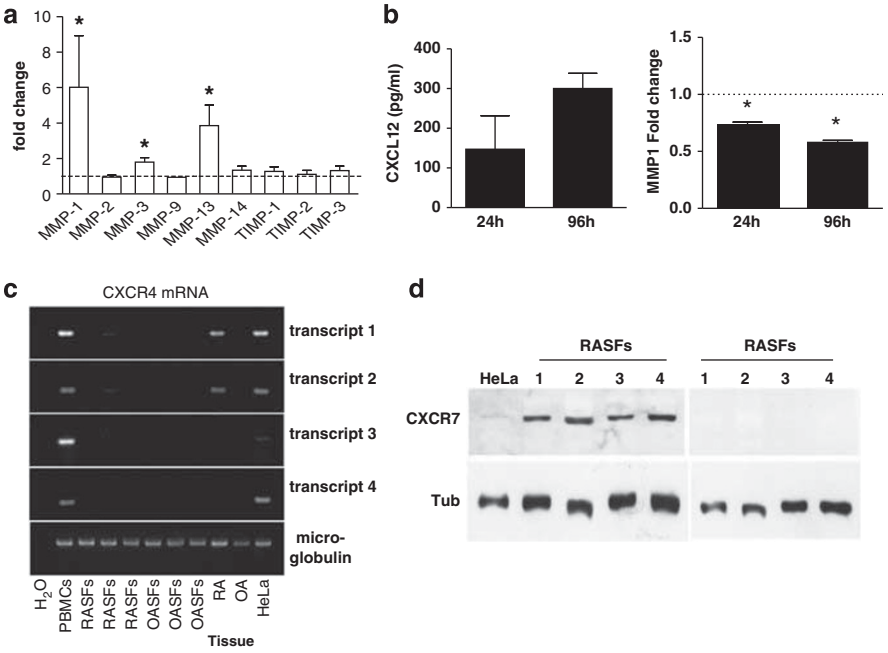


**Figure 4** Overexpression of CXCL12 induces MMPs expression. (**a**) Expression of MMP-1, MMP-3 and MMP-13 transcripts are significantly increased after stimulation of RASFs with CXCL12 ($n=6$). Data are presented as $\times$ fold change after stimulation relative to unstimulated cells. (**b**) Time-dependent release of CXCL12 protein in cell culture supernatants and inhibition of MMP1 expression using CXCL12 blocking antibodies ($n=3$) (**c**) Expression of the four known CXCR4 mRNA transcripts 1–4 by conventional reverse transcriptase PCR. (**d**) Western blot showing the expression of CXCR7 protein in HeLa cells and RASFs obtained from four different patients; patient's samples were loaded twice on a 10% gel and the membrane was cut. The left side was incubated with anti-CXCR7 antibodies, the right side with anti-CXCR7 antibodies pre-incubated with CXCR7 synthetic peptide; α-tubulin (tub) served as a loading control. *$P<0.05$.

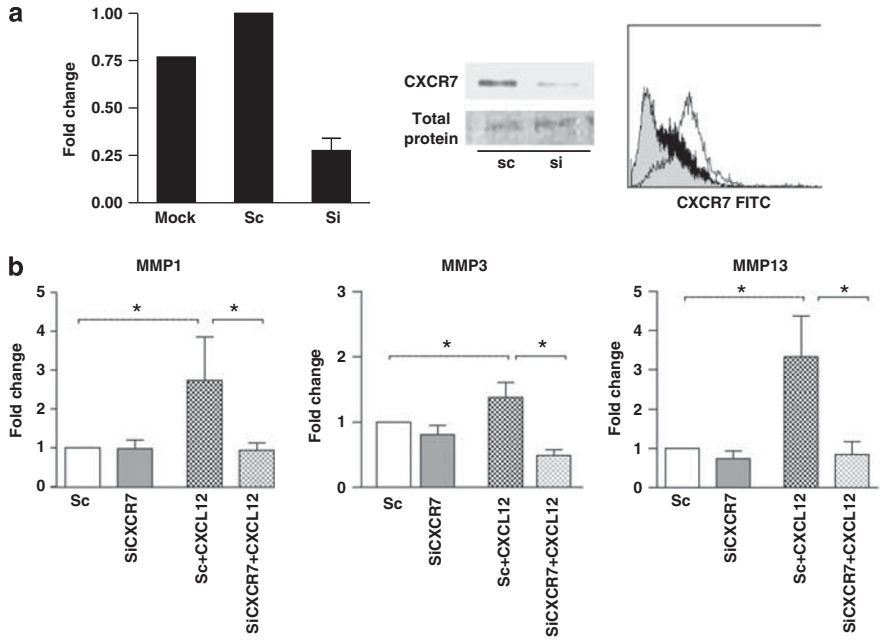


**Figure 5** CXCR7 is functional in SFs. (**a**) Expression of CXCR7 after silencing was measured on mRNA level (left panel) and on protein level (right panels). Western blot and fluorescence-activated cell sorting analysis confirmed silencing of CXCR7 after transfection with siRNA when compared with cells transfected with control siRNA. Gray filled line: rabbit IgG; black filled line: CXCR7 siRNA-transfected RASFs; black open line: scrambled control siRNA-transfected RASFs. (**b**) MMP-1, MMP-3 and MMP-13 levels in control transfected RASFs (sc) and CXCR7-silenced RASFs (siCXCR7) with and without CXCL12 stimulation ($n=7$). *$P<0.05$

Transcriptional regulation studies of the CXCL12 promoter revealed the presence of E-box consensus sequences, which are binding sites for multiple transcription factors such as bHLH, NFAT, SP1 and HIF1α.[34–36] The low-methylation levels observed in the CXCL12 promoter in RASFs could change the binding of these transcription factors

and thereby increase gene expression. In healthy fibroblasts, the CXCL12 promoter was strongly methylated in our study. In cancers, however, it has been shown that DNA hypermethylation of the CXCL12 promoter in colon carcinomas, mammary carcinomas and the MCF7 breast cancer cell line promotes tumor metastasis.[37–39]

In the current study basal levels of CXCL12 were higher in RASFs compared with OASFs. Even though these changes were not dramatic, we believe that they are of clinical significance as CXCL12 expression positively correlated with levels of C-reactive protein. CXCL12 is expressed at high levels in RA, and it has been implicated in a number of pathogenic events such as recruitment and persistence of inflammatory cells in the synovium, as well as production of cytokines and matrix-degrading enzymes.[16,17,20,23,40] The role of CXCL12 in cartilage destruction was further supported by *in vivo* experiments, which showed that CXCL12 antagonists inhibit fibroblast-induced cell infiltration in immunodeficient mice.[41]

CXCL12 can signal via CXCR4 and CXCR7. Expression of CXCR4 has been detected in synovium of healthy individuals as well as in OA and RA patients.[20,42] Controversial data are published regarding the expression of CXCR4 in SFs. Although Garcia-Vicuna *et al* detected CXCR4 in SFs, Kanbe *et al* did not.[16,23] We also could not detect mRNA for CXCR4 in SFs of RA or OA patients. As pancreatic cancer cells do not express CXCR4 due to promoter hypermethylation, CXCR4 silencing might also occur by DNA methylation in SFs.[43] CXCR7, previously named RDC1 was first classified as an orphan receptor as no ligand could be found.[44] Recent publications have then related this receptor to cell recruitment, migration and proliferation after binding of CXCL12 in various cell types.[9,45,46] In the present study, we show that functional CXCR7 is also expressed in SFs and that it has a role in the induction of MMP production via CXCL12. Based on our data we hypothesize that although CXCR4 might be responsible for CXCL12-mediated cell trafficking into RA joints, CXCR7 mediates CXCL12 signaling in resident SFs. The exact mechanism of signaling after binding of CXCL12 to CXCR7 is not clear yet.[9,45] Recent studies found an association between CXCR7 and the adaptor protein β-arrestin2.[47–49] Interactions of arrestins with different receptors have been shown to facilitate activation of the MAPK cascade, a signaling pathway, which is known to regulate also MMPs.[50]

High production of MMPs by RASFs has been identified as a major mechanism of joint destruction in RA.[51] In particular, MMP-1 and MMP-13 were reported to have a crucial role in the invasive properties of RASFs as shown in the severe combined immunodeficiency mouse co-implantation model.[52,53] Expression of MMPs is regulated by multiple pathways. DNA hypomethylation modulates expression of different MMPs in cancer cells. In OA chondrocytes the adipocytokine leptin has not only been found to be upregulated by promoter DNA hypomethylation but also to stimulate expression of MMP-13.[54] Also, a variety of cytokines and chemokines stimulate the expression of MMPs.[55,56] The chemokine CXCL12 was reported to induce the expression of MMP-3 in chondrocytes and of MMP-9 in osteoclasts.[16,22] Furthermore, increased gelatinase and collagenase activity was described after CXCL12 stimulation in SFs.[23] Our present data suggest that the increased collagenase activity after CXCL12 stimulation stems from increased transcription of MMP-1 and MMP-13. On the other hand, CXCL12 stimulation neither changed the quantity of MMP-2 nor of MMP-9 transcripts, pointing to indirect regulation of gelatinase activity by CXCL12. As MMP-3 was described to contribute to the activation of pro-MMP-9 and we found that MMP-3 is more abundant in CXCL12-stimulated cells, it is probable that CXCL12 increases gelatinase activity via upregulation of MMP-3.[57,58] Based on the differential induction of specific MMPs after stimulation with CXCL12 it can be concluded that CXCL12 does not induce MMPs via the activator protein-1 (AP-1), since all of the measured MMPs contain an activator protein-1 site in their promoter regions. Differences among the MMP promoters have been described and include not only binding sites for different transcription factors but also variability in the number and arrangement of binding sites, which strongly increases the complexity of MMP regulation.[59]

In summary, our data show that basal expression of the chemokine CXCL12 is increased in RASFs due to a defect in gene regulation by DNA methylation. In RA joints, accumulated CXCL12 produced by RASFs might lead to increased expression of MMPs, which mediate joint destruction. In addition to the previously reported global hypomethylation, we identified CpG island-specific hypomethylation of CXCL12, which is involved in the intrinsic activation of RASFs and thereby in the perpetuation of RA.

## Materials and methods

*Patients and tissue preparation*

Synovial tissues were obtained from trauma patients (NSFs, $n=2$), RA ($n=14$) and OA ($n=11$) patients undergoing joint replacement surgery at the Schulthess Clinic Zurich after written consent (patient's characteristics are shown in Table 1). All RA patients fulfilled the American College of Rheumatology criteria for the classification of RA.[60] Normal lung fibroblasts were isolated from open lung biopsies of patients suspected with cancer but with a negative diagnosis. SFs and normal lung fibroblasts were isolated and cultured as described previously.[61] SFs were used in passages 4 and 5 for experiments.

*Bisulfite sequencing*

Genomic DNA was prepared from SFs using the QiAmp DNA blood Mini kit (Qiagen, Hombrechtikon, Switzerland). The DNA (1 μg) was bisulfite modified using the EpiTect bisulphite kit (Qiagen). Two rounds hemi-nested PCR amplification of bisulfite-modified DNA (2 μl) was performed using the AmpliTaq Gold polymerase (Applied Biosystems, Rotkreuz, Switzerland). The PCR cell cycle program was 95 °C 4 min; 95 °C 30 s, 52 °C 90 s, 72 °C 2 min × 5; 95 °C 30 s, 52 °C 90 s, 72 °C 90 s × 25; 72 °C 4 min. Primers were designed for two regions of the CXCL12 CpG island promoter. The forward 5′-GTTTGTGATTAG TTTATTTTATTA-3′, reverse 5′-CTAAATAAAAACCAAT AAAAAAC-3′ and hemi-reverse 5′-AAAAAATCCTAC TTTCTATAC-3′ bisulfite sequencing primers amplified

**Table 1** Characteristics of the study patients

| *Diagnosis* | *Patient number* | *Age (years)* | *Gender* | *Disease duration (years)* | *NSAIDs* | *DMARDs* | *RF pos (>20 I.U.)* |
|---|---|---|---|---|---|---|---|
| RA | 1 | 55 | F | 18 | + | Steroids | + |
| | 2 | 64 | F | 9 | – | MTX hydroxychloroquine steroids | + |
| | 3 | 70 | M | 4 | + | Steroids | + |
| | 4 | 65 | F | 15 | – | MTX | + |
| | 5 | 52 | F | 19 | – | Actemra, MTX | + |
| | 6 | 69 | F | 28 | – | Humira | + |
| | 7 | 45 | F | 17 | – | Salazopyrin, Arava | + |
| | 8 | 65 | M | 15 | – | MTX | + |
| | 9 | 52 | F | 21 | – | Arava | NA |
| | 10 | 66 | F | 46 | – | MTX | + |
| | 11 | 67 | F | 37 | – | Mabthera | + |
| | 12 | 66 | F | 16 | – | Arava, Prednison | + |
| | 13 | 73 | F | 7 | – | Prednison | + |
| | 14 | 79 | F | 30 | – | Prednison | NA |
| OA | 1 | 95 | F | NA | – | – | NA |
| | 2 | 70 | F | NA | + | – | NA |
| | 3 | 53 | F | NA | + | – | NA |
| | 4 | 61 | F | NA | – | – | NA |
| | 5 | 71 | M | NA | – | – | NA |
| | 6 | 73 | F | NA | – | – | NA |
| | 7 | 70 | M | NA | – | – | NA |
| | 8 | 82 | F | NA | – | – | NA |
| | 9 | 62 | M | NA | – | – | NA |
| | 10 | 79 | M | NA | – | – | NA |
| | 11 | 72 | F | NA | – | – | NA |

Abbreviations: DMARDs, disease-modifying antirheumatic drugs; F, female; M, male; MTX, methotrexate; NA, not assessed; NSAIDs, non-steroidal anti-inflammatory drugs; OA, osteoarthritis; RA, rheumatoid arthritis; RF pos, rheumatoid factor positivity.

the region −741 to −477 bp and the forward 5′-GTTTTT TATTGGTTTTTATTTAGTTTT-3′, reverse 5′-TACCTCCA CCCCCACTATAT-3′ and hemi reverse 5′-GAGTTTGAG AAGGTTAAAGGT-3′ bisulfite sequencing primers amplified the region −244 to +272 bp. The computer software MethPrimer predicted CpG islands with the following criteria: island size >100, GC content >50% and Obs/exp >0.6 and designed bisulfite primers for DNA methylation analysis.[62] The PCR purified fragments were cloned using the Qiagen PCR cloning handbook according to manufacturer's instructions (Qiagen). Positive clones were sequenced (Microsynth, Balgach, Switzerland). The data were analyzed using the BiQ analyzer software.[63]

*McrBC digestion and quantitative real-time PCR*

The CXCL12 promoter DNA methylation was determined by methylation-sensitive McrBC-PCR assay. Genomic DNA (1 μg) was sonicated using the Diagenode bioruptor (Diagenode, Liege, Belgium) (10 min, 15 s on and 15 s off). The sonicated DNA was digested with 10 units of McrBC or mock undigested in a 50 μl reaction mixture containing 1 × NEB2 buffer, 10 mM GTP and 0.1 mg ml$^{-1}$ bovine serum albumin. The restriction digestion reaction was incubated at 37 °C overnight and then the reaction was deactivated with heating at 65 °C for 20 min. The McrBC-treated DNA and mock samples were purified using Qiagen PCR purification protocol kit. Quantitative SYBR green PCR using the CXCL12 promoter (−741 to −477) primers: forward 5′-CACCATT GAGAGGTCGGAAG-3′, reverse 5′-AATGAGACCCG TCTTTGCAG-3′, was carried out with the McrBC digested and mock samples. McrBC cleaves methylated DNA strands and inhibits PCR amplification. In contrast, unmethylated DNA prevents McrBC cleavage and can be measured by quantitative PCR. Mock-undigested DNA is considered as total amount of DNA used in the reaction. Methylated DNA will have decreased Ct values after McrBC digestion. The real-time PCR results were normalized with the mock-treated sample DNA (deltaCt) and presented as fold induction of PCR recovery after digestion with McrBC.

*Stimulations*

RASFs and OASFs were seeded at low density 24 h before treatment. RASFs were stimulated with 100 ng ml$^{-1}$ recombinant CXCL12 (Peprotech, London, UK). OASFs were treated with 0.5 μM or 1 μM 5-azacytidine (Sigma, Buch, Switzerland) for 6 days. The cell culture medium was changed every 24 h and replaced with new 5-azaC.

*Transfection of CXCR7 siRNA*

siRNA for CXCR7 and double-stranded siRNA without homology to mammalian genes (negative control) (both from Qiagen) were used for silencing experiments. Also, transfection without siRNA (mock) was performed to determine whether the experimental setup causes unspecific effects. The transfections were done by electroporation (Nucleofector, Lonza, Cologne, Germany) using transfection reagents for primary mammalian fibroblasts (Basic Nucleofector Kit; Lonza) at a concentration of 0.9 μg siRNA/$5 \times 10^5$ cells. Transfected RASFs were cultured for 48 h before efficiency of knockdown was measured by real-time PCR, fluorescence-activated cell sorting and western blot, or before stimulations were begun.

*RNA isolation and quantitative real-time PCR*
Total RNA was isolated using the RNeasy Miniprep kit (Qiagen) including DNase treatment. RNA was reverse transcribed using random hexamers and multiscribe reverse transcriptase (both Applied Biosystems). Samples without addition of reverse transcriptase served as negative control (non-RT). Relative quantification of mRNA levels by TaqMan/SYBRGreen real-time PCR was done using eukaryotic 18S ribosomal RNA as endogenous control (Applied Biosystems). The differences of the comparative threshold cycles (Ct) of sample and 18S complementary DNA were calculated (dCt). Relative expression levels were calculated following the formula ddCt = dCt (sample stimulated) − dCt (sample unstimulated). Relative expression was calculated using the expression $2^{-ddCt}$. The sequence of the primers used for measuring MMP-1, MMP-2, MMP-3, MMP-9, MMP-13, MMP-14, TIMP-1, TIMP-2, TIMP-3 were previously described.[64]

*PCR*
mRNA was reverse transcribed using oligo dT and moloney murine leukemia virus reverse transcriptase (both from Applied Biosystems). PCR was performed on a C1000 Thermal Cycler (Bio-Rad laboratories, Hercules, CA, USA) with the following primer pairs and protocols designed to detect the four different CXCR4 mRNAs as published on the National Center for Biotechnology Information AceView database. CXCR4 transcript 1: forward primer 5′-GGAAAAGATGGGGAGGAGAG-3′, reverse primer 5′-CACTTCCAATTCAGCAAGCA-3′; CXCR4 transcript 2: forward primer 5′-CAGCAGGTAGC AAAGTGACG-3′, reverse primer 5′-GTAGATGGTGGG CAGGAAGA-3′; CXCR4 transcript 3: forward primer 5′-AAGGGTCACCGAAAGGAGTT-3′, reverse primer 5′-GAAGAGACCGGTGGTCTGAG-3′; CXCR4 transcript 4: forward primer 5′-GTTAAGCGCCTGGTGACTGT-3′, reverse primer 5′-GGTAACCCATGACCAGGATG-3′. 94 °C 5 min, 40 cycles with 94 °C 30 s, 58 °C 30 s, 72 °C 30 s and a final elongation of 5 min with 72 °C. β-microglobulin: forward primer 5′-AAGATTCAGGTTTACTC ACGTC-3′, reverse primer 5′-TGATGCTGCTTACATG TCTCG-3′. 94 °C 5 min, 35 cycles with 94 °C 30 s, 55 °C 30 s, 72 °C 30 s, final elongation 72 °C 5 min. Reaction products were separated on a 1.5% agarose gel and signals were visualized using ethidium bromide.

*Western blot analysis*
Whole cell lysates were prepared by lysing cells in 2× Laemmli buffer. Proteins were separated on a 10% SDS-polyacrylamide gel and transferred to Protran nitrocellulose membranes (Schleicher & Schüll, Dassel, Germany). Membranes were blocked for 1 h at room temperature in 5% non-fat dry milk with 0.05% Tween 20 in Tris-buffered saline and were incubated overnight with polyclonal rabbit anti-human CXCR7 (2.8 μg ml$^{-1}$; Abcam, Cambridge, UK). To ensure specificity of the CXCR7 antibodies, blots were incubated with CXCR7 antibodies, which were pre-incubated for 1 h at 37 °C with or without 10 μg ml$^{-1}$ of a synthetic CXCR7 peptide (10 μg ml$^{-1}$; Abcam). Afterwards, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Jackson Immunoresearch, Suffolk, UK). Bound antibodies were visualized using enhanced chemiluminescence (Amersham Bioscience, Otelfingen, Switzerland). Equal protein loading was confirmed using mouse anti-human α-tubulin antibodies or Ponceau solution (Sigma-Aldrich, Basel, Switzerland).

*Flow cytometry*
RASFs were detached from the culture flask with accutase (PAA Laboratories, Linz, Austria), washed with phosphate-buffered saline/1% fetal calf serum and incubated with 20 μg ml$^{-1}$ of rabbit anti-human CXCR7 (Abcam) or of rabbit IgG for 45 min at 4 °C. Cells were washed with phosphate-buffered saline/1% fetal calf serum and subsequently incubated with fluorescein isothiocyanate-labeled goat anti-rabbit IgG for 45 min at 4 °C. Washing steps were repeated twice before analysis of the cells in the flow cytometer (FACSCalibur). Data were processed using CellQuest software (BD Biosciences, San Diego, CA, USA).

*Enzyme-linked immunosorbent assay*
RASFs were seeded at $1 \times 10^5$ per ml Dulbecco's modified Eagle's medium and CXCL12 was measured after 24 h (Figure 1b) or at $2 \times 10^5$ per ml Dulbecco's modified Eagle's medium and CXCL12 was measured after 24 and 96 h (Figure 5c) in the supernatants with the DuoSet ELISA development system (R&D Systems, Abingdon, UK) according to the manufacturer's instructions.

*CXCL12 blockade*
RASFs ($2 \times 10^5$) were incubated with 1 μg ml$^{-1}$ neutralizing anti-human CXCL12 antibodies (R&D Systems) or IgG control. Fresh antibodies or control was added after 48 h. Cells were lysed after 24 h or 96 h.

*Statistical analysis*
All data are expressed as mean ± s.e.m. Statistical analysis was performed using GraphPad Prism software, version 4.03 (GraphPad System, San Diego, CA, USA). For analysis between different groups the Mann–Whitney *U*-test was used and for paired analysis Wilcoxon signed-rank test was used. *P*-values $<0.05$ were considered significant.

## Conflict of interest

The authors declare no conflict of interest.

## Acknowledgements

We thank Mrs Maria Comazzi and Mr Ferenc Pataky for their excellent technical work. We also thank our collaborators Professor C Buckley and Dr A Filer for providing us with the human lung fibroblast cultures. This work was supported by the Articulum Fellowship Program (YR), the Institute of Arthritis Research, Epilanges, Switzerland, and by the European Community's FP6 (Autocure) and FP7 funding (Masterswitch). This publication reflects only the author's views. The European Community is not liable for any use that may be made of the information herein.

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