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Altered Expression of MicroRNA in Synovial Fibroblasts and Synovial Tissue in Rheumatoid Arthritis

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***Objective.* MicroRNAs (miRNA) have recently emerged as a new class of modulators of gene expression. In this study we investigated the expression, regulation, and function of miR-155 and miR-146a in rheumatoid arthritis (RA) synovial fibroblasts (RASFs) and RA synovial tissue.**

***Methods.* Locked nucleic acid microarray was used to screen for differentially expressed miRNA in RASFs treated with tumor necrosis factor α (TNFα). TaqMan-based real-time polymerase chain reaction was applied to measure the levels of miR-155 and miR-146a. Enforced overexpression of miR-155 was used to investigate the function of miR-155 in RASFs.**

***Results.* Microarray analysis of miRNA expressed in RASFs treated with TNFα revealed a prominent up-regulation of miR-155. Constitutive expression of both miR-155 and miR-146a was higher in RASFs than in those from patients with osteoarthritis (OA), and expression of miR-155 could be further induced by TNFα, interleukin-1β, lipopolysaccharide, poly(I-C), and bacterial lipoprotein. The expression of miR-155 in RA synovial tissue was higher than in OA synovial tissue. Enforced expression of miR-155 in RASFs was found to repress the levels of matrix metalloproteinase 3 (MMP-3) and reduce the induction of MMPs 3 and 1 by Toll-like receptor ligands and cytokines. Moreover, compared with monocytes from RA peripheral blood, RA synovial fluid monocytes displayed higher levels of miR-155.**

***Conclusion.* This study provides the first description of increased expression of miRNA miR-155 and miR-146a in RA. Based on these findings, we postulate that the inflammatory milieu may alter miRNA expression profiles in resident cells of the rheumatoid joints. Considering the repressive effect of miR-155 on the expression of MMPs 3 and 1 in RASFs, we hypothesize that miR-155 may be involved in modulation of the destructive properties of RASFs.**

MicroRNAs (miRNA) are a recently discovered class of small, evolutionarily conserved noncoding RNAs that function as posttranscriptional repressors of gene expression. These molecules are generated from long primary transcripts via sequential processing by Drosha enzyme in the nucleus and by Dicer enzyme in the cytoplasm, producing ~21 nucleotide–long mature, functionally active miRNA (1). The miRNA are highly conserved among mammals (2). Gene mapping and in silico studies have estimated that miRNA account for 3% of the human genome, and are predicted to regulate ~30% of protein-coding genes (3).

The mode of action of miRNA involves binding to partially complementary sites in the 3′-untranslated regions of their target messenger RNA (mRNA). Following miRNA:mRNA duplex formation, posttranscriptional regulation is mediated by either target transcript degradation or inhibition of translation, or a combination of both (4). Patterns of miRNA expression are strictly regulated and highly specific to each cell type as

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well as to each developmental stage (5). A number of studies have demonstrated that some miRNA target large numbers of transcripts (6). In addition, several studies have shown that an individual mRNA can be targeted by numerous miRNA (7).

The miRNA control basic biologic functions, such as embryogenesis, organogenesis, proliferation, apoptosis, stress, and the antiviral response (8). Increasing evidence has linked miRNA regulatory activities with human diseases, most notably cancer. Abnormal patterns of miRNA have been found in human tumors, and aberrant expression of miRNA has been implicated in oncogenesis (9). In addition, miRNA have been found to control hematopoiesis (10), metabolism (11), and cardiac hypertrophy (12). It has been found that miRNA modulate T cell selection and T cell receptor sensitivity (13) as well as Treg cell development (14), which may suggest that miRNA are also involved in the development of autoimmunity. Recently, altered levels of miRNA have been linked to chronic inflammatory skin diseases (15). However, the potential role of miRNA in the pathogenesis of inflammatory and autoimmune arthritis has not yet been investigated.

Among the ~500 human miRNA identified thus far, miR-155 stands out as an miRNA that has been implicated in a large number of biologic activities. Initially, miR-155 was characterized as an oncogenic miRNA and was shown to be dysregulated in a large number of human neoplastic diseases (16). Subsequently, miR-155 was shown to be involved in the modulation of hematopoiesis, and very recently, its importance in the normal function of the immune system has been demonstrated (17–19).

Rheumatoid arthritis (RA) is a chronic inflammatory disorder resulting in irreversible joint damage. As demonstrated by experiments in the human cartilage–SCID mouse coimplantation model, synovial fibroblasts derived from patients with RA (RASFs) are critically involved in the processes of joint destruction (20). RASFs display increased expression of proinflammatory genes and matrix-destructive enzymes, as well as numerous alterations in cell signaling (21). However, the primary molecular events that initiate and direct the activation of RASFs remain elusive, thereby preventing the development of an early intervention that could inhibit the destructive properties of RASFs.

Based on the fundamental posttranscriptional regulatory activities that miRNA exert on gene expression and the evidence indicating their critical role in many biologic processes, we hypothesized that changes in the expression and function of certain miRNA in RA patients may play an important role in disease pathogenesis. Herein we demonstrate that RASFs have constitutively altered levels of 2 miRNA, miR-155 and miR-146a, and that proinflammatory stimuli abundant in the RA joints can regulate expression of these miRNA. Furthermore, we show the increased expression of miR-155 and miR-146a in the RA synovium. Finally, we provide evidence of a functional role of miR-155 as a modulator of matrix metalloproteinase 3 (MMP-3) expression and MMP-1 expression in RASFs.

PATIENTS AND METHODS

Preparation of synovial tissue and synovial fibroblasts. Synovial tissue specimens were obtained during synovectomy or joint replacement surgery from patients with RA and from patients with osteoarthritis (OA), after informed consent was obtained from all patients. The study was approved by the local ethics committee. All RA patients fulfilled the American College of Rheumatology (formerly, the American Rheumatism Association) criteria for the classification of RA (22). Synovial tissue used for RNA isolation was collected in RNAlater (Ambion-Applied Biosystems, Rotkreuz, Switzerland) immediately after surgery and stored at −20°C. To isolate synovial fibroblasts, synovial tissue specimens were minced and digested with Dispase at 37°C for 60 minutes. After washing, cells were grown in Dulbecco's minimum essential medium (Gibco-Invitrogen, Basel, Switzerland) supplemented with 10% fetal calf serum, 2 m*M* L-glutamine, 50 IU/ml penicillin/streptomycin, 0.2% Fungizone, and 10 m*M* HEPES (all reagents provided by Gibco-Invitrogen). Cultures of RASFs and OASFs were maintained at 37°C in a humidified atmosphere of 5% CO_2. All synovial fibroblasts between passages 4 and 6 were subjected to experimental procedures.

Preparation of monocytes from synovial fluid and peripheral blood. Synovial fluid samples were obtained from patients with RA during joint aspiration, and cells were collected by centrifugation. Peripheral blood mononuclear cells (PBMCs) were obtained from the blood of patients with RA and from healthy donors, and isolated by standard Ficoll density-gradient centrifugation. CD14+ cells were isolated from synovial fluid cells and from PBMCs using CD14 MACS MicroBeads according to the manufacturer's protocol (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of the CD14+ cell fraction, as assessed by flow cytometry, was consistently more than 90%.

Reagents and stimulation assays. Cultured synovial fibroblasts were grown in 12-well culture plates (6×10^4 cells/well) and stimulated for 8 hours or 24 hours with the following agents: 20 μg/ml poly(I-C) (InvivoGen, San Diego, CA), 100 ng/ml lipopolysaccharide (LPS) from *Escherichia coli* (List Biological Laboratories, Campbell, CA), 300 ng/ml bacterial lipoprotein (bLP; InvivoGen), 1 ng/ml recombinant interleukin-1β (IL-1β; R&D Systems, Abingdon, UK), and 10 ng/ml tumor necrosis factor α (TNFα; R&D Systems).

Microarray experiments. Total RNA was isolated from RASFs treated for 8 hours with TNFα (10 ng/ml) or from untreated cells, using the mirVana miRNA Isolation Kit (Ambion-Applied Biosystems). RNA quality was determined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA), and 5 μg of total RNA was further enriched in small RNAs ($\leq$200 nucleotides) using the mirVana miRNA Isolation Kit according to the manufacturer's protocol. The miRNA were labeled with the NCode miRNA Labeling System (Invitrogen, Basel, Switzerland). Briefly, a poly(A) tailing reaction was performed and capture sequences were ligated to the poly(A) products. Tagged miRNA derived from both samples were then purified, pooled, and hybridized to a MiRCURY locked nucleic acid (LNA) microarray (Exiqon, Vedbaek, Denmark) containing 454 LNA-modified oligonucleotide probes for human, mouse, and rat miRNA as annotated in the miRBase release 8.0 (23,24).

Hybridization conditions and subsequent washing steps were performed as recommended by Exiqon. After washing, Alexa Fluor 3 and Alexa Fluor 5 capture reagents were hybridized, and the microarrays were scanned with the GenePix Microarray Scanner (Molecular Devices, Sunnyvale, CA). GenePix Pro 5.0 software was used for image acquisition, normalization, and data analysis. Spots with signal-to-noise ratios of $\geq$3 in either the 532-nm or 635-nm channel were classified as present, and thus included in the subsequent analyses. The miRNA displaying $\geq$2-fold changes in signal intensity between TNFα-treated and untreated RASFs were considered to be differentially expressed.

RNA isolation and TaqMan-based real-time polymerase chain reaction (PCR) analysis of miRNA. Synovial tissue was homogenized with TissueLyser (Qiagen, Basel, Switzerland) prior to RNA extraction. Total RNA was isolated from synovial tissue, synovial fibroblasts, and monocyte lysates using the mirVana miRNA Isolation Kit. TaqMan miRNA assays (Applied Biosystems) were used for semiquantitative determination of the expression of miR-155 and miR-146a (25). First, 10 ng of total RNA was reverse-transcribed using miR-155– or miR-146a–specific stem-loop reverse transcription (RT) primers, MultiScribe reverse transcriptase, RT buffer, dNTPs, and RNase inhibitor (Applied Biosystems) in the GeneAmp 9700 PCR system (Applied Biosystems), according to the manufacturer's instructions. Real-time PCR was performed on the resulting complementary DNA (cDNA) using miR-155– or miR-146a–specific TaqMan primers and TaqMan Universal PCR Master Mix in a 7500 real-time PCR system (Applied Biosystems).

The expression of let-7a and the U6B small nuclear RNA (RNU6B) was used as endogenous control for data normalization. The comparative threshold cycle (C_t) method was used for relative quantification of the miRNA. Differences in the C_t values (ΔC_t) between the tested miRNA and the let-7a or RNU6B cDNA were calculated to determine the relative expression levels, using the following formula: $\Delta\Delta C_t = \Delta C_t$ of the RA sample $-$ ΔC_t of the OA sample. The fold change between tested tissue samples was calculated according to the formula $2^{-\Delta\Delta C_t}$. The same formulas were applied for analysis of relative expression of miR-155 and miR-146a in stimulated RASFs compared with control RASFs.

Transfection and functional assays for miR-155. RASFs (n = 3) were transfected in 12-well plates (5×10^4 cells/well) using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol, with 100 n*M* (final concentration) of synthetic miR-155 precursory molecule (pre–miR-155) or a scrambled control (pre-miR negative control #1; Ambion-Applied Biosystems). Twenty-four, 48, and 72 hours after transfection, expression levels of the MMPs and of IL-6 were measured. In separate experiments, RASFs (n = 3) were transfected with pre–miR-155 or scrambled control, which was carried out 24 hours prior to stimulation with LPS, IL-1β, or TNFα. Twenty-four hours after stimulation, expression levels of the MMPs and of IL-6 were analyzed and compared.

Semiquantitative TaqMan real-time PCR assays (Applied Biosystems) were used to analyze the relative expression of mRNA transcripts for MMPs 1, 3, 9, and 13. The C_t values for each MMP transcript were normalized to the C_t values for the corresponding 18S ribosomal RNA. Enzyme-linked immunosorbent assay (ELISA) was performed to detect MMP-3 protein expression, using the DuoSet ELISA Development System (R&D Systems, Minneapolis, MN), and to detect IL-6 expression, using the OptEIA kit (BD PharMingen, San Diego, CA) according to the manufacturer's instructions.

Statistical analysis. The Mann-Whitney U test and, where appropriate, the Wilcoxon rank sum test were used for statistical evaluation of the data. Analyses were performed using SPSS software (SPSS, Chicago, IL). *P* values less than 0.05 were considered significant.

RESULTS

Expression of miR-155 in RASFs and regulation by TNFα, IL-1β, and Toll-like receptor (TLR) ligands. To simulate the inflammatory milieu in a joint of a patient with RA, we treated RASFs in vitro with TNFα (10 ng/ml) for 8 hours, and then assessed miRNA expression using an miRNA LNA microarray. We observed prominent up-regulation of miR-155 after TNFα stimulation (results not shown). This finding, together with previously published reports of the effects of miR-155 on cell proliferation and on the function of the immune system, prompted us to study the expression and regulation of miR-155 in cultured synovial fibroblasts.

To confirm the stimulatory effect of TNFα on miR-155 expression and to further investigate the regulation of miR-155 by other proinflammatory mediators known to be critically involved in the development of inflammation and destruction of the rheumatoid joints, we stimulated RASFs with TNFα, IL-1β, and TLR ligands (26) and measured miR-155 levels by semiquantitative TaqMan miRNA assay. We found that, similar to the stimulatory effects of TNFα, IL-1β as well as the TLR ligands LPS, poly(I-C), and bLP markedly induced miR-155 expression in RASFs after 8 hours of stimula-

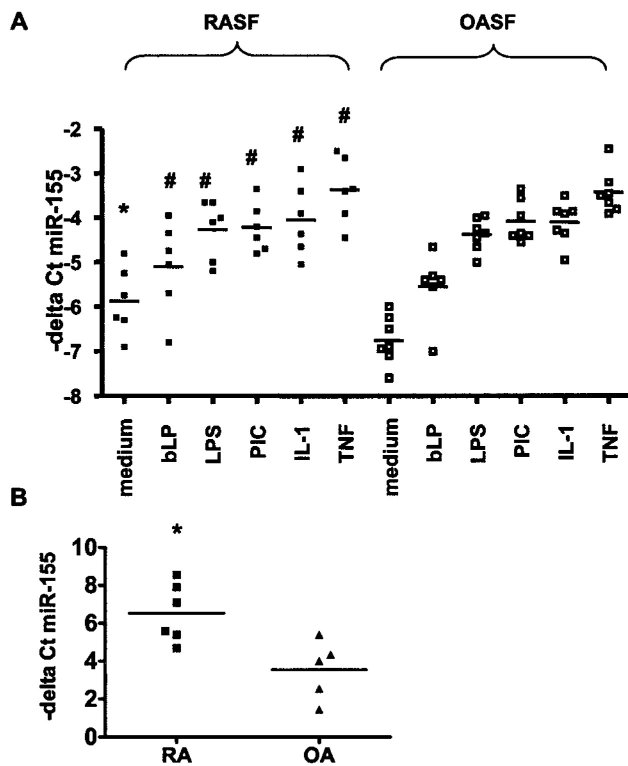

Figure 1. Overexpression of miR-155 in **A,** cultures of rheumatoid arthritis (RA) and osteoarthritis (OA) synovial fibroblasts (RASFs and OASFs, respectively) and **B,** RA and OA synovial tissue. **A,** RASFs (n = 6) and OASFs (n = 7) were treated with the indicated stimuli (proinflammatory cytokines and Toll-like receptor ligands) or left untreated (medium). Results are the difference in comparative threshold cycle (ΔC_t) between miR-155 and the internal control, let-7a. * = $P < 0.05$ versus OASF medium; # = $P < 0.03$ versus RASF medium. **B,** Expression of miR-155 in RA (n = 6) and OA (n = 5) synovial tissue was assessed as the ΔC_t between miR-155 and the internal control, U6B small nuclear RNA. * = $P < 0.02$ versus OA synovial tissue. Bars indicate the mean. bLP = bacterial lipoprotein; LPS = lipopolysaccharide; PIC = poly(I-C); IL-1 = interleukin-1β; TNF = tumor necrosis factor α.

tion (n = 6) (Figure 1A). The up-regulation of miR-155 upon stimulation of RASFs, as compared with that in unstimulated control cultures, was a mean ± SEM 5.7 ± 0.4-fold with TNFα stimulation, 3.5 ± 0.1-fold with IL-1β, 3.2 ± 0.2-fold with poly(I-C), 3.1 ± 0.4-fold with LPS, and 1.9 ± 0.3-fold with bLP ($P < 0.03$).

We next investigated the response of miR-155 to stimulation of OASFs. The absolute expression levels of miR-155 in OASFs (n = 7) upon stimulation with cytokines and TLR ligands were not significantly different from those in RASFs, indicating that both cell types up-regulate miR-155 to a similar extent. Interestingly, levels of miR-155 in unstimulated RASF cultures were twice as high as those in unstimulated OASF cultures ($P < 0.05$) (Figure 1A).

TNFα proved to be the most potent stimulus of miR-155 expression, both in RASFs and in OASFs. IL-1β, poly(I-C), and LPS up-regulated expression of miR-155 to a similar magnitude, whereas bLP showed only very modest stimulatory effects.

Increased expression of miR-155 in RA synovial tissue. Considering the stimulatory effects of TNFα and IL-1β on the expression of miR-155 and the high abundance of these cytokines in rheumatoid joints, we analyzed the expression of miR-155 in the RA synovium. The samples of synovial tissue from RA patients proved to be highly enriched in miR-155, with levels of expression being 8-fold higher than those found in noninflamed synovia from patients with OA ($P < 0.02$) (Figure 1B). Taken together, our findings document a consistent up-regulation of miR-155 expression in RASFs as well as in RA synovial tissue.

Down-regulation of MMP-3 in RASFs following overexpression of miR-155. To address the function of miR-155 in RASFs, we performed gain of function experiments by transfecting RASFs with precursory miR-155 molecules, which are known to increase the cellular levels of mature miR-155. Effective up-regulation of miR-155 in RASFs as a result of transfection with pre–miR-155 was confirmed, on the level of mature miR-155, by TaqMan analysis (results not shown).

In this context, we chose to investigate the expression of MMPs 1, 3, 9, and 13 and the cytokine IL-6 as markers of the destructive and inflammatory properties of RASFs (27,28). We found that enforced expression of miR-155 in RASFs resulted in a substantial reduction in the constitutive expression of MMP-3 as compared with that in cells transfected with scrambled control. The expression of MMP-3 transcripts in RASFs after transfection with pre–miR-155, relative to that in scrambled control–transfected cells, was a mean ± SEM 0.4 ± 0.03 after 48 hours and 0.3 ± 0.13 after 72 hours (Figure 2A). Accordingly, overexpression of miR-155 in RASFs also inhibited the secretion of MMP-3 protein into the culture supernatants, by a mean ± SEM 58 ± 9% after 48 hours and 58 ± 6% after 72 hours, as compared with the levels in scrambled control–transfected cells (Figure 2B).

Furthermore, enhanced expression of miR-155 in RASFs potently blocked the induction of both MMP-3 and MMP-1 by cytokines and TLR ligands. The overexpression of miR-155 in RASFs 24 hours prior to stimulation with LPS reduced the expression of MMP-3

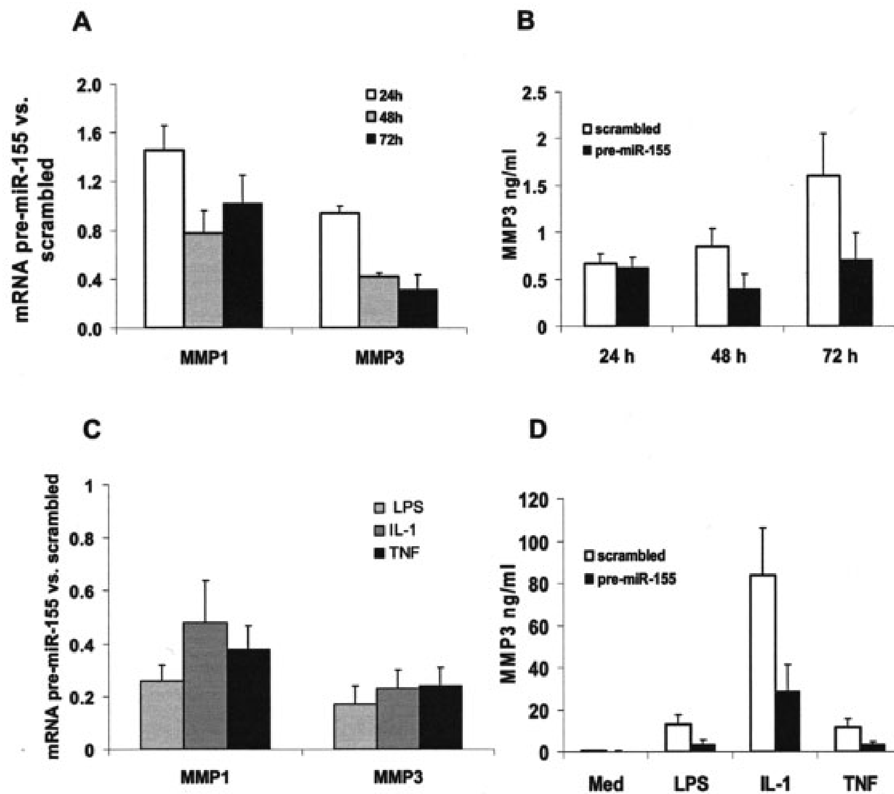

Figure 2. Down-regulation of matrix metalloproteinase 1 (MMP-1) and MMP-3 expression by miR-155 in rheumatoid arthritis synovial fibroblasts (RASFs). RASFs (n = 3) were transfected with synthetic miR-155 precursory molecule (pre–miR-155) or scrambled control prior to being assessed for constitutive expression of MMP-1 and MMP-3 mRNA (**A**) and MMP-3 protein (**B**) in the culture supernatants after 24, 48, and 72 hours. In addition, RASFs (n = 3) were transfected with pre–miR-155 or scrambled control 24 hours prior to stimulation with lipopolysaccharide (LPS), interleukin-1β (IL-1β), or tumor necrosis factor α (TNFα), and levels of MMP-1 and MMP-3 mRNA (**C**) and MMP-3 protein (**D**) were assessed after stimulation. Results in **A** and **C** are the mean and SEM expression relative to scrambled control–transfected cells (set at 1.0). Results in **B** and **D** are the mean and SEM ng/ml.

mRNA after 24 hours to a mean ± SEM 0.17 ± 0.06, relative to that in scrambled control–transfected cells (Figure 2C). The values for relative expression of MMP-3 mRNA in RASFs transfected with pre–miR-155 and stimulated with IL-1β and TNFα were 0.23 ± 0.07 and 0.24 ± 0.07, respectively (Figure 2C). In accordance with the changes observed on the mRNA level, enforced expression of miR-155 in RASFs blocked the production of MMP-3 protein in response to stimulation with LPS, IL-1β, and TNFα by a mean ± SEM 79 ± 7%, 71 ± 9%, and 77 ± 5%, respectively, as compared with the levels in scrambled control–transfected cells (Figure 2D).

Interestingly, increased expression of miR-155 also reduced the induction of MMP-1 mRNA by cytokines and TLR ligands. The relative expression of MMP-1 mRNA in RASFs transfected with pre–miR-155 prior to stimulation with LPS was a mean ± SEM 0.26 ± 0.19. The respective values for cells stimulated with IL-1β and TNFα were 0.48 ± 0.2 and 0.38 ± 0.09 (Figure 2C). The constitutive expression of MMP-1 as well as the constitutive and stimulated expression of MMPs 9 and 13 and IL-6 were not influenced by overexpression of miR-155 (results not shown).

Increased levels of miR-155 in CD14+ cells in RA synovial fluid. Monocytes/macrophages are abundantly present in the synovium of patients with RA. We therefore analyzed the expression of miR-155 in CD14+ cells in samples of synovial fluid and peripheral blood from patients with RA. Interestingly, CD14+ cells in the RA synovial fluid expressed 4.4-fold higher levels of

miR-155 as compared with the levels in peripheral blood CD14+ cells ($P < 0.03$) (Figure 3). No difference in miR-155 expression in peripheral blood CD14+ cells was observed between RA patients and normal healthy donors (Figure 3), indicating that the observed changes in miR-155 expression are restricted to local monocytes in the inflamed joints.

Increased expression of miR-146a in RASFs and up-regulation by IL-1β and LPS. Since we could demonstrate TLR-dependent regulation of miR-155 in synovial fibroblasts, we were intrigued by a study describing the induction of another miRNA, miR-146a, in human monocytes in response to TLR ligands, which was similarly found to modulate innate immune responses (29). We therefore extended our analysis to include miR-146a. In cultured RASFs, constitutive levels of miR-146a were found to be 4-fold higher than those in OASFs, with the difference reaching statistical significance ($P < 0.001$) (Figure 4A).

As discussed above, stimulation of RASFs with proinflammatory mediators up-regulated the expression of miR-155. To determine the effect of these inflammatory molecules on miR-146a expression in RASFs, the cells were stimulated with IL-1β, LPS, TNFα, poly(I-C), and bLP prior to analysis of miR-146a expression. Only IL-1β and LPS significantly induced the expression of miR-146a, whereas TNFα, poly(I-C), and bLP did not. The up-regulation of the levels of miR-146a in RASFs upon stimulation with IL-1β and upon stimulation with LPS was a mean $\pm$ SEM 5.6 $\pm$ 1.3-fold and 2.9 $\pm$ 0.3-fold, respectively, as compared with the levels in untreated cells ($P < 0.04$ for both). In contrast to a recent study describing LPS as the most potent inducing agent of miR-146a in human monocytes (29), we found that in RASFs, IL-1β was the most potent stimulus. Interestingly, IL-1β– and LPS-stimulated RASFs displayed 3.5-fold and 4.5-fold higher levels of miR-146a, respectively, than those displayed by OASFs stimulated in the same manner ($P < 0.001$) (Figure 4A).

Similar to miR-155, the expression of miR-146a was up-regulated in RA synovium compared with OA synovium. These differences between RA and OA synovium did not reach statistical significance (Figure 4B).

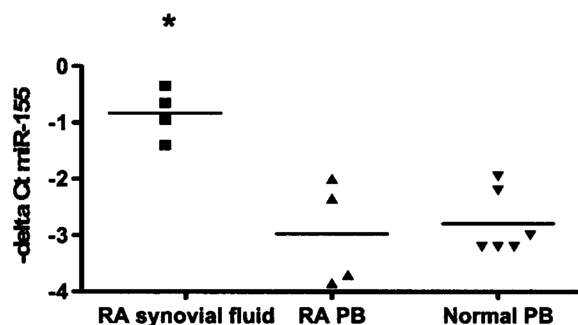

Figure 3. Increased expression of miR-155 in rheumatoid arthritis (RA) synovial fluid CD14+ cells compared with RA peripheral blood (PB) CD14+ cells. Expression of miR-155 was assessed in monocytes derived from synovial fluid (n = 4) and peripheral blood (n = 4) from RA patients as well as peripheral blood from normal healthy donors (n = 6). Results are the difference in comparative threshold cycle (ΔC_t) between miR-155 and the internal control, let-7a. Bars show the mean. * = $P < 0.03$ versus RA PB.

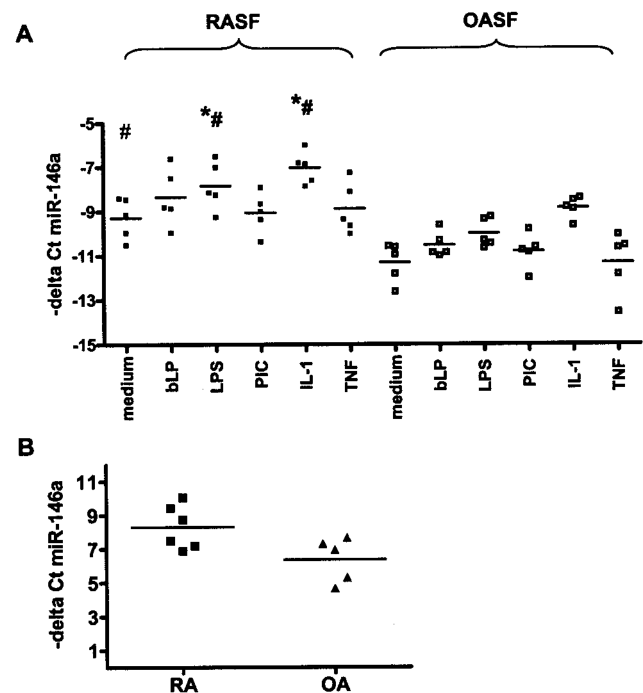

Figure 4. Overexpression of miR-146a in **A,** cultures of rheumatoid arthritis (RA) and osteoarthritis (OA) synovial fibroblasts (RASFs and OASFs, respectively) and **B,** RA and OA synovial tissue. **A,** RASFs (n = 6) and OASFs (n = 7) were treated with the indicated stimuli (proinflammatory cytokines and Toll-like receptor ligands) or left untreated (medium). Results are the difference in comparative threshold cycle (ΔC_t) between miR-146a and the internal control, let-7a. * = $P < 0.04$ versus RASF medium; # = $P < 0.001$ versus the corresponding OASF treatment groups. **B,** Expression of miR-146a in RA (n = 6) and OA (n = 5) synovial tissue was assessed as the ΔC_t between miR-146a and the internal control, U6B small nuclear RNA. Bars indicate the mean. bLP = bacterial lipoprotein; LPS = lipopolysaccharide; PIC = poly(I-C); IL-1 = interleukin-1β; TNF = tumor necrosis factor α.

DISCUSSION

In addition to the abundance of protein-coding genes implicated in the pathogenesis of RA, the

miRNA, which function as posttranscriptional repressors of the genome, need to be taken into account when elucidating molecular mechanisms of the disease. Herein we report overexpression of 2 miRNA, miR-155 and miR-146a, in synovial fibroblasts and synovial tissue derived from patients with RA. Moreover, we show that the expression of both miR-155 and miR-146a is up-regulated in synovial fibroblasts following stimulation with proinflammatory mediators. Furthermore, it is demonstrated that enforced expression of miR-155 in RASFs represses the production of MMP-3 and counteracts the induction of MMPs 1 and 3 by proinflammatory cytokines and TLR ligands.

Mature miR-155 is generated from a precursor form known as BIC RNA, encoded by the *bic* gene and known to be linked with lymphoma (30). Under normal conditions, miR-155 has been described to be expressed by human T cells, B cells, monocytes, and endothelial cells (29,31–33). Under pathologic conditions, miR-155 has been associated with a number of human neoplastic disorders. Studies have shown that miR-155 accumulates in chronic lymphocytic leukemia (34), lymphomas (30,35), breast cancer, and pancreatic cancer (36,37). Our findings extend the information on miR-155 expression in human cells by demonstrating its presence in synovial fibroblasts.

The present results indicate that the constitutive expression of miR-155 and miR-146a is increased in RASFs as compared with OASFs. Although the mechanisms leading to these alterations are unclear, we found that proinflammatory mediators abundantly present in the joints of RA patients could efficiently stimulate the expression of these miRNA. These findings strongly suggest that long-term exposure of synovial fibroblasts to the inflammatory milieu may persistently alter miRNA expression in these cells.

Up-regulation of miR-155 and miR-146a resulting from stimulation with TLR ligands appears to be a common response of fibroblasts and monocytes. Murine macrophages up-regulate miR-155 after exposure to poly(I-C) and TNFα (38), whereas human monocytes increase miR-146a after LPS stimulation (29). We found increased levels of miR-155 in monocytes derived from the synovial fluid of RA patients as compared with those derived from the RA peripheral blood. We have previously shown the presence of endogenous TLR ligands, such as double-stranded RNA derived from necrotic cells, in the joints of patients with RA (39). We therefore propose that TLR ligands as well as proinflammatory cytokines in inflamed joints influence the expression of miR-155 and, possibly, the expression of other miRNA.

Diverse cellular activities of miR-155 have thus far been described. Most importantly, miR-155 is involved in the process of oncogenesis (40). Under physiologic conditions, this miRNA has been shown to be involved in the differentiation of hematopoietic progenitor cells (17,40). Recent experiments with miR-155–knockdown mice described an important role for miR-155 in the normal function of the immune system. Mice deficient in miR-155 showed defects in T cell–dependent antibody responses, whereby the functions of germinal center B cells, T helper cells, and dendritic cells were specifically impaired (18,19).

The fact that, in RASFs, miR-155 is regulated by TLR ligands and cytokines suggests that miR-155 may modulate cellular responses to these stimuli. Indeed, we could show that transfection with miR-155 altered the response of RASFs to LPS, IL-1β, and TNFα, which was manifested as changes in the expression of MMP-1 and MMP-3. Constitutive levels of MMP-3 as well as the magnitude of induction of both MMP-3 and MMP-1 following stimulation with cytokines and TLR ligands were decreased as a consequence of enhanced expression of miR-155. These results indicate that miR-155 functions in RASFs as a repressor of MMP-3 and MMP-1.

Based on our observations documenting the increased levels of miR-155 in RASFs and the stimulatory effects of inflammatory mediators on miR-155 expression, as well as the miR-155–dependent repression of the stimulated expression of MMPs 3 and 1 in RASFs, it can be hypothesized that miR-155 has a counter-regulatory role in the process of tissue destruction in arthritic joints. We propose that miR-155 might function in a specific cellular and inflammatory context as a protective miRNA that locally down-regulates the expression of certain MMPs, thereby controlling excessive tissue damage due to inflammation. However, the elucidation of the functional role of miR-155 in the pathogenesis of RA will require further studies, including experiments with animal models of arthritis.

In contrast to the relatively well-described functional role of miR-155, the mRNA targets of miR-155 are poorly characterized. To date, angiotensin II receptor is the only gene identified as being targeted by miR-155 (41). Whether miR-155 directly targets MMP-3 and MMP-1 transcripts or, in an indirect manner, down-modulates their expression via targeting of signaling molecules involved in the transcriptional control of these MMPs requires further investigation. Neither MMP-3 nor MMP-1 has been predicted (by miRGen analysis) to be a direct target of miR-155. However, a recent study

by Tili et al raised the possibility of an indirect action of miR-155 on the control of expression of MMP-3 and MMP-1 (42). That study demonstrated that miR-155 most probably targets proteins involved in LPS signaling, such as the Fas-associated death domain, IKB kinase ϵ, and the TNF receptor superfamily–interacting serine-threonine kinase 1. Thus, synergistic down-regulation of several signal-transducing proteins could account for the strong inhibitory effect observed in our study. Interestingly, a similar function has been described for miR-146a in human monocytes stimulated with LPS (29). That study demonstrated that miR-146a controls TLR and cytokine signaling via targeting of the IL-1 receptor–associated kinase and TNF receptor–associated factor 6.

Taken together, our study findings reveal a dysregulation of the expression of the miRNA miR-155 and miR-146a in synovial tissue, synovial fibroblasts, and monocytes in the rheumatoid joints. In addition, our results document the profound influence of the inflammatory milieu on miRNA expression in synovial cells. Our functional studies showed that miR-155 controls the expression of MMP-3 and MMP-1 in RASFs, suggesting that miR-155 may be involved in the modulation of tissue destruction in RA and, at the same time, raising the possibility of a therapeutic potential of miRNA-based approaches for the treatment of joint destruction in RA.

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AUTHOR CONTRIBUTIONS

Dr. Stanczyk had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Stanczyk, Pedrioli, Brentano, Sanchez-Pernaute, S. Gay, Kyburz.

Acquisition of data. Stanczyk, Pedrioli, Kolling.

Analysis and interpretation of data. Stanczyk, Pedrioli, Brentano, R. E. Gay, Detmar, S. Gay, Kyburz.

Manuscript preparation. Stanczyk, Pedrioli, Sanchez-Pernaute, Detmar, Kyburz.

Statistical analysis. Stanczyk.

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