MIF allele-dependent regulation of the MIF coreceptor CD44 and role in rheumatoid arthritis

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Fibroblast-like synoviocytes mediate joint destruction in rheumatoid arthritis and exhibit sustained proinflammatory and invasive properties. CD44 is a polymorphic transmembrane protein with defined roles in matrix interaction and tumor invasion that is also a signaling coreceptor for macrophage migration inhibitory factor (MIF), which engages cell surface CD74. High-expression *MIF* alleles (*rs5844572*) are associated with rheumatoid joint erosion, but whether MIF signaling through the CD74/CD44 receptor complex promotes upstream autoimmune responses or contributes directly to synovial joint destruction is unknown. We report here the functional regulation of CD44 by an autocrine pathway in synovial fibroblasts that is driven by high-expression *MIF* alleles to up-regulate an inflammatory and invasive phenotype. MIF increases CD44 expression, promotes its recruitment into a functional signal transduction complex, and stimulates alternative exon splicing, leading to expression of the CD44v3–v6 isoforms associated with oncogenic invasion. CD44 recruitment into the MIF receptor complex, downstream MAPK and RhoA signaling, and invasive phenotype require MIF and CD74 and are reduced by MIF pathway antagonists. These data support a functional role for high-*MIF* expression alleles and the two-component CD74/CD44 MIF receptor in rheumatoid arthritis and suggest that pharmacologic inhibition of this pathway may offer a specific means to interfere with progressive joint destruction.

immunogenetics | autoimmunity | MIF | CD44 | CD74

The pathologic hallmark of rheumatoid arthritis (RA) is a synovial pannus that comprises proliferating and invasive fibroblast-like synoviocytes (FLSs), infiltrating leukocytes, and an associated neoangiogenic response (1, 2). Rheumatoid FLSs destroy cartilage and underlying bone by producing matrix metalloproteinases (MMPs), inflammatory and growth-promoting cytokines, and prostaglandins (2). Rheumatoid synoviocytes also resist apoptosis and show increased adhesive and invasive properties; for instance, when implanted into immunodeficient mice, they readily migrate to distant tissue sites (3). The tumor-like features of these stromal lineage cells persist during long-term culture and may result from epigenetic and genetic alterations, including mutations in the tumor suppressor *p53* that augment prosurvival pathways (2, 4, 5).

Macrophage migration inhibitory factor (MIF) is an immunoregulatory cytokine that is expressed by different cell types and inhibits activation-induced apoptosis to sustain the survival and inflammatory activation of monocytes/macrophages (6, 7). Human genetic studies have established the presence of functional polymorphisms in the *MIF* promoter that occur commonly in the population, with high-expression alleles linked to the severity of rheumatoid joint erosions (8, 9). Immunoneutralization or genetic deletion of MIF also inhibits arthritis development and joint destruction in different experimental models of disease (10–14). MIF is expressed in elevated levels in the plasma and synovium of RA patients (8, 15), where it induces sustained MAPK activation (16, 17), suppresses the proapoptotic action of p53 (11, 18), and increases the production of arachidonic acid (19), which enhances the translational stability of proinflammatory cytokine mRNAs and contributes to high levels of prostaglandin release (17). MIF also is produced by rheumatoid T lymphocytes, where it has been shown to stimulate MMP expression in synovial fibroblasts (20) and up-regulate RANKL to promote osteoclastogenesis (21).

MIF initiates signal transduction by binding to cell surface CD74 (22), leading to the intracytoplasmic phosphorylation of its coreceptor CD44 and activation of Src-family kinases (23). CD44 is a polymorphic glycoprotein that mediates cell–cell adhesion and cell–matrix interactions, and it has been implicated in cellular homing, tumor invasiveness and metastasis, and angiogenesis (24–26). The CD44 gene comprises 19 exons, of which 10 participate in alternative splicing to produce variants with an extended ectodomain structure (e.g., CD44v1–10) (27). Oncogenic activation initiates alternative splicing, and the CD44v3–v6 isoforms, in particular, have been implicated in enhancing cellular migration, adhesion, and invasion by mechanisms that involve increased matrix interaction and the creation of neodomains for growth factors and MMPs (27). Whether CD44 expression, signal transduction, or alternative splicing is functionally regulated by high-genotypic *MIF* expression and has a direct pathogenic role in rheumatoid pannus formation is unknown. The precise mechanisms underlying MIF's role in autoimmune tissue damage are of interest and are being focused by the entry into clinical testing

Significance

High-expression alleles of the cytokine macrophage migration inhibitory factor (MIF) are associated with severe joint destruction in autoimmune arthritis, but the mechanism for this effect is unknown. High-genotypic *MIF*-expressing joint fibroblasts produce high levels of MIF under inflammatory stimulation to up-regulate the surface expression of the MIF signaling coreceptor CD44 and promote its alternative splicing into invasive, tumor-associated isoforms, which contribute to the invasive and tissue-destructive character of the rheumatoid joint synovium. These findings support a precision medicine approach to the treatment of rheumatoid arthritis by pharmacologically targeting the MIF pathway in high-genotypic *MIF*-expressing patients.

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Conflict of interest statement: R.B. and J.B. are listed as co-inventors on patents describing MIF inhibitors.

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of anti-MIF and -CD74 antibodies, with the objective that therapeutic intervention may be guided by an individual's *MIF* genotype (28–30).

Results

CD44 Expression Is Enhanced in Rheumatoid Synovium and Synovial Fibroblasts. The two-component MIF receptor comprising the CD74 MIF binding protein and the CD44 signal transducer is expressed on numerous cell types, including monocytes/macrophages and stromal cells (31), and prior studies support the expression of a signaling-competent MIF receptor on FLSs (16, 19). We confirmed the synovial tissue expression of CD74 and CD44 by immunohistochemical staining of arthroplasty specimens obtained from patients with RA or osteoarthritis (OA), a degenerative joint disease in which invasive pannus does not develop. Positive immunoreactivity for CD74 (Fig. S1 *A–C*) and CD44 (Fig. S1 *E–G*) was observed in the hyperplastic synovial tissue, with the most prominent staining evident in the fibroblast-like synovial lining and stromal cells and the infiltrating mononuclear leukocytes. Only weak immunoreactivity for CD74 and CD44 was apparent in the lining layer of synovia obtained from subjects with OA (Fig. S1 *D* and *H*), which is consistent with the less-inflammatory nature of OA compared with RA (1, 2).

We next compared the cellular expression of CD44 by early passage FLSs isolated from the synovia of joints affected by RA (RA-FLSs) or OA (OA-FLSs). Western blot analysis revealed the presence of CD44 in both RA- and OA-FLSs, and with the level of immunoreactive protein approximately fourfold higher in RA-FLSs than in OA-FLSs (Fig. S2*A*). The addition of synovial fluid from RA patients to cultured OA-FLSs markedly increased CD44 expression, suggesting that proteins elaborated in the inflammatory milieu up-regulate the expression of CD44 (Fig. S2*B*). The addition of IL-1β and TNF, two inflammatory cytokines present in high levels in RA synovial fluid (1), increased the expression of CD44 in cultured RA- and OA-FLSs (Fig. S2*C*). IL-1β and TNF also increased CD44 expression in human endothelial cells, indicating that the proinflammatory induction of CD44 is not restricted to FLSs.

CD44 Expression Is Dependent on MIF and Up-Regulated in High-Genotypic *MIF*-Expressing Synovial Fibroblasts. Western blot analysis of RA-FLSs showed that MIF dose-dependently increased CD44 protein expression when added in concentrations present in RA synovial fluid (32), and this up-regulating effect was comparable to that observed after addition of IL-1β or TNF (Fig. 1 *A–C*). IL-1β and TNF can act as MIF secretogogues, and both cytokines exert a proproliferative action on FLSs that can be inhibited by the addition of a neutralizing anti-MIF mAb (16). Anti-MIF blocked the IL-1β– or TNF-induced expression of CD44, which suggests an autocrine pathway for MIF release followed by up-regulation of CD44 protein expression (Fig. 1 *B* and *C*). We confirmed the IL-1β– and MIF-mediated induction of CD44 surface expression in RA-FLSs by flow cytometry (Fig. 1*D*, *Left*). Cultured fibroblasts secrete low amounts of MIF constitutively as a homeostatic survival signal (33), and we observed CD44 surface expression to be reduced in murine embryonic fibroblasts genetically deficient in MIF ($Mif^{-/-}$) or the MIF binding receptor CD74 ($Cd74^{-/-}$) (Fig. 1*D*, *Center* and *Right*). These data indicate that baseline CD44 surface expression is dependent both on MIF and its interaction with the MIF-binding receptor CD74.

We also explored the regulatory influence of proinflammatory cytokines on CD74 surface expression. CD74 is up-regulated by IFN-γ in monocytes/macrophages (22), and we observed a similar up-regulatory effect of IFN-γ on CD74 in cultured FLSs (Fig. 1*E*). By contrast, neither MIF, IL-1β, nor TNF up-regulated CD74 expression on FLSs, and IFN-γ showed no direct effect on surface CD44 expression.

MIF is encoded in a functionally polymorphic locus (*rs5844572*), with variant alleles distinguished by a four-nucleotide microsatellite (−794 $CATT_{5-8}$) in the gene promoter. *MIF* expression increases with CATT repeat number, so that individuals with the $CATT_5$ (5-CATT) allele are low *MIF* expressers, and those

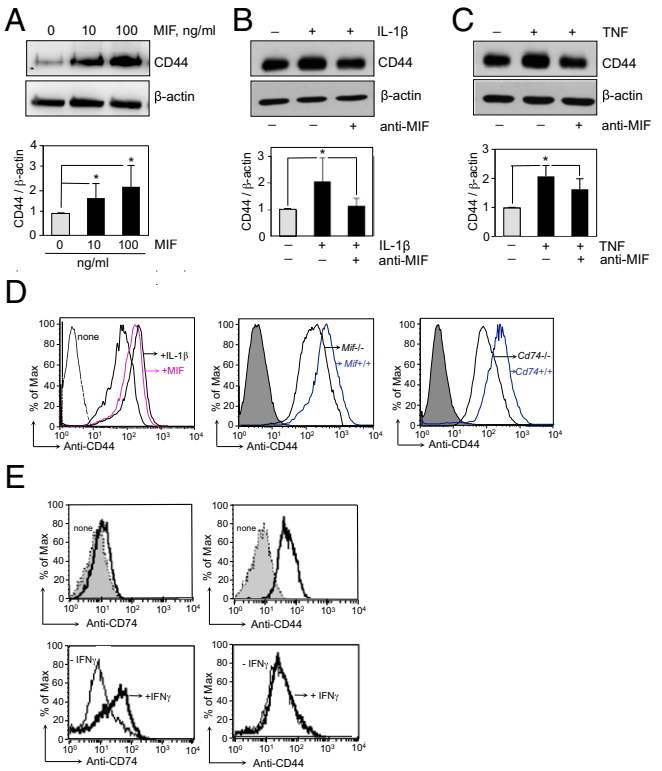

Fig. 1. Inflammatory expression of CD44 in FLSs is MIF-dependent. (*A*) Dose-dependent induction of CD44 in RA-FLSs cocultured with MIF for 24 h. (*B* and *C*) IL-1β (*B*) or TNF (*C*) induction of CD44 in RA-FLSs is blocked by anti-MIF. Cytokines were added at a concentration of 10 ng/mL for 24 h in the presence of anti-MIF mAb (IIID.9; 5 μg/mL) or an IgG1 isotypic control (–). (*D*) Cell surface CD44 expression measured by flow cytometry in RA-FLSs stimulated for 24 h with IL-1β (10 ng/mL) or MIF (10 ng/mL) (*Left*) and reduced expression in *Mif* (*Center*) or *Cd74* (*Right*) gene-deficient mouse fibroblasts. $Mif^{+/+}$ and $Cd74^{+/+}$ denote wild-type controls. (*E*) Synovial fibroblast cell surface expression of CD74 (*Left*) and CD44 (*Right*) after stimulation with IFN-γ (10 ng/mL, 48 h) showing inducibility of CD74, but not CD44. Data are representative of three RA-FLS lines studied (none refers to isotype control). Data in *A–D* are representative of three independent experiments with similar results. Histograms show the ratio of densitometric scanning of CD44 to β-actin for experiments with three different cell lines. $^*P < 0.05$ by two-tailed Student's *t* test for the comparisons shown.

with 6-, 7-, and 8-CATT alleles are proportionally higher *MIF* expressers (29, 34, 35). High-expression *MIF* alleles (>5-CATT) confer an increased risk for rheumatoid or juvenile inflammatory arthritis, and in those individuals with established RA, the presence of a low expression (5-CATT) *MIF* genotype protects from the development of severe erosive disease with a reported odd ratio of 8.2 (8, 9, 36).

Cultured RA-FLSs express MIF mRNA and produce MIF protein in a *MIF* promoter $CATT_{5-8}$ length-dependent manner (35). We confirmed a *MIF* genotype-dependent increase in MIF production by a panel of early passage cultured RA-FLSs (Fig. 2*A*) and analyzed cellular CD74 and CD44 expression by flow cytometry (Fig. 2*B*). Cell surface CD74 expression did not vary as a function of *MIF* genotype; however, the expression of CD44 was greater in synoviocytes with high-expression (>5-CATT) vs. low-expression (5-CATT) *MIF* genotypes. These data support the finding that *MIF* expression increases CD44, but not CD74, expression in RA-FLSs.

To independently assess the relationship between MIF and CD44 expression in RA synovia, we examined the expression levels of the two genes in a previously collected registry of 83 rheumatoid synovial tissue samples obtained at arthroplasty and subjected to whole-tissue microarray analysis. As shown in Fig. 2*C*,

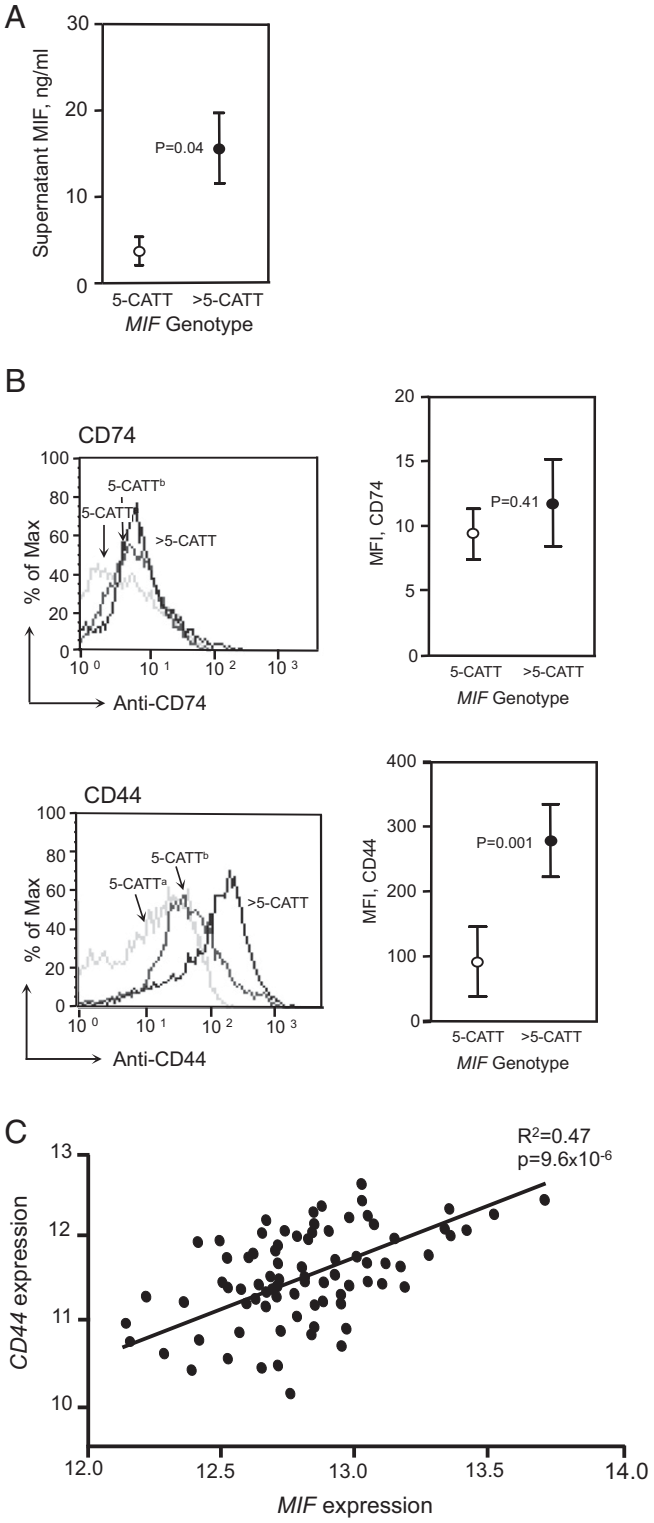

Fig. 2. Human *MIF* genotype-dependent expression of the MIF signaling coreceptor CD44. (*A*) MIF content measured by ELISA in 72-h supernatants harvested from cultures of early passage RA-FLSs ($n = 4$ patient-derived lines per genotype, mean ± SD with $P = 0.04$ by two-tailed Student's t test). (*B*) Flow cytometry analysis of cell surface CD74 and CD44 expression in three patient-derived early passage RA-FLS lines. The 5-CATT^a and -CATT^b denote two genotypic low *MIF* expresser lines (−794 $CATT_5$), and >5-CATT denotes a representative high *MIF* expression (−794 $CATT_{6,7}$) RA-FLS line. Plots are representative of a total of four lines studied per genotypic group. Mean fluorescence intensity (MFI) of cell surface CD74 and CD44 staining in four low-*MIF* expresser (5-CATT) and four high-*MIF* expression (>5-CATT) RA-FLS lines (mean ± SD by two-tailed Student's t test) is shown. (*C*) Correlation plot for CD44 vs. MIF mRNA expression in RNA samples obtained from the joint synovia of 83 subjects with RA. The expression scores were calculated from a quartile-trimmed mean of the normalized probe set values.

there was a significant correlation between the tissue expression of *MIF* and *CD44* ($R^2 = 0.47$, $P = 9.6 \times 10^{-6}$). A similar relationship between *MIF* and *CD74* expression was not detected. This correlated expression supports the functional, autocrine/paracrine role of MIF in promoting the inflammatory expression of CD44 in rheumatoid synovium.

Inflammatory Prostaglandin Production by Synovial Fibroblasts Is MIF Receptor (CD74/CD44) and MIF Genotype-Dependent. Prior reports indicated that the inflammatory activation of synovial fibroblasts leading to cyclooxygenase-2 (COX-2) expression and prostaglandin (PGE_2) production is MIF- and CD74-dependent, including under conditions of direct IL-1β stimulation (19, 22). We tested the requirement for the MIF coreceptor CD44 in COX-2 expression by transfecting human synovial fibroblasts with a CD44 siRNA. CD44 expression was observed to be essential for MIF-stimulated COX-2 expression (Fig. 3 *A* and *B*).

IL-1β is considered to have a dominant role in the induction of synovial prostaglandin production (37). Given evidence for an intermediary autocrine/paracrine function for MIF in this action of IL-1β (16, 19) (Fig. 1*B*), and the observation that CD44 expression levels correlate with MIF production (Fig. 2*C*), we examined the impact of IL-1β stimulation on inflammatory prostaglandin production in low- vs. high-genotypic *MIF*-expressing synovial fibroblasts. We verified the reported requirement for MIF engagement of CD74 in IL-1β–stimulated COX-2 expression (19) (Fig. 3*C*), and observed a $CATT_{5-8}$ length-dependent increase in COX-2 expression and PGE_2 production in low- vs. high-genotypic *MIF*-expressing synovial fibroblasts (Fig. 3 *D* and *E*).

MIF Enhances Cell Surface CD74–CD44 Interaction. Beyond the impact of MIF on CD44 expression, we addressed whether MIF influences the formation of competent CD74/CD44 signal transduction complexes. Prior confocal microscopy and coimmunoprecipitation studies supported the direct interaction of MIF with its binding receptor CD74 and with cell-surface contacts between CD74 and its signaling coreceptor CD44 (22, 23, 38). Gene deletion and reconstitution studies also have established the requirement for CD74 and CD44 coexpression in MIF-dependent MAPK activation and prosurvival functions (23, 38). It is unknown, however, whether CD74 and CD44 exist in preformed receptor heterodimers or whether MIF engagement of CD74 promotes CD44 recruitment into a functional signaling unit. We analyzed cells transfected with recombinant CD74 and CD44 fused in their intracytoplasmic domains to yellow or cyan fluorescent proteins (YFP or CFP, respectively) for measurement of fluorescence resonance energy transfer (FRET) as a sensitive indicator of protein proximity (Fig. 4*A*). Although a low baseline level of FRET was detectable in CD74/CD44-coexpressing cells, the addition of MIF markedly increased FRET (Fig. 4 *B* and *C*), suggesting that MIF binding to CD74 promotes CD44 recruitment into apposition with CD74. This enhancement in FRET interaction was dependent on the CD44 intracellular domain, which undergoes MIF- and CD74-dependent phosphorylation (23), because FRET enhancement was not observed with an intracellular domain-truncated CD44Δ67-CFP construct lacking this domain (Fig. 4*D*). Notably, recombinant elimination of CD74 Ser-202 (YFP-CD74ΔCS)—which is the site of a posttranslational chondroitin sulfate modification hypothesized to facilitate CD74/CD44 membrane interactions (39)—did not influence on MIF's ability to

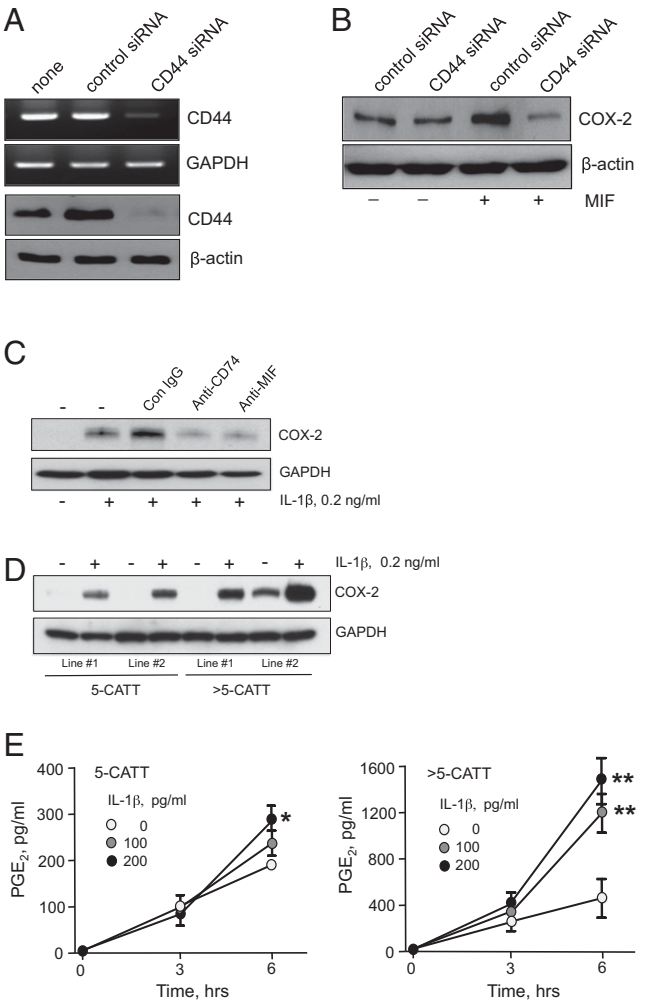

Fig. 3. *MIF* genotype and CD44-dependent expression of COX-2 and PGE_2 in RA-FLSs. (*A* and *B*) Effective siRNA-mediated knockdown of CD44 mRNA and protein in RA-FLSs (*A*) and resulting reduction in COX-2 expression after stimulation with MIF (*B*; 10 ng/mL, 12 h). Data shown are representative of three RA-FLS lines studied. (*C*) IL-1β–stimulated COX-2 expression in RA-FLSs is blocked by anti-MIF or -CD74. Data are representative of three RA-FLS lines that were stimulated for 8 h. (*D*) Increased IL-1β–inducible COX-2 expression in high-genotypic (>5-CATT) vs. low-genotypic (5-CATT) *MIF* expression RA-FLS cell lines (Western blot is representative of two replicates). (*E*) Increased IL-1β–induced PGE_2 production in high-genotypic (>5-CATT) vs. low-genotypic (5-CATT) *MIF* expression RA-FLS cell lines. Supernatant PGE_2 concentration was measured by ELISA in three RA-FLS lines per genotypic group. $^*P < 0.05$; $^{**}P < 0.01$ vs. untreated cells at 6 h by two-tailed Student's *t* test (mean ± SD).

promote the recruitment of CD44 into a MIF/CD74 signaling complex or induce downstream ERK1/2 phosphorylation (Fig. 4 *D* and *E*).

We tested the impact on MIF-dependent FRET and signal transduction of two small-molecule pharmacologic MIF modulators: MIF098 and MIF020 (40, 41). These molecules bind specifically to the MIF/CD74 interface to either reduce (e.g., MIF098) or increase (e.g., MIF020) its K_D with CD74, leading to corresponding antagonist or agonist effects on signal transduction and biologic action (42, 43). The addition of the MIF antagonist MIF098 and the MIF agonist MIF20 to YFP–CD74/CD44–CFP-expressing cells modulated FRET efficiency and ERK1/2 phosphorylation in the direction predicted from the pharmacologic action of these small molecules (Fig. 4 *F* and *G*).

MIF Stimulates CD44 Alternative Splicing. CD44 is encoded by a multiexon gene that can undergo alternative splicing to produce translational extensions of its extracellular domain. The CD44v3 and CD44v6 exons confer increased migratory and invasive properties to oncogenically transformed cells by introducing adhesion and MMP docking domains into the 361-amino acid "standard" or hematopoietic isoform of CD44 (27). These isoforms have been detected in rheumatoid synovia as well as in cultured RA-FLSs (44, 45). We asked whether the increased expression of CD44 observed in cultured RA-FLSs was accompanied by a shift to the CD44v3/v6 splice variants. Using exon-specific quantitative PCR primers, we observed increased expression of the CD44v3 isoform in synovial fibroblasts obtained from RA vs. OA synovia (Fig. 5*A*). By contrast, the relative expression level of the CD44v6 isoform was similar between these cell types. The addition of anti-MIF to synovial fibroblasts, which constitutively secrete MIF in culture (Fig. 2*A*), reduced the expression of both the CD44v3 and CD44v6 isoforms in RA-FLSs, but not OA-FLSs (Fig. 5*B*). IL-1β addition increased the expression of the CD44v3 and CD44v6 isoforms in RA-FLSs, as well as the CD44v3 isoform in OA-FLSs. The ability of IL-1β to induce CD44 alternative splicing was not inhibited by anti-MIF, however, suggesting that this action of IL-1β proceeds independently of autocrine/paracrine MIF release. We additionally confirmed the ability of MIF and IL-1β to up-regulate the cell surface expression of the CD44v3 and CD44v6 protein isoforms by Western blot (Fig. 5*C*) and flow cytometry (Fig. 5*D*).

The RNA-binding proteins transformer 2α (Tra2α) and Tra2β are primary regulators of alternative splicing and are induced in invasive carcinomas that express CD44 variant isoforms (46). We found MIF stimulation to up-regulate Tra2α, but not Tra2β, expression in RA-FLSs, as well as in primary human skin fibroblasts (Fig. 5*E*). These data suggest a direct upstream role for MIF in the induction of the Tra2α RNA-splicing protein necessary for CD44 exon rearrangement.

MIF-CD74/CD44 Axis Promotes Synovial Fibroblast Adhesion and Migration. Rheumatoid synovial fibroblasts show enhanced cellular adhesiveness, which may reflect the ability of CD44 to form a cocomplex with $\alpha_v\beta_3$ integrin (47). MIF up-regulates $\alpha_v\beta_3$ integrin expression in chondrosarcomas (48, 49), and the addition of MIF to RA-FLSs also increased $\alpha_v\beta_3$ integrin expression together with cell adhesiveness (Fig. 6 *A* and *B*). Genetic knockdown of CD44 decreased the MIF-induced increase in synovial fibroblast adhesion, supporting a role for CD44 in promoting adhesive character in RA-FLSs (Fig. 6*C*).

A distinctive feature of signal transduction through the CD74/CD44 receptor complex is sustained ERK1/2 activation, which requires Rho GTPases (17, 50). Rho GTPases regulate cytoskeletal dynamics and cell movement, and their activation is reduced in $Mif^{-/-}$ macrophages, which exhibit reduced adhesion, migratory activity, and actin polymerization and stress fiber formation (50, 51). High-genotypic *MIF*-expressing RA-FLSs (>5-CATT) showed increased migration and invasive activity compared with low-genotypic *MIF*-expressing cells (5-CATT) (Fig. 7*A*), and this effect was associated with increased baseline activation of Rho GTPase (Fig. 7*B*). Prior studies supported an important role for MIF engagement of CD74 in chemotactic responses (51), and we found pharmacologic MIF antagonism with MIF098 to reduce synovial fibroblast chemokinesis and membrane invasion (Fig. 7*C*). MIF098 also reduced the chemotactic response to exogenously added MIF (Fig. 7*D*) and IL-1β (Fig. 7*E*), which is a strong pathologic stimulus for synovial fibroblast migration and invasion (2, 52). IL-1β–induced Rho GTPase activation, in turn, required MIF, CD74, and CD44, as assessed by antibody neutralization (Fig. 7*F*), RNA interference (Fig. 7*G*), and pharmacologic MIF antagonism (Fig. 7*H*).

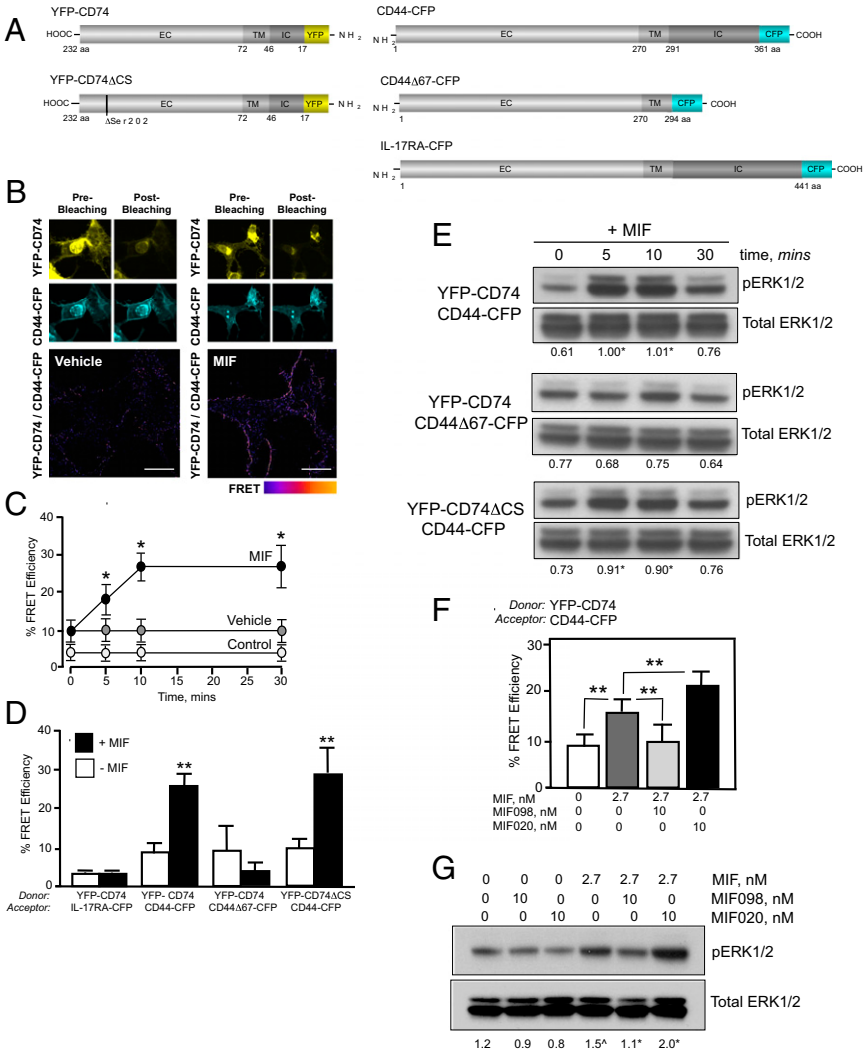

Fig. 4. MIF enhances cell surface CD74/CD44 interaction and signal transduction. (*A*) Schematic diagram of the recombinant protein constructs used for FRET and signal transduction studies. EC, TM, and IC denote the extracellular, transmembrane, and intracellular domains, respectively. CD74 and CD44 are type II and I membrane proteins, respectively, with CD74 shown in reverse N-terminal orientation relative to CD44. CD74ΔCS denotes site-directed deletion of the Ser-202 site of chondroitin sulfate (CS) modification, and CD44Δ67 denotes a CD44 with a 67-amino acid deletion of its intracytoplasmic signaling domain (amino acids 295–361). The IL-17 receptor (IL-17RA-CFP) was used as a negative FRET control. (*B*) Fluorescence images of $CD74^{null}CD44^{null}$ COS7/M6 cells transfected with YFP–CD74 as acceptor and CD44–CFP as donor that show localization and FRET efficiency before and after acceptor photobleaching and in the absence and presence of MIF (100 ng/mL, 10 min). Images are representative of at least 20 cells examined. (Scale bars: 1 μm.) (*C*) Time-dependent increase in FRET efficiency in YFP–CD74 and CD44–CFP COS7/M6 cell transfectants stimulated with MIF (100 ng/mL) or vehicle. COS7/M6 cells transfected with YFP–CD74 and IL-17RA–CFP served as background control. (*D* and *E*) MIF-stimulated FRET efficiency (*D*) and ERK1/2 phosphorylation (*E*) in COS7/M6 cells transfected with truncated CD44Δ67–CFP in place of full-length CD44–CFP donor or mutant YFP–CD74ΔCS in place of the YFP–CD74 acceptor. MIF was added at a concentration of 100 ng/mL in *D* and *E* and for 10 min in *D*. Separate experiments demonstrated equivalent cellular expression of recombinant mutant constructs as recombinant wild-type proteins. Numerical values in Western blots denote densitometric scanning of the ratio of pERK1/2 to total ERK1/2 phosphorylation in three separate Western blotting studies. $*P < 0.05$ vs. time 0 by two-tailed Student's *t* test. (*F* and *G*) MIF-stimulated FRET efficiency (*F*) and ERK1/2 phosphorylation (*G*) in YFP–CD74 and CD44–CFP COS7/M6 cell transfectants in the presence of the small-molecule MIF antagonist MIF098 or agonist MIF020. MIF was added at a concentration of 100 ng/mL (2.7 nM) for 5 min. Separate experiments demonstrated no impact of MIF098 or MIF020 alone in the absence of MIF stimulation. Numerical values in Western blots denote the ratio of densitometric scanning of pERK1/2 to total ERK1/2 phosphorylation in three separate Western blotting studies. $*P < 0.05$ vs. time 0 (*C* and *E*) or 0 nM MIF098/MIF020 (*G*); $**P < 0.01$ vs. –MIF (*D*) or as indicated (*F*); $\wedge P < 0.05$ vs. –MIF (*G*), all by two-tailed Student's *t* test.

Discussion

Functional promoter polymorphisms in *MIF* occur commonly in the population (minor allele frequency > 5%), and among patients with RA, high-genotypic *MIF* expressers have more severe joint erosion (8, 9). MIF binds to and activates CD74, which is expressed on numerous cell types together with its signaling coreceptor, CD44. Both proteins undergo protein kinase A-mediated phosphorylation of their intracytoplasmic domains, and CD44 phosphorylation, in turn, leads to the recruitment of Src-family kinases and guanine nucleotide exchange factors (23, 31, 38). In addition to initiating MIF-dependent signal transduction pathways responsible for proliferation and cell survival, CD44 is a polymorphic and multifunctional cell surface protein with biologic activities relevant to rheumatoid synovial pathology. Matrix interaction, cellular migration, and the presentation of growth factors and MMPs are enhanced by alternative splicing and the introduction of extended extracellular domains (24, 27). Notably, the expression in transformed cells of extended isoforms, particularly CD44v3–v6, contributes to tumor progression and correlates with metastatic disease in several human cancers (26, 53, 54). A shift toward the expression of CD44 splice variants has been reported in systemic lupus erythematosus (SLE) (55) and RA (44), including within the synovium of rheumatoid joints (45). Experimental studies support a role for CD44 in promoting leukocyte migration into synovia, and anti-CD44 administration ameliorates swelling and the development of articular deformities in mouse models (44, 56, 57). The increased migration and adhesion of SLE T cells also is mediated by CD44 and can be inhibited by downstream inhibition of Rho kinase (58).

The present findings support a direct role for MIF in upregulating the expression of its signaling coreceptor, CD44. We confirmed prior observations that rheumatoid synovia, which exhibit a profound inflammatory and hyperplastic character, express higher levels of CD44 compared with OA synovia (45). Enhanced expression of CD44 was noted in cultured rheumatoid synovial fibroblasts, and levels increased in response to rheumatoid synovial fluid and the proinflammatory cytokines IL-1β and TNF, which are produced in high levels in the joint and can function as MIF secretogogues (16, 19). By contrast, baseline expression of the ligand-binding component of the MIF receptor complex, CD74, was not influenced by these innate cytokines, but was increased by IFN-γ, which is derived from T lymphocytes and natural killer cells, and is a strong stimulus for CD74 upregulation in monocytes/macrophages (22). The direct role of MIF/CD74 in CD44 expression was verified genetically in $Mif^{-/-}$

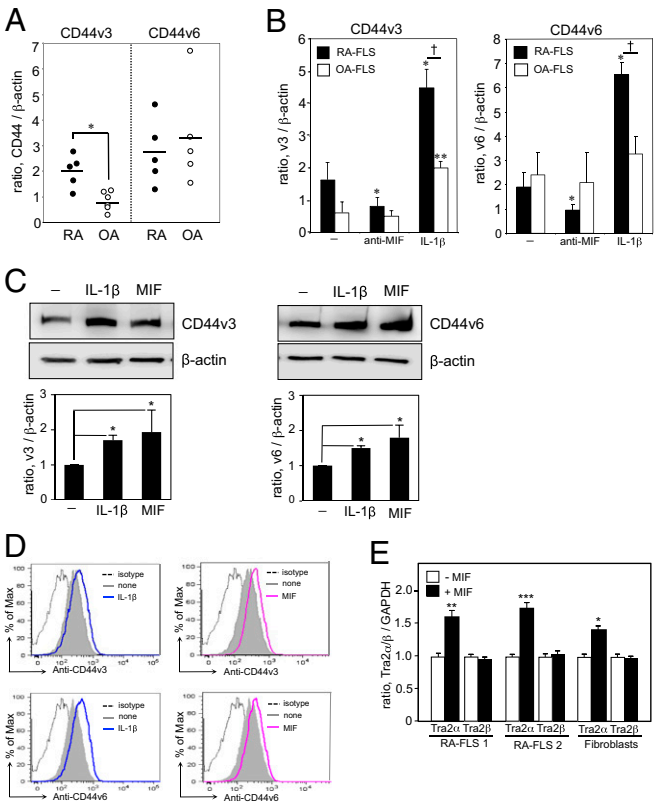

Fig. 5. MIF promotes CD44 alternative splicing and expression of the extended CD44v3 and CD44v6 isoforms, and induces the splicing factor Tra2α. (*A*) Basal expression of CD44v3 and CD44v6 isoforms in RA- vs. OA-FLSs measured by quantitative PCR. (*B*) Effect of anti-MIF antibody and recombinant IL-1β on the expression of CD44v3 and CD44v6 in RA- and OA-FLSs quantified by quantitative real-time PCR. Cells were pretreated with anti-MIF antibody (5 μg/mL) or IL-1β (10 ng/mL) for 24 h. $*P < 0.05$; $**P < 0.01$ vs. untreated cells; $^{\dagger}P < 0.005$ between RA- and OA-FLSs (two-tailed Student's *t* test). (*C* and *D*) Inducible expression of CD44v3 and CD44v6 isoforms by MIF and IL-1β. The RA-FLSs were stimulated with IL-1β (10 ng/mL) or MIF (10 ng/mL) for 24 h, and the alternatively spliced proteins were quantified by specific antibody for Western blot (*C*) and flow cytometry analysis (*D*). Histograms show densitometry for Western blot analysis of three RA-FLS cell lines (mean ± SD). (*E*) Quantitative PCR measurement of Tra2α and Tra2β mRNA content in two RA-FLS and one primary human skin fibroblast line after 24-h stimulation with MIF (10 ng/mL). Data are mean ± SD of three measurements. $*P < 0.05$; $**P < 0.01$; $***P < 0.005$ vs. no MIF addition by two-tailed Student's *t* test.

and *Cd74*$^{-/-}$ murine fibroblasts, which showed reduced CD44 expression compared with wild-type fibroblasts.

Synovial fibroblasts express *MIF* mRNA and produce MIF protein in a *MIF* −794 $CATT_{5-8}$ length-dependent manner (35). An analysis of rheumatoid synovial fibroblast lines stratified by *MIF* genotype revealed higher CD44 cell surface expression in high-genotypic *MIF*-expressing cells (>5-CATT) compared with low-genotypic *MIF*-expressing cells (5-CATT). In a gene expression dataset of 83 rheumatoid synovial tissues, a close correlation between *MIF* and *CD44* mRNA levels also was observed, supporting MIF's role in up-regulating the expression of its signaling coreceptor in vivo.

Rheumatoid synovial fibroblasts express COX-2 and produce high levels of PGE_2 in response to IL-1β stimulation (37). We verified the requirement for MIF and CD74 in IL-1β–induced COX-2 expression (19) and found CD44 to be a necessary receptor component for this MIF-dependent response. High-genotypic *MIF*-expressing synovial fibroblasts showed an increase in COX-2 protein content and produced greater amounts of PGE_2 than low-genotypic *MIF*-expressing cells, supporting the role of the MIF-CD74/CD44 signaling axis in the inflammatory prostaglandin effector response.

In addition to up-regulating cellular CD44 expression, MIF also increased the membrane association of CD74 and CD44 to enhance signal transduction. Measurements of MIF-induced CD74–CD44 FRET showed that this association requires the CD44 intracellular domain, which undergoes changes in serine phosphorylation in response to MIF/CD74 engagement (23), and to be unaffected by the extracellular chondroitin sulfate modification of CD74, which had been proposed to facilitate CD74–CD44 membrane interactions (39). These observations indicate that, in contrast to other cytokine signaling complexes, such as the heterodimeric IL-17 receptor (59), the CD74/CD44 MIF receptor does not exist in preformed receptor complexes. Instead, MIF binding to CD74 initiates CD44 association with CD74 to create functional signal transduction units that induce downstream MAPK phosphorylation. MIF-dependent CD74–CD44 association, as assessed by FRET and ERK1/2 phosphorylation, also was modulated by two recently developed pharmacologic agents that bind at the interface of MIF with CD74 to either increase or decrease MIF binding affinity and produce corresponding agonist or antagonist effects in vivo (41–43, 60).

The observation that MIF increases both CD44 expression and its interaction with CD74 at the plasma membrane to enhance signal transduction dovetail with genetic studies that have linked high-expression *MIF* alleles with more severe joint erosion (8). Moreover, the finding that MIF itself can induce splicing factor

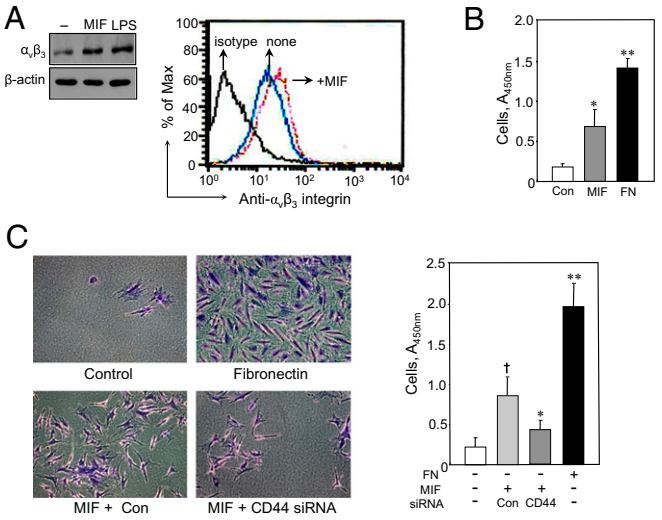

Fig. 6. MIF increases synovial fibroblast adhesion via CD44. (*A*) MIF increases $\alpha_v\beta_3$ integrin expression in RA-FLSs. Cells (1×10^5) were stimulated with MIF (10 ng/mL) or LPS (100 ng/mL) for 24 h, and $\alpha_v\beta_3$ integrin expression was assessed by Western blot analysis and flow cytometry. Data are representative of experiments performed in three RA-FLS lines with similar results. (*B*) Enhanced adhesion of MIF-stimulated RA-FLSs in vitro. The 96-well assay plates were coated with albumin (Con), MIF (100 ng/mL), or fibronectin (FN; 5 μg/mL) before the addition of RA-FLSs at 37 °C for 3 h. Attached cells were quantified by hexosaminidase assay. Values are the mean ± SD of triplicate experiments performed on three representative RA-FLS lines. $*P < 0.05$; $**P < 0.01$ vs. untreated cells by two-tailed Student's *t* test. (*C*) CD44 siRNA-mediated suppression of MIF-induced FLS adhesion. MIF (100 ng/mL) and FN (5 μg/mL) were used for coating plates. After 24 h of transfection with control (Con) or CD44 siRNA, the RA-FLSs were added to the plates, fixed with 8% (vol/vol) paraformaldehyde, stained with crystal violet, and enumerated by microscopy (100×). Representative images are shown, and quantification reflects 10 high-power fields per experimental condition from three RA-FLS cell lines studied. $*P < 0.05$; $**P < 0.01$ vs. untreated cells; $^{\dagger}P < 0.05$ vs. CD44 siRNA, all by two-tailed Student's *t* test.

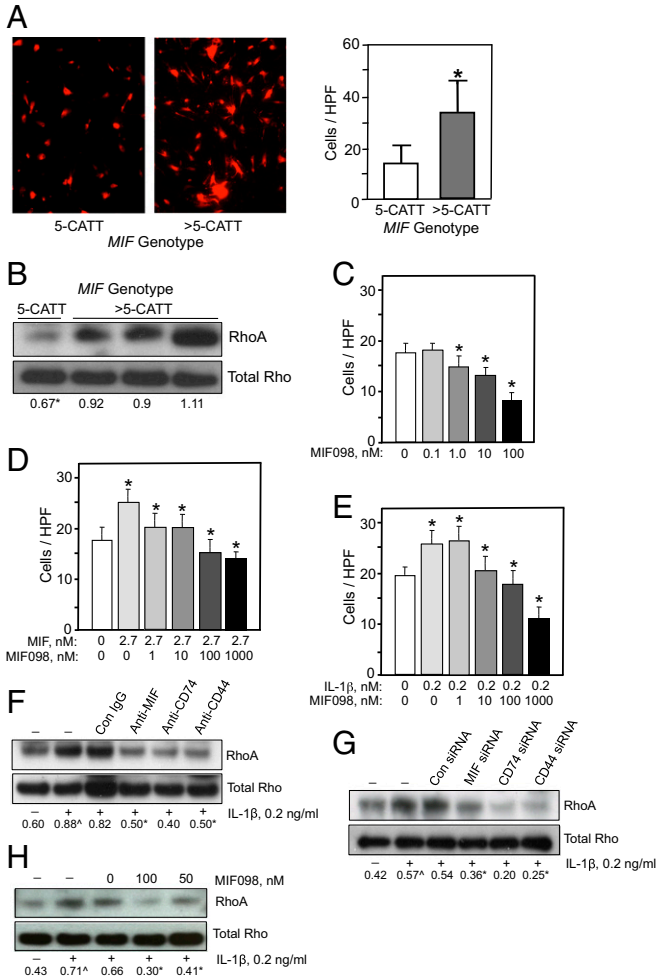

Fig. 7. *MIF* genotype-dependent invasive activity of synovial fibroblasts and inhibition by pharmacologic MIF antagonism. (*A*) Representative images and quantification of membrane permeation by low-genotypic *MIF*-expressing (5-CATT) and high-genotypic *MIF*-expressing (>5-CATT) RA-FLSs. Early passage RA-FLS lines were added to collagen-coated Millicell membranes and quantified 48 h later by fluorescence staining of lower membrane surfaces. Data are summed from triplicate measurements of three individual 5-CATT and four individual >5-CATT RA-FLS lines (mean ± SD). $*P < 0.05$ by two-tailed Student's *t* test. (*B*) Increased RhoA GTPase expression in high-genotypic *MIF*-expressing vs. low-genotypic *MIF*-expressing RA-FLSs. RhoA and total Rho content was measured by Western blotting as described in *Materials and Methods*. The 5-CATT line is representative of three RA-FLS lines analyzed, and numerical values denote the densitometric ratio of RhoA to total Rho of three separate Western blotting studies. $*P < 0.05$ for 5-CATT vs. >5-CATT (two-tailed Student's *t* test). (*C*) Dose-dependent inhibition of spontaneous RA-FLS migration and membrane invasion by the small-molecule MIF antagonist MIF098. MIF098 or vehicle control (0 nM) were added for 48 h. Data are triplicate measurements of one representative >5-CATT RA-FLS line (of three lines studied) and are expressed as mean ± SD. $*P < 0.05$ by two-tailed Student's *t* test. (*D* and *E*) Reduction of MIF (*D*) or IL-1β–stimulated RA-FLS migration and membrane invasion by MIF098 (*E*). RA-FLSs were stimulated with MIF (100 ng/mL = 2.7 nM) together with added MIF098 for 48 h. Data are triplicate measurements of one representative >5-CATT RA-FLS line (of three lines studied) and are expressed as mean ± SD. $*P < 0.05$ by two-tailed Student's *t* test. (*F*) Western blot analysis of synovial fibroblast content of RhoA and total Rho after stimulation with IL-1β in the presence of control IgG (Con), anti-MIF, -CD74, or -CD44 for 4 h (each antibody at 100 ng/mL). (*G*) Western blot of activated and total Rho in synovial fibroblasts transfected overnight with control, MIF, CD74, or CD44 siRNAs and stimulated for 4 h with IL-1β. (*H*) Dose-dependent inhibition of stimulated RhoA activation by MIF098. MIF098 or vehicle control were added for 4 h at the time of IL-1β stimulation. For *F*–*H*, the numerical values below the Western blots denote the ratio of densitometric scanning of RhoA to total Rho in three separate Western blotting studies. $*P < 0.05$ vs. Con IgG (*F*), Con siRNA (*G*), or 0 nM MIF098 (*H*); $^P < 0.05$ vs. no IL-1β addition, all by two-tailed Student's *t* test.

expression unifies these observations with the invasive character of rheumatoid synovial fibroblasts and their enrichment for the protumorigenic CD44v3 and CD44v6 isoforms (42, 43).

We also confirmed a direct role for MIF in the adhesiveness of rheumatoid synovial fibroblasts, which is considered important for the formation of invasive pannus (2). CD44 mediates matrix adhesion by forming a cocomplex with $\alpha_v\beta_3$ integrin (47), and MIF was found to up-regulate $\alpha_v\beta_3$ integrin expression and matrix adhesion. Enhanced adhesion, in turn, was dependent on CD44 expression. Increased migration and invasive phenotype also was a feature of high-genotypic *MIF*-expressing synovial fibroblasts, and this effect was associated with enhanced RhoA GTPase activation and required the MIF/CD74/CD44 signaling axis. The pharmacologic MIF antagonist MIF098, which reduces the K_D for MIF interaction with CD74, decreased invasion stimulated by MIF or IL-1β and decreased MIF/CD74/CD44-dependent RhoA activation.

The present studies add to a closer understanding of the cytokine network within the rheumatoid joint. Both TNF and IL-1β induce MIF secretion from preformed cellular stores (19, 61), and we observed the actions of IL-1β on CD44 expression, COX-2 activation, and synovial fibroblast migration/invasion to occur by the intermediation of MIF. Like MIF, IL-1β stimulation also led to CD44 alternative splicing, although IL-1β's regulatory action in this respect was not blocked by anti-MIF. Conceivably, CD44 alternative splicing and the precise profile of expressed isoforms arise from multiple signal transduction pathways to influence cellular phenotype (27).

In summary, these studies identify the MIF coreceptor CD44 to be expressed by rheumatoid synovial fibroblasts in a *MIF* allele-dependent fashion and to undergo functional regulation and activation by autocrine/paracrine MIF. MIF increases cellular CD44 expression, promotes its recruitment into a CD74/CD44 signal transduction complex, and induces alternative splicing to produce the tumor-related CD44v3/v6 isoforms. Genotypic high-*MIF*-expressing synovial fibroblasts thus show an enhanced proinflammatory and invasive phenotype that is mediated by CD44, which may explain, in part, different clinical courses and treatment responses among RA patients. Pharmacologic MIF antagonism, particularly in high-genotypic *MIF* expressers, could have a specific ameliorative effect in rheumatoid joint pathology by targeting CD44 and its pathologic actions on synovial fibroblasts.

Materials and Methods

Immunohistochemical Staining. The 5-μm sections of paraffin-embedded synovial tissue specimens were obtained at arthroplasty from four RA and three OA patients that met the classification criteria endorsed by the American College of Rheumatology/European League Against Rheumatism (62). Specimens were treated with pepsin for 30 min and blocked with 1% BSA for 30 min at room temperature. Tissue sections then were stained with monoclonal anti-CD74 (LN-2; Santa Cruz Biotechnology; sc-6262), monoclonal anti-CD44 (BD Biosciences; C-26), or an isotype control antibody in a humidified chamber at 4 °C. Slides were washed three times in Tris-buffered saline and incubated with biotinylated anti-mouse IgG (Vector Laboratories) for 30 min at room temperature. CD74- or CD44-positive cells were detected by using peroxidase-conjugated streptavidin (Vector Laboratories) followed by 3′3-diaminobenzidine tetrahydrochloride (Vector Laboratories). The sections were counterstained with hematoxylin.

Isolation and Culture of FLSs, Endothelial Cells, and MEFs. FLSs were obtained from the synovial tissues of RA or OA patients as described (63) and cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (vol/vol) FBS (Gibco BRL). All experimental studies were restricted to early passage cells (passage number ≤ 3). Genotyping for the functional *MIF* promoter microsatellite was performed as described (64). Human endothelial vascular

endothelial cells were isolated from normal-term umbilical cord veins and maintained in M199 medium containing 20% (vol/vol) FBS as described (65). Mouse fibroblasts were isolated from the embryos of wild-type, *Mif*$^{-/-}$, and *Cd74*$^{-/-}$ mice (all in the C57BL/6 background) and cultured in DMEM supplemented with 10% (vol/vol) FBS (18, 22).

Flow Cytometry, Western Blotting, and ELISA Analyses. Cultured cells (typically 4–5 × 10^5) were harvested by brief incubation with trypsin/EDTA, washed, and stained with monoclonal anti-CD74 (LN2; Santa Cruz), monoclonal anti-CD44 (HCAM; Thermo Fisher; 156-3c11), monoclonal anti-CD44v3 (3G5; R&D Systems; 3660-CD), monoclonal anti-CD44v6 (2F10; R&D Systems), and monoclonal anti-$\alpha_v\beta_3$ antibodies (LM609; Chemicon) for 30 min at room temperature followed by washing twice in PBS containing 1% FBS and incubated with Alexa 488-conjugated goat anti-mouse IgG (Invitrogen). Fluorescence was measured by a FACScan cytometer (Becton Dickinson).

Cytokine stimulation studies used individual synovial fibroblast lines cultured in DMEM, 1% FBS, and antibiotic/antimycotic supplement (Gibco) for 24 h in the absence or presence of added cytokine. Human MIF (native sequence) was produced recombinantly as described (final endotoxin content <0.04 U/μg) (66). Human TNF and IL-1β were obtained from R&D Systems. For Western blot analysis, electrophoresis was performed by using SDS/PAGE, and blots were transferred to nitrocellulose membranes. Membranes were incubated with antibodies directed against CD44, CD44v3, CD44v6, pERK1/2 (Cell Signaling; 4377S), total ERK1/2 (Santa Cruz Biotechnology; sc-94), COX-2 (Santa Cruz; sc-1745), GAPDH (Santa Cruz; sc-365062), and β-actin (Sigma-Aldrich). Protein visualization was performed by the enhanced chemiluminescent technique (Thermo Fisher).

RNA and protein expression levels in FLSs were determined by reverse-transcription PCR and Western blot analysis, respectively. For PCR assay, the following specific primers were used: CD44: 5′-CAGACCTGCCCAATGCCTTT-GATGGACC-3′, CD44 sense; 5′-CAAAGCCAAGGCCAAGAGGGATGCC-3′, CD44 antisense. PCR amplification was conducted over 25 cycles of denaturation at 94 °C for 30 s, annealing at 62 °C for 60 s, and elongation at 72 °C for 30 s. GAPDH or β-actin mRNA expression was used as an internal control with the following primers: 5′-AAAGGGCCCTGACAACTCTTT-3′, GAPDH sense, and 5′-GGTGGTCCAGGGGTCTTACT-3′, GAPDH antisense; and 5′-CCTGTACGC-CAACACAGTGC-3′, β-actin sense, and 5′-ATACTCCTGCTTGCTGATCC-3′, β-actin antisense (35).

For the down-regulation of MIF, CD74, or CD44 transcripts, FLSs were transfected by using Lipofectamine RNAiMAX (Life Technologies) with the corresponding siRNAs: control (D-001210), hMIF (M-011335), hCD74 (M-001210-03-05) (all from Dharmacon), and hCD44 siRNA (AM16708) (Ambion). The transfected cells were cultured in FBS-free DMEM for 4 h and stimulated with or without IL-1 β (201-LB; R&D) for 2 h. Rho activation was measured as described by Swant et al. (50) by pulldown of cell lysates using Rho-binding domain of rhotekin agarose beads and Western blot detection with anti-RhoA antibody (Santa Cruz; sc-418). Immunoneutralization studies used the following monoclonal antibodies: anti-MIF (IIID.9) (67), anti-CD74 (LN-2; Santa Cruz; sc-6262), anti-CD44 (HCAM; Thermo Fisher; 156-3c11), or an isotypic control IgG_1 (HB49) for 4 h. Supernatant PGE_2 content was measured by ELISA (R&D Systems).

Synovial Tissue Expression Analysis. A previously collected human synovial tissue registry was accessed for comparative mRNA expression analysis and comprised samples obtained in accord with a Yale University Institutional Review Board-approved protocol for subjects undergoing arthroplasty and/or synovectomy and fulfilling American College of Rheumatology/European League Against Rheumatism criteria (68). Synovial samples were processed for expression analysis on GeneChip Human Genome U133 Plus 2 Affymetrix arrays, and the accessed data are in the Gene Expression Omnibus database (accession no. GSE48780).

Real-Time PCR for CD44v3 and CD44v6 Isoforms. Total RNAs were extracted by using the RNeasy mini kit (Qiagen) according to the manufacturer's instructions. Each of the total RNAs was reverse-transcribed to cDNA by using the iScript cDNA synthesis kit (Bio-Rad), and real-time PCR was carried out by using the iQ SYBR Green Supermix (Bio-Rad) according to the manufacturer's protocol. The expression level of each target was normalized to the endogenous reference gene β-actin, calculated as $2^{-\Delta Ct} \times 100$ where $\Delta C_t = C_{t\ (sample\ mRNA)} - C_{t\ (\beta\text{-}acitn\ mRNA)}$. The specific primers were designed based on cDNA sequences in GenBank. The following primers were used: 5′-ACGTCT TCAAATACCATCTC-3′, CD44v3 sense, and 5′-CCAAGATGATCAGCCATTCTGG-3′, CD44v3 antisense; and 5′-CAG GCAACTCC-TAGTAGTAC-3′, CD44v6 sense, and 5′-CCAAGATGATCAGCCATTCT-GG-3′, CD44v6 antisense. Quantitative PCR for the splicing factors Tra2α and Tra2β used the following primers: 5′-GATCTCGTTCCTACAGCCCA-3′, Tra2α sense, and 5′-ATTAACTGCCACCTTCCACC-3′, Tra2α antisense; and 5′-GGCGGTCACCTTCTCC-TTAC-3′, Tra2β sense, and 5′-CACTGTCATCAGAGCAAGCC-3′, Tra2β antisense. Gene expression of each target was normalized to the reference gene GAPDH.

Fluorescence-Resonance Transfer Studies. The expression plasmid encoding the type II transmembrane protein CD74 fused to YFP (YFP-CD74) has been described (69). The YFP-CD74ΔCS plasmid, which lacks the requisite codon (Ser-202) for chondroitin sulfate (CS) modification, was engineered by site-directed mutagenesis of YFP-CD74 by using the following primers: 5′-CTGGAGGACCC-GACT-GGGCTGGGT-3′ and 5′-ACCCAGCCCAGTCGGGTCCTCAC-G-3′. The different CD44-CFP–expressing plasmids were constructed as follows. The CD44-CFP plasmid was cloned by inserting a CD44 cDNA amplified from the pTracer-CD44 plasmid (23), which encodes the 361 aa standard (hematopoietic) form of CD44, into pAmCyan1-N1, by using the following primers: 5′-TCCGCTC-GAGCGATGCACAGGAGGAGA-3′ and 5′-AGCGAATTCCGTCACATGG-GGACT-GGGCC-3′. The CD44 intracellular (cytoplasmic) domain-truncated CFP fusion plasmid (CD44Δ67-CFP) was similarly constructed from the corresponding pTracer-CD44Δ67 plasmid (23). A functionally expressed IL-17R–CFP plasmid was kindly provided by Sarah Gaffen, University of Pittsburgh, Pittsburgh, for use as a negative control (70).

Transfection of COS-7/M6 cells, which express neither CD74 nor CD44 (23), was performed by using Lipofectamine 2000 (Invitrogen). Transfected cultures were rendered quiescent by culture in OPTI-MEM (Gibco) for 16 h before stimulation with recombinant MIF (100 ng/mL). The small molecule MIF antagonist: 3-(3-hydroxybenzyl)-5-methylbenzooxazol-2-one (MIF098) (40) and MIF agonist: 4-(4-(pyridin-3-yl)-1H-1,2,3-triazol-1-yl) phenol (MIF20) (41) were dissolved in 10% (vol/vol) DMSO stock solutions before addition to transfected cell cultures for FRET and signal transduction analysis. Vehicle controls used 10% (vol/vol) DMSO at identical dilution (<0.1% final).

FRET analysis was conducted as described (71). Briefly, FRET was determined by the acceptor photobleaching technique: photobleaching of the acceptor (YFP) yields a significant increase in donor (CFP) fluorescence. Images were acquired by using a Leica TCS SP2 confocal microscope equipped with a 63× oil immersion objective (NA = 1.4), and 458- and 514-nm laser lines were used to excite the CFP and YFP fluorophores, respectively. After acquisition of prebleached CFP and YFP images, YFP in the selected regions of interest was bleached by at least 70–85% using repetitive scanning with a 514-nm laser operated at full power, and post-bleach CFP and YFP images were acquired. FRET efficiency was calculated from the acquired images by using a FRETcalc plugin for ImageJ software. FRET calculation between CFP and YFP of each transfectant was determined as the mean value of >20 cells having both CFP and YFP signals, which was randomly selected.

The impact of the small-molecule MIF modulators MIF098 (40, 60) and MIF020 (41, 42) on CD74/CD44 ERK1/2 phosphorylation was assessed in COS7-M6 cells transfected with YFP-CD74 and CD44-CFP that were prepared 16 h previously and cultured in DMEM for 4 h. Compounds were added for 2 h before Western blot analysis.

Cell Adhesion and Invasion Assays. For adhesion, RA-FLSs (3 × 10^4) were added into 24-well plates and incubated for 3 h at 37 °C in a 5% (vol/vol) CO_2 humidified incubation. Nonadherent cells were removed by gently washing the wells two times with PBS. Adherent cells were fixed with 4% (wt/vol) paraformaldehyde for 15 min at room temperature, followed by rinsing with PBS and staining with 0.4% crystal violet. After extensive rinsing, the dye was released from the cells by the addition of 33% acetic acid, and the plates were read in a microplate reader (Molecular Devices) at 405 nm. Digital imaging of crystal violet-labeled cells, which appear violet color under a light microscope, was used to quantify attached cells.

Invasion assays were performed in 24-well plates and collagen-precoated Millicell inserts (Millicell-PCF, 8 μm; EMD Millipore). Early passage RA-FLSs were trypsinized, washed, and resuspended in DMEM/0.1% FBS at a concentration of 1 ×10^5 cells per mL before adding of 0.3 mL of the cell suspension to the Millicell inserts. The plates then were placed under 5% (vol/vol) CO_2 humidified incubation at 37 °C for 48 h after adding 0.6 mL of growth medium (DMEM/1% FBS) into the wells together with MIF, MIF098, MIF020, or vehicle control. Inserts then were removed and washed, and the cells on the lower membrane surface were stained for 20 min with CyQuant GR dye 1:75 (Chemicon; catalog no. ECM520) for 20 min. Membranes were examined by fluorescence microscopy (Zeiss; ebg100; A_{80-520} nm), and digital imaging of labeled cells was used to quantify attached cells.

Statistical Analysis. Data are expressed as the mean ± SD. Comparisons of the numerical data between groups were performed by the paired or unpaired Mann–Whitney *u* test of the Student's *t* test (two-tailed). A P value < 0.05 was considered statistically significant.

Study Approval. The use of discarded surgical specimens from arthroplasties and peripheral blood for monocyte cultivation were approved by the Institutional Review Boards of the Catholic Medical Center (Seoul; no.

CUMC09U034) and Yale University. Informed consent was obtained for the procurement and analysis of these specimens.

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