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RasGRP4 is Aberrantly Expressed in the Fibroblast-like Synoviocytes of Patients with Rheumatoid Arthritis and Controls their Proliferation

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ABSTRACT

Objective. **Ras guanine nucleotide-releasing protein-4 (RasGRP4) is a calcium-regulated guanine nucleotide exchange factor and diacylglycerol/phorbol ester receptor not normally expressed in fibroblasts. While RasGRP4-null mice are resistant to arthritis induced by anti-glucose-6-phosphate isomerase autoantibodies, the human relevance of these data are unknown. We therefore evaluated the importance of RasGRP4 in the pathogenesis of human and rat arthritis.**

Methods. **Synovial tissue from rheumatoid arthritis (RA) and osteoarthritis (OA) patients were evaluated immunohistochemically for the presence of RasGRP4 protein. Fibroblast-like synoviocytes (FLSs) were isolated from the synovial biopsies, and the expression of RasGRP4 was evaluated by a real-time quantitative polymerase chain reaction assay. The proliferation potency of FLSs was evaluated by exposing the cells to a RasGRP4-specific small interfering RNA (siRNA). Finally, the ability of RasGRP4-specific siRNAs to hinder type-II collagen-induced arthritis in rats was evaluated to confirm the importance of the signaling protein in the disease.**

Results. **Unexpectedly, RasGRP4 protein was detected in the synovium's hyperplastic lining where proliferating FLSs preferentially reside. The FLSs isolated from tissues obtained from a subpopulation of RA patients expressed much more RasGRP4 than those from examined OA patients. Moreover, the level of its transcript was correlated with the cell's proliferation rate. The ability of cultured FLSs to divide was diminished when given RasGRP4-specific siRNAs. The intra-articular injection of RasGRP4-specific siRNAs also dampened experimental arthritis in rats.**

Conclusion. **RasGRP4 is aberrantly expressed in FLSs and helps regulate their growth. This intracellular signaling protein is therefore a candidate target for dampening proliferative synovitis and joint destruction.**

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by inflammation of the joints (1). Although disease-modifying anti-rheumatic drugs (DMARDs) including biologics are often used to treat patients with RA, remission by the simplified disease activity index occurs in <50% of the treated patients (1-3). Also problematic are the high costs of the existing therapeutics, as well as the increased susceptibility of the treated patients to opportunistic infections.

Fibroblast-like synoviocytes (FLSs) are key players in the inflammation and destruction of the joints of RA patients (4). Since FLSs increase their expression of matrix metalloproteinases (MMPs) when they encounter tumor necrosis factor-α (TNF-α) (5) and other proinflammatory factors, one of the ways anti-TNF-α therapy is effective in RA is by diminishing the ability of FLSs and other cell types in the arthritic joint to produce the zymogen forms of MMPs and aggrecanases which proteolytically destroy cartilage's extracellular matrix (6). Of all the cells implicated in RA pathogenesis, FLSs are perhaps the least well understood. Indeed, there is no currently approved therapeutic for RA that targets this cell directly. Thus, a better understanding of the role(s) of FLSs in the pathogenesis of RA is needed, especially the factors and mechanisms that control their growth.

Mast cells (MCs) also have been linked with the inflammation and damage to the joints in RA patients (7-9). Experimental arthritis was markedly reduced in MC-deficient $Kit^{W}/Kit^{W\text{-}v}$ and $Kitl^{S}/Kitl^{Sl\text{-}d}$ mice (10, 11) and in transgenic C57BL/6 (B6) mice lacking MC-restricted tryptase•heparin complexes (12, 13). Inflammatory arthritis is dependent on proinflammatory cytokines. In that regard, TNF-α, interleukin (IL)-1β, and IL-6 released from activated MCs, macrophages, and other cell types in the arthritic joint induce nearby FLSs to proliferate and increase their expression of pro-MMPs and pro-aggrecanases which are activated by varied MC proteases.

Ras guanine nucleotide-releasing protein-4 (RasGRP4) is a calcium-regulated guanine nucleotide exchange factor (GEF) and diacylglycerol/phorbol ester receptor (14). It was initially cloned from IL-3-developed mouse bone marrow-derived MCs (mBMMCs), and every examined MC in mice, rats, and humans expressed this intracellular signaling protein (14-16). While peripheral blood monocytes (17) and neutrophils (18) also express RasGRP4, fibroblasts normally do not (14, 16, 19).

Four members of the RasGRP family of signaling proteins exist in mice and humans, and dysregulation of RasGRP expression can have profound consequences *in vivo* and *in vitro.* For example, forced expression of RasGRP1 (20, 21), RasGRP2 (22), RasGRP3 (23), or RasGRP4 (14, 16, 19) in normal fibroblasts led to decreased contact inhibition and increased proliferation when the transfectants were subsequently exposed to low levels of phorbol myristate acetate (PMA). These findings raised the possibility that the presence of a RasGRP in the wrong cell type can cause dysregulation of its growth rate, thereby increasing its susceptibility to transformation. In that regard, the insertion of mouse leukemia viruses into the 5'-flanking regions or introns of the RasGRP1 gene, the RasGRP2 gene, or at a chromosome 7A3-B1 site near where the RasGRP4 gene resides can lead to myeloid leukemia, B-cell lymphomas, or T-cell lymphomas in mice (24, 25). In support of the pro-oncogenic activity of this family of GEFs, many of the human RasGRP4 ESTs in GenBank's database originated from clear cell tumors of the kidney. Moreover, a RasGRP4 cDNA was isolated from a patient with acute myelogenous leukemia (19).

Using a homologous recombination approach, Adachi, Stevens, and their coworkers recently created a RasGRP4-null B6 mouse line (26). The MCs in the tissues of these animals were morphologically normal but functionally defective in terms of their ability to exocytose many of the cell's mediators when activated. The finding that calcium ionophore- or PMA-treated mBMMCs developed from the RasGRP4-null mice contained fewer TNF-α and

IL-1β transcripts than similarly treated WT mBMMCs, raised the possibility that RasGRP4 participates in signaling pathways that result in the generation and/or release of varied pro-inflammatory mediators from MCs and possibly other cell types in the synovium which are essential for joint inflammation and destruction. The RasGRP4$^+$ cells in the mouse's synovium that are essential in K/BxN arthritis induced by anti-glucose-6-phosphate isomerase autoantibodies have not yet been identified. Nevertheless, the finding that experimental arthritis could not be induced in RasGRP4-null B6 mice documented for the first time an essential role for this GEF in the effector phase of IgG/C5a-mediated murine arthritis model (26). In support of these mouse data, we identified defective isoforms of RasGRP4 in the peripheral blood mononuclear cells of a subset of patients with RA (17).

Because MCs play prominent roles in one experimental arthritis model, we assumed that the inability to induce K/BxN arthritis in RasGRP4-null mice was primarily a consequence of dysregulation of the pro-inflammatory MCs in the animal's synovium. Unexpectedly, we now show that the most of the RasGRP4$^+$ cells in the human arthritic joint are cadherin-11$^+$ FLSs rather than MCs, and that the aberrant expression of this GEF in FLSs contributes to their proliferation, and thereby the development of arthritis. RasGRP4 is therefore important in both MC- and FLS-dependent inflammatory arthritis.

PATIENTS AND METHODS

Preparation of synovial tissue and FLSs. Synovial tissue specimens were acquired during synovectomy or joint replacement surgery from 10 patients with RA and 10 patients with OA, after written informed consent. The characteristics and clinical information on these patients are summarized in Table 1. This study was approved by the Human Ethics Committee of the Hokkaido University Hospital, and every RA patient fulfilled the American College of Rheumatology criteria for the classification of RA (27). Tissue was harvested and

collected in phosphate buffered saline (PBS). A portion of the obtained synovial tissue in each instance was evaluated histochemically and immunohistochemically. Depending on the amount of tissue obtained, another portion in each tissue specimen was utilized for generating the FLSs that were investigated in this study.

As previously reported (28), the synovium was removed, minced, and placed in 10 ml Hanks' Balanced Salt Solution containing type-I collagenase (Sigma). After a 2-hour digestion at 37°C, each digest was sequentially passed through a metal mesh and then a nylon mesh with 100-μm pores. The liberated cells were collected by centrifugation, and placed in a 75-cm^2 culture flask containing 15 ml of Iscove's modified Dulbecco's medium (IMDM, Sigma) supplemented with 10% heat-inactivated FBS, 100 IU/ml penicillin, and 100 μg/ml streptomycin (Sigma). The liberated cells were then cultured at 37°C in a humidified atmosphere of 5% CO_2. Passage 3-6 FLSs were subjected to the experimental procedures noted below. Other investigators previously showed their FLSs express heat shock protein-47 (HSP-47)/SERPINH1 (29). As assessed immunohistochemically, >97% of the cells in our FLS cultures expressed this collagen chaperone protein and serine protease inhibitor. In contrast, none of the cells in the cultures expressed the macrophage/monocyte protein CD14. In addition, none of the cells became metachromatic when stained with toluidine blue, as do MCs (data not shown).

Immunohistochemistry of human synovial tissue. The synovial tissues from our arthritis patients were placed in Tissue Fixative (Genostaff), embedded in paraffin, and sectioned at 6 μm. The resulting sections were deparaffinized, washed in ethanol, and rehydrated in PBS. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in PBS. The treated sections were rinsed, incubated for 10 minutes with G-Block (Genostaff), and then incubated overnight in PBS containing anti-human RasGRP4 antibody (Abcam), a polyclonal goat anti-human cadherin-11 antibody (R&D Systems), a monoclonal

mouse anti-human CD14 antibody (Abcam), a mouse monoclonal anti-human CD68 antibody (Dako), or a mouse monoclonal anti-human HSP-47 antibody (Enzo Life Sciences). Normal rabbit IgG (Dako), goat IgG (Santa Cruz Biotech.), mouse IgG2a (R&D Systems), mouse IgG (Santa Cruz Biotech.), or mouse IgG2b (R&D Systems) served as the respective negative controls. After a wash, the secondary biotin-labeled anti-rabbit, anti-goat, or anti-mouse antibody (Dako) was added. Horseradish peroxidase labeled streptavidin (Nichirei) was employed to detect the antigen. Some sections were stained with hematoxylin-eosin or toluidine blue.

Synovial tissues from nine RA and five OA patients were evaluated immunohistochemically for the presence of RasGRP4 protein, and the RasGRP4$^+$ area in the cadherin-11$^+$ lining portion of the joint of each patient was quantified. Patient characteristics are summarized in Table 1. Since the lining area increased in parallel to the amount of FLS hyperplasia, the RasGRP4$^+$ lining area was adjusted to the linear horizontal length (mm^2/mm) of the analyzed lining (29).

Real-time quantitative reverse transcription polymerase chain reaction analyses (qPCR). Total RNA was obtained using TRIzol RNA Reagent (Life Technologies), according to the manufacturer's instructions. The RNA samples were reverse-transcribed using SuperScript VILO (Invitrogen). A standard qPCR approach was then used to monitor the levels of the RasGRP4 transcript in the FLSs from 10 patients with RA and 10 with OA. In these experiments, the level of the RasGRP4 transcript was normalized to that of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) transcript with an Applied Biosystems 7500 Real-Time PCR System and TaqMan MGB primers specific for the transcripts that encode RasGRP4 (primer set Hs01073180_m1) and GAPDH (primer set Hs99999905_m1). Relative quantification was performed using the comparable cycle threshold (CT) method in which ΔCT is the level of the RasGRP4 transcript in the RNA sample relative to that of the

GAPDH transcript. The difference in the expression of the RasGRP4 transcripts among each sample was defined as fold changes in mRNA levels by $2^{-\Delta\Delta CT}$.

Cell proliferation assay, and the effect of TNF-α on the expression of RasGRP4 in cultured human FLSs. A qPCR approach was used to evaluate the ability of TNF-α to regulate the expression of RasGRP4 in cultured FLSs (n = 6). After washing with PBS, 7.5×10^5 FLSs in 15 ml IMDM medium (supplemented with 100 IU/ml penicillin, 100 µg/ml streptomycin, and 10% heat-inactivated FBS) were added. Seventeen hours later, TNF-α (Sigma) was added to the culture medium to achieve 10 or 100 ng/ml concentrations of the cytokine. After a 48-hour incubation at 37°C, the untreated and TNF-α-treated FLSs were lysed following the addition of 750 µl TRIzol.

In replicated cultures, the proliferation of the TNF-α-treated FLSs also was evaluated using the tetrazolium/formazan assay. After washing with PBS, 3×10^3 FLSs in 100 µl IMDM containing 10% heat-inactivated FBS, penicillin, and streptomycin were added to each well of a "Celltiter 96 Proliferation Assay Kit" (Promega). Different concentrations (0, 10 or 100 ng/ml) of human TNF-α (Sigma) were added to the medium. Following a 5-day incubation at 37°C, 100 µl of the dye solution in the assay kit was added to each well and the treated cells were incubated for another 4 hours at 37°C. Each reaction was terminated using the kit's stop solution, and the resulting optical density at 570 nm was measured. These proliferation assays were done in a triplicate, and the experiments were carried out before the FLSs became confluent in the culture dishes.

Small interfering RNA (siRNA) knockdown of RasGRP4 levels in cultured human FLSs. A siRNA approach was used to diminish the levels of the RasGRP4 transcript, and thereby protein, in the FLSs from our RA patients (n = 4). After washing the FLSs with PBS, 7.5×10^5 cells in 15 ml IMDM containing 10% heat-inactivated FBS were added. The next day, the medium was changed to Opti-MEM I medium (Life Technologies), and the cells

were transfected with a solution containing 10 nM siRNA (Applied Biosystems) with 3 nM Lipofectamine RNAiMAX (Life Technologies). For these experiments, the company's Silencer select negative control No. 1 siRNA was used, as were the Silencer-Select Pre-designed siRNAs **s41861** and **s41862** that targeted exons 12 and 10, respectively in the normal, full-length isoform of human RasGRP4 (14). After a 6-hour incubation at 37°C, the medium were changed to siRNA-free IMDM containing 10% heat-inactivated FBS. After a 48-hour incubation at 37°C, the treated FLSs were collected, total RNA was isolated. The levels of the transcripts that encode RasGRP4, Receptor Activator of Nuclear Factor κB Ligand (RANKL, primer set Hs00243522_m1), IL-6 (primer set Hs00985639_m1), vascular endothelial growth factor (VEGF, primer set Hs00900055_m1), MMP-1 (primer set Hs00899658 m1), MMP-3 (primer set Hs00968305_m1) and microsomal PGE synthase-1 (miPGES-1, primer set Hs01115610_m1) in the treated human FLSs were quantified and normalized against that of the house-keeping transcript that encodes GAPDH. TaqMan primers specific for each transcript were used.

Cell proliferation assays also were performed on replicate siRNA-treated FLSs from our RA patients (n = 4). After washing with PBS, 1×10^5 FLSs in 100 μl Opti-MEM I medium was added to each well of a Celltiter 96-well proliferation assay kit. After a 48-h incubation at 37°C, the tetrazolium/formazan proliferation assay was performed.

Importance of RasGRP4 in the collagen-induced arthritis (CIA) experimental model in rats. Using the disease model developed by Earp and coworkers (30), CIA was induced in fifteen 7-week old female Lewis rats by immunizing each animal at the base of the tail with 200 μl of a 1 mg/ml solution of porcine type-II collagen (Chondrex) dissolved in 0.05 M acetic acid and emulsified 1:1 in Incomplete Freund's Adjuvant (Chondrex). Seven days later the animals received a booster injection of the collagen antigen in Incomplete Freund's Adjuvant emulsion. The experimental protocol was approved by the Animal Ethics

Committee at Hokkaido University. Five rats in each experimental group received on day 14 an intra-articular injection into both the right and left ankle joints a 50-μl solution that contained a 10-μM siRNA-atelocollagen (Koken) complex. The five rats in the first group received the negative control siRNA–atelocollagen complex. We used atelocollagen, because siRNA-atelocollagen complexs are resistant to nucleases and are efficiently transduced into cells, thereby allowing long-term gene silencing (31). The five rats in the second group received siRNA **s139320①** which targeted the sixth exon of rat RasGRP4 gene. Finally, the five rats in the third group received siRNA **s139321②** which targeted the twelfth exon of the rat RasGRP4 gene.

On days 0, 7, 14, 17, 21, 24, 28, 31, and 35, the control and RasGRP4-specific siRNA-treated arthritic animals were examined for visual signs of disease, defined as macroscopic evidence of increased paw thickness and ankle diameter. Arthritis was scored from 0 (no erythema and swelling) to 4 (erythema and severe swelling that encompassed the ankle, foot, and digits or ankylosis of the limb) (32). The accuracy of the scoring system was verified by micro-computerized tomography (micro-CT, R_mCT2, Rigaku) coupled with histological analysis of representative arthritic limbs. Radiographic severity of the CIA rats was assessed blindly on day 35. Micro-CT of the ankles was performed on all animals. The treated rats were given a score of 0 (normal joint) to 3 (severe cartilage and bone erosions) for each ankle, based on the extent of soft tissue swelling, joint space narrowing, bone destruction, and periosteal new bone formation (33).

Rats were sacrificed on day 35 for histopathologic and immunohistochemical analyses. The ankles were decalcified, embedded in paraffin, and serial sections were stained with RasGRP4 antibody, hematoxylin-eosin, or toluidine blue for microscopic examination. Inflammation was scored on a 0 (no inflammation) to 3 (severe inflamed joint) scale

depending on the number of inflammatory cells in the synovial cavity (exudate) and synovial tissue (infiltrate). Exudate and inflammatory infiltrate were scored individually. Cartilage destruction was scored on a scale of 0–3, where 0 corresponds to no cartilage loss and 3 corresponds to complete loss of articular cartilage. Loss of bone was scored on a scale of 0 (no damage) to 5 (loss of cartilage and bone) scale (34). Anti-hRasGRP4 antibody was also used to evaluate the effects of siRNAs used in this rat CIA model. The RasGRP4$^+$ lining area was adjusted to the linear horizontal length (mm^2/mm) of the analyzed lining as done in human synovial tissues.

Statistical analysis. For cross-sectional analyses, quantitative variables were compared by Welch's t test. Changes in quantitative variables after siRNA therapy were tested by Kruskal-Wallis test with post-hoc test. Correlation between different numerical variables was analyzed by Pearson's test. *P* value less than 0.05 were considered significant.

RESULTS

Immunohistochemistry of human synovial tissue. As assessed immunohistochemically with anti-RasGRP4 antibody, the toluidine blue$^+$ MCs in the arthritic human synovium contained RasGRP4 protein (Figure 1A), as anticipated. While most of the CD14$^+$/CD68$^+$ mature macrophages in the synovium were not abundant in the RasGRP4-positive area (Figures 1C and D) as also anticipated, the GEF was prominently expressed in the hyperplastic lining areas of the RA synovium (Figures 1B, C, D, and Figure 2), with lower levels in OA synovium (Figure 2). Others reported that FLSs express cadherin-11 (35) and HSP-47 (29). Most of the RasGRP4$^+$ cells in the synovium of our RA patients unexpectedly were cadherin-11$^+$/HSP-47$^+$ FLSs rather than MCs. Moreover, the RasGRP4$^+$ areas were significantly increased in the synovial tissues of our RA patients

compared with those of our OA patients (Figure 2D). The clinical features of our arthritis patients are summarized in Table 1.

Quantitation of the levels of the RasGRP4 transcript in FLSs derived from RA and OA patients. The levels of the RasGRP4 transcripts were quantified in early passages of cultured FLSs from patients with RA or OA using a qPCR approach. The FLSs from some RA patients contained substantial amounts of RasGRP4 mRNA (Figure 3A), thereby supporting the immunohistochemical data of the synovial tissues from these patients. Nucleotide sequence analysis of the primary qPCR product from patient RA3 confirmed that the evaluated transcript in the mRNA assay encoded full-length RasGRP4, rather than its homologous family members RasGRP1, RasGRP2, or RasGRP3. The FLSs from 5 out of 6 RA patients that had been treated with biologics contained lower amounts of RasGRP4 mRNA. In that regard, RA patients 3 and 4 had been given the anti-IL-6 receptor tocilizumab, whereas RA patients 5-8 had been given anti-TNF-α agents (patient 5 and 7 with infliximab, patient 6 with golimumab, and patient 8 with etanercept). The levels of the RasGRP4 transcript were correlated with the rate of proliferation of the FLSs ($R^2 = 0.67$, $P < 0.01$) (Figure 3B).

TNF-α induced RasGRP4 expression in FLS and the steady-state proliferation of FLSs is dependent, in part, on RasGRP4. As previously reported (4, 36, 37), TNF-α-treated FLSs proliferated significantly faster than FLSs that did not encounter this pro-inflammatory cytokine (Figure 3D). Because the levels of the RasGRP4 transcript were significantly higher in TNF-α-treated FLSs (Figure 3C), a siRNA approach was used to determine whether or not these observations were directly linked. The levels of the RasGRP4 transcript in FLSs-treated with one of two different siRNAs specific for human RasGRP4 were significantly decreased compared with the levels of the RasGRP4 transcript in untreated FLSs or cells given a control siRNA (Figure 4A). The FLSs isolated from our RA patients

also decreased their proliferation activity when exposed to the RasGRP4-specific siRNAs (Figure 4B).

FLSs-derived RANKL, IL-6, VEGF-A, MMP-1, MMP-3, and miPGES-1 work in syngery to promote the development of RA (38). Decreased RasGRP4 expression in the RasGRP4-specific siRNA-treated FLSs did not lead to decreased levels of the transcripts that encode RANKL, IL-6, VEGF-A, and MMP-3 (Figure 4 C), suggesting that RasGRP4 is not essential for the expression of those four proteins at the mRNA level in the arthritic joint. In contrast, the levels of the MMP-1 and miPGES-1 transcripts were significantly decreased in those FLSs given a RasGRP4 siRNA (Figure 4C), implying that the latter two proteins were under the control of this signaling protein.

Assessment of CIA in rats. Intra-articular injection of siRNAs specific for rat RasGRP4 significantly improved the clinical arthritis score, as well as the ankle diameter, of the arthritic rats given type-II collagen compared with that of the control group of animals (Figure 5A). Micro-CT revealed that the arthritic rats given a RasGRP4-specific siRNA had significantly less erosion scores relative to replicate animals given a control siRNA (Figure 5 B). Anti-hRasGRP4 antibody recognized rat mast cells as well as proliferative lining area in arthritic synovial tissue, but control IgG did not (data not shown). Thus, this antibody cross-reacted with rat RasGRP4 in immunohistochemistry, although the signal intensities were somewhat weaker when compared with those seen in human tissues. The $RasGRP4^{+}$ area in the synovial lining area was significantly decreased in the relevant tissues of those rats which received the intra-articular injection of the RasGRP4-specific siRNA-atelocollagen complexes, compared with those rats which received the intra-articular injection of control siRNA (Figure 5C). Arthritic rats which received the RasGRP4-specific siRNA had significantly lower exudate, inflammatory cell infiltrate, cartilage destruction, and histological bone-erosion scores in the ankle joints relative to the arthritic animals which

received the control siRNA (Figure 5D). On the other hand, there was no histological difference in the knee joints (data not shown).

DISCUSSION

The FLS is the major cell type in the synovium that invades the joints of patients with RA. This mesenchymal cell plays significant adverse roles in the initiation and perpetuation of destructive joint inflammation. Cadherin-11 is an adhesion protein that participates in the homotypic aggregation of FLSs (35). Izquierdo and coworkers (29) reported that the collagen-specific molecular chaperone HSP-47 is another marker for the FLSs in human synovial tissue. FLS expansion paralleled the activity and temporal progression of RA, and could be partially reversed by anti-TNF-α therapy. Our analysis revealed that many of the cadherin-11^{+}/HSP-47^{+} FLSs in the hyperplastic lining area of the diseased joints of a subset of RA patients unexpectedly contained high levels of RasGRP4 protein. Moreover, the RasGRP4^{+} lining area was significantly increased in the synovial tissues of the RA patients compared with those of OA patients. Although monocytes contain RasGRP4 mRNA, this signaling protein and its transcript decreases in amount when these immature progenitors differentate into mature macrophages or osteoclasts. As expected, very few $CD14^{+}$/$CD68^{+}$ macrophages in the arthritic synovium contained dectable amounts of RasGRP4 protein.

Normal fibroblasts do not express RaGRP4. Indeed, recombiant mouse and human were initially generated in transfected RasGRP$4^{-/-}$ mouse 3T3 fibroblasts to evaluate their ability to activate Ras (39). Fibroblasts are particularly abundant in human skin, and none of the 210,759 human skin-derived ESTs in GeneBank's current EST database originated from the human *RasGRP4* gene (see GeneBank Hs130434). Thus, the finding that the majority of the RasGRP4^{+} cells in synovial tissue from our RA patients were FLSs was unanticipated.

The levels of the RasGRP4 transcript in the FLSs of some of our RA patients were considerably higher than those in every OA patient. The levels of the RasGRP4 transcript in FLSs stimulated with TNF-α also were significantly higher than the levels of untreated control cells, and the rate of proliferation of cultured FLSs was significantly increased when these mesenchymal cells encountered TNF-α. This finding might be one of the reasons why the FLSs from those RA patients that had been previously treated with an TNF-α inhibitor tended to express less RasGRP4.

The abnormally high rate of proliferation of the FLSs at the synovial intimal lining layer is a prominent feature of RA. The correlation between the levels of the RasGRP4 transcript and the proliferation rate of FLSs support the importance of TNF-α and the downstream signaling protein RasGRP4 in unchecked growth of the FLSs in the joints of some patients with RA. The Mitogen-activated Protein Kinase (MAPK) cascade, which is stimulated by engagement of cytokine and Toll-like receptors, is the most extensively studied pathway in FLSs. MAPK signaling involves the activation of three levels of kinases. The top tier includes MAPK kinase kinase. The middle level includes MAPK kinase, and the distal level comprises Extracellular Signal-regulated Kinase (ERK), p38 MAPKs, and the c-Jun N-terminal kinase (JNK) (40). ERK1/2 is also important in MMP-1 (41) and COX-2-dependent PGE_2 generation in FLSs (42). ERK1/2 signaling has been implicated in the chronic aspects of RA (40). Because RasGRP4 is a GEF that activates Ras (14), it therefore was not surprising that the levels of its transcript were correlated with the proliferation potency of the FLSs.

The FLSs from a subset of RA patients decreased their proliferation when exposed to a RasGRP4-specific siRNA. FLSs express RANKL, IL-6, VEGF, MMP-1, MMP-3, and miPGES (37). Although the levels of the transcripts that enclode RANKL, IL-6, VEGF, and MMP-3 were unaltered in the FLSs that had been exposed to the RasGRP4-specific siRNA,

the levels of the MMP-1 and miPGES-1 transcripts were significantly decreased in these cells. MMP-1 is a neutral protease expressed by stromal cells, and it participates in the destruction of cartilage and remodeling of its damaged extracellular matrix (41). miPGES is an enzyme that acts downstream of COX-2; it catalyzes the final step of PGE_2 biosynthesis. Proinflammatory cytokine-activated cells (e.g., synovial cells, chondrocytes, and macrophages) are the primary sources of PGE_2 in the inflamed joints of RA patients. COX-2 has been targeted for the treatment of RA by using non-steroidal anti-inflammatory drugs including selective COX-2 inhibitors (38).

We showed CIA rats that had received an intra-articular injection of a RasGRP4-specific siRNA significantly improved their arthritis and prevented joint destruction, in agreement with the recent finding that RasGRP4-null C57BL/6 mice are resistant to experimental K/BxN arthritis (26). Thus, the importance of RasGRP4 in a second well-established experimental arthritis model has now been shown.

In the present study, we demonstrated that inhibition of RasGRP4 resulted in reduced proliferation of FLSs *in vitro* and in diminished arthritis and joint destruction in a rat disease model. Thus, RasGRP4 is an attractive treatment target in RA patients.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article. Dr. Yasuda had full access to all of the data in the study and takes responsibility.

Study conception and design. Kono, Yasuda, and Stevens.

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Table 1

Characteristics of the RA and OA patients used in this study

	Histological examination		Examination of cultured FLS	
	RA	OA	RA	OA
Gender, female/male	7/2	5/0	8/2	10/0
Age at the operation, years	70 (58-78)	73 (63-80)	66 (33-78)	72 (58-80)
Duration of disease, years	11 (5-48)	NA	10.5 (2-48)	NA
Serum CRP level, mg/dl	0.16 (0.02-5.69)	0.05 (0.02-0.32)	0.12 (0.02-5.69)	0.1 (0.02-0.61)
MMP-3, mg/dl	95.7 (44.8-449.9)	NA	90.5 (31.3-449.9)	NA
RF, U/ml	12 (1.1-82.8)	NA	16.4 (1.1-82.8)	NA
ACPA, U/ml	115 (4.5-300)	NA	115 (4.5-300)	NA
Swollen joint count	3 (1-6)	NA	3 (1-6)	NA
Tender joint count	2 (0-16)	NA	3 (0-16)	NA
DAS28–ESR	4.4 (2.4-5.0)	NA	4.3 (2.4-5.0)	NA
HAQ–DI	1 (0-1.75)	NA	1 (0-1.75)	NA
Methotrexate use, n (%)	5 (55)	0 (0)	5 (50)	0 (0)
Biologics agent use, n (%)	4 (44, TCZ 22, IFX 22)	0 (0)	6 (60, TCZ 20, IFX 20, ETN 10, GLM 10)	0 (0)

NA, not available; CRP, C reactive protein; RF, rheumatoid factor; ACPA, anti–citrullinated peptide Ab; ESR, erythrocyte sedimentation rate; HAQ-DI, health assessment questionnaire disability index; TCZ, tocilizumab; IFX, infliximab; ETN, etanercept; and GLM, golimumab.

FIGURE LEGENDS

Figure 1. RasGRP4 protein is abundant in synovial MCs, but not synovial macrophages. **A**, Serial sections of the human synovium were stained with toluidine blue or anti-RasGRP4 antibody. The red arrowheads highlight toluidine blue$^+$/RasGRP4$^+$ MCs in the synovium of these patients. Original magnification ×400. **B**, **C**, and **D**, Serial sections were stained with control, anti-RasGRP4, anti-CD14, or anti-CD68 antibody. While RasGRP4 protein was abundant in synovial MCs and in an undefined cell type, this GEF was not abundant in CD14$^+$/CD68$^+$ macrophages. **B**, Original magnification ×400. **C** and **D**, Original magnification ×600.

Figure 2. The FLSs in the hyperplastic lining area of the synovium of a subset of RA patients contain appreciable amounts of RasGRP4 protein. **A-C**, Serial sections from different RA (**A** and **C**) and OA (**B** and **C**) patients were stained with anti-RasGRP4 (**A**, **B**, and **C**), anti-cadherin-11 (**A** and **B**), or anti-HSP-47 (**C**) antibody. The caderin-11$^+$/HSP-47$^+$ FLSs in hyperplastic lining areas of the synovium of a number of RA patients often contained immunoreactive RasGRP4 protein. **D**, The cadherin-11$^+$ lining area of the synovium of our RA patients (n = 9) contained more RasGRP4 protein than the corresponding area of our OA patients (n = 5). Data are expressed as the mean ± SD. $^{**}P < 0.01$ versus OA by Welch's t test. ×400.

Figure 3. Evaluation of RasGRP4 mRNA levels in FLSs and proliferation of these cells. **A**, The levels of the RasGRP4 transcript were high in the FLSs from RA patients 1, 2, 3, and 10. RA patients 3 and 4 had been given tocilizumab (TCZ), whereas RA patient 5 and 7 infliximab (IFX), patient 6 golimumab (GLM), and patient 8 etanercept (ETN). Bio; biologics. **B**, The presence of the RasGRP4 transcript was correlated with the rate of proliferation of these FLSs. The black diamonds (♦) and white circles (○) in **B** correspond to the FLSs from RA (n = 6) and OA (n = 4) patients, respectively. **C**, Evaluation of RasGRP4 mRNA levels in FLSs (n = 6) stimulated with TNF-α. **D**, Proliferation of FLSs (n = 6) stimulated with TNF-α by Welch's t test. Data are expressed as the mean ± SEM (**A**) or SD (**C** and **D**). P: passage, RQ: relative quantification, and R: correlation coefficient. $^{**}P < 0.01$ versus Control (C) or MOCK (D).

Figure 4. The effect of knockdown of RasGRP4 levels in FLSs from RA patients (n = 4). **A-B**, The effects of siRNAs on RasGRP4 mRNA levels in the FLSs from RA patients (**A**) and their proliferation rates (**B**) were evaluated. **C**, The levels of the transcirpts that encode RANKL, IL-6, VEGF, MMP-1, MMP-3, and miPGES-1 were measured in FLSs that were treated with a control siRNA or one of two siRNA specific for human RasGRP4. Data are expressed as the mean ± SD. $^{*}P < 0.05$; $^{**}P < 0.01$ versus MOCK by Welch's t test.

Figure 5. Intra-articular injection of a RasGRP4-specific siRNA hinders the development of CIA in rats. **A**, CIA was induced in rats, and the arthritis scores and ankle diameters of the resulting animals were monitored for 35 days. One group (n = 10 legs) of animals received the control siRNA (■). The second (n = 10 legs) and third (n = 10 legs) groups received the RasGRP4-specific siRNAs s139320/siRNA① (39320/ss139321/siRNA② (●), respectively.

B, Micro-CT of representative instances in which arthtitic rats the control siRNA, siRNA①, or siRNA②. The erosion scores of each group of animals was quantitated. Shown are the mean data ± SEM of 10 legs per group. **C**, Hematoxylin-eosin staining and immunohistochemistry using anti-hRasGRP4 antibodies: representative instances of arthritic rats that received the control siRNA, siRNA①, and siRNA② are shown. RasGRP4 positive area/horizontal length of the analyzed lining was compared among these three groups. **D**, Exudate scores, inflammatory cell infiltrate scores, cartilage destruction and bone erosion scores of arthritic rats treated with control siRNA or RasGRP4 siRNAs. Data were expressed as the mean ± SEM. $*P < 0.05$; $**P < 0.01$ versus siRNA Control. Ti, tibia; Ta, talus.

Figure 1.

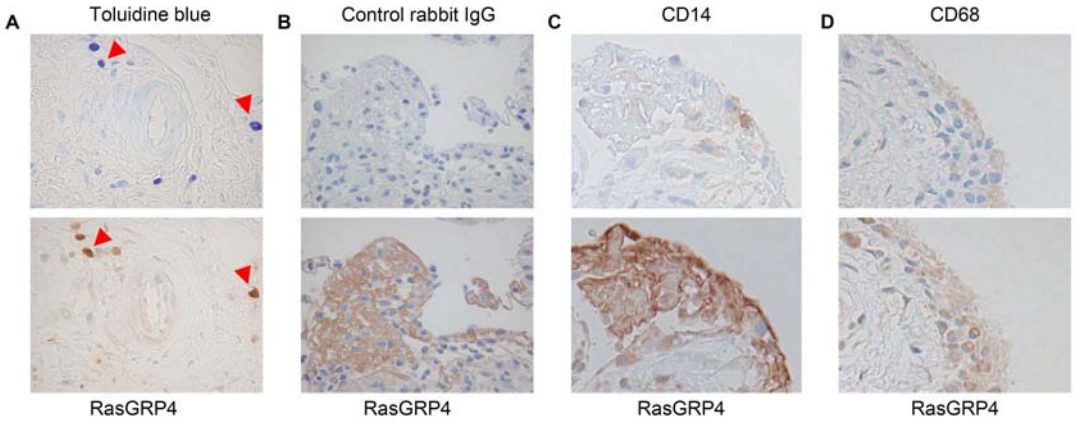

A
Toluidine blue
B
Control rabbit IgG
C
CD14
D
CD68
RasGRP4
RasGRP4
RasGRP4
RasGRP4

Figure 2.

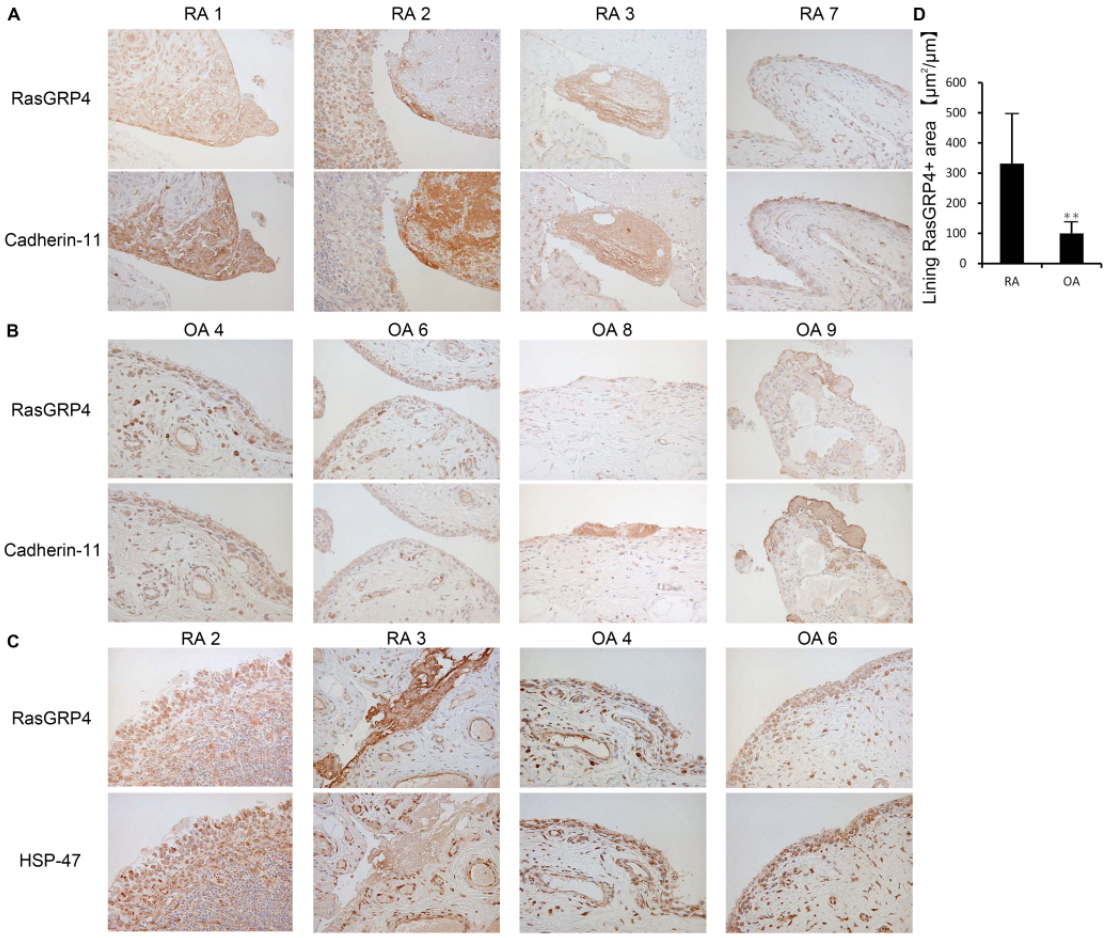

A
RA 1
RA 2
RA 3
RA 7
RasGRP4
Cadherin-11
B
OA 4
OA 6
OA 8
OA 9
RasGRP4
Cadherin-11
C
RA 2
RA 3
OA 4
OA 6
RasGRP4
HSP-47
D
Lining RasGRP4+ area 【μm²/μm】
600
500
400
300
200
100
0
**
RA
OA

Figure 3.

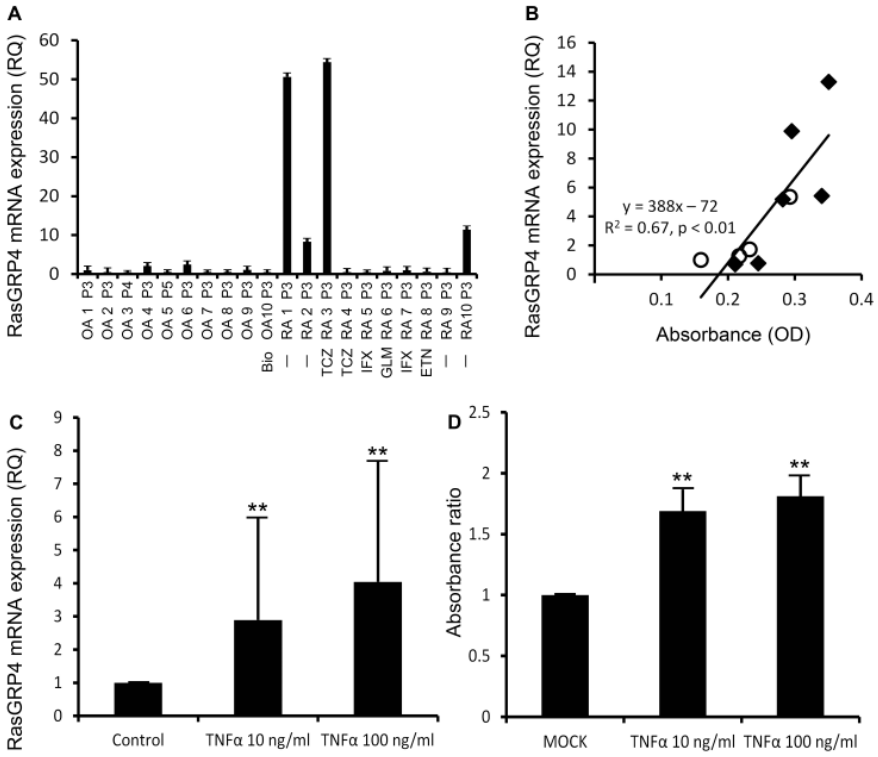

A
RasGRP4 mRNA expression (RQ)
OA 1 P3
OA 2 P3
OA 3 P4
OA 4 P3
OA 5 P5
OA 6 P3
OA 7 P3
OA 8 P3
OA 9 P3
OA10 P3
RA 1 P3
RA 2 P3
RA 3 P3
RA 4 P3
RA 5 P3
RA 6 P3
RA 7 P3
RA 8 P3
RA 9 P3
RA10 P3
Bio
TCZ
TCZ
IFX
GLM
IFX
ETN
B
RasGRP4 mRNA expression (RQ)
y = 388x – 72
R² = 0.67, p < 0.01
Absorbance (OD)
C
RasGRP4 mRNA expression (RQ)
Control
TNFα 10 ng/ml
TNFα 100 ng/ml
D
Absorbance ratio
MOCK
TNFα 10 ng/ml
TNFα 100 ng/ml

Figure 4.

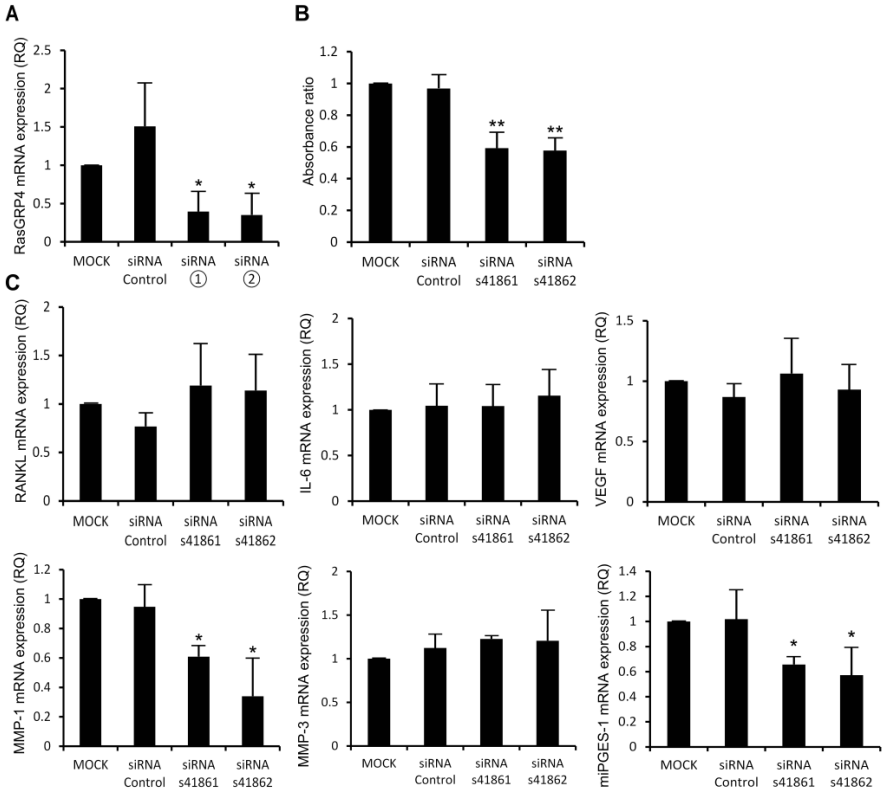
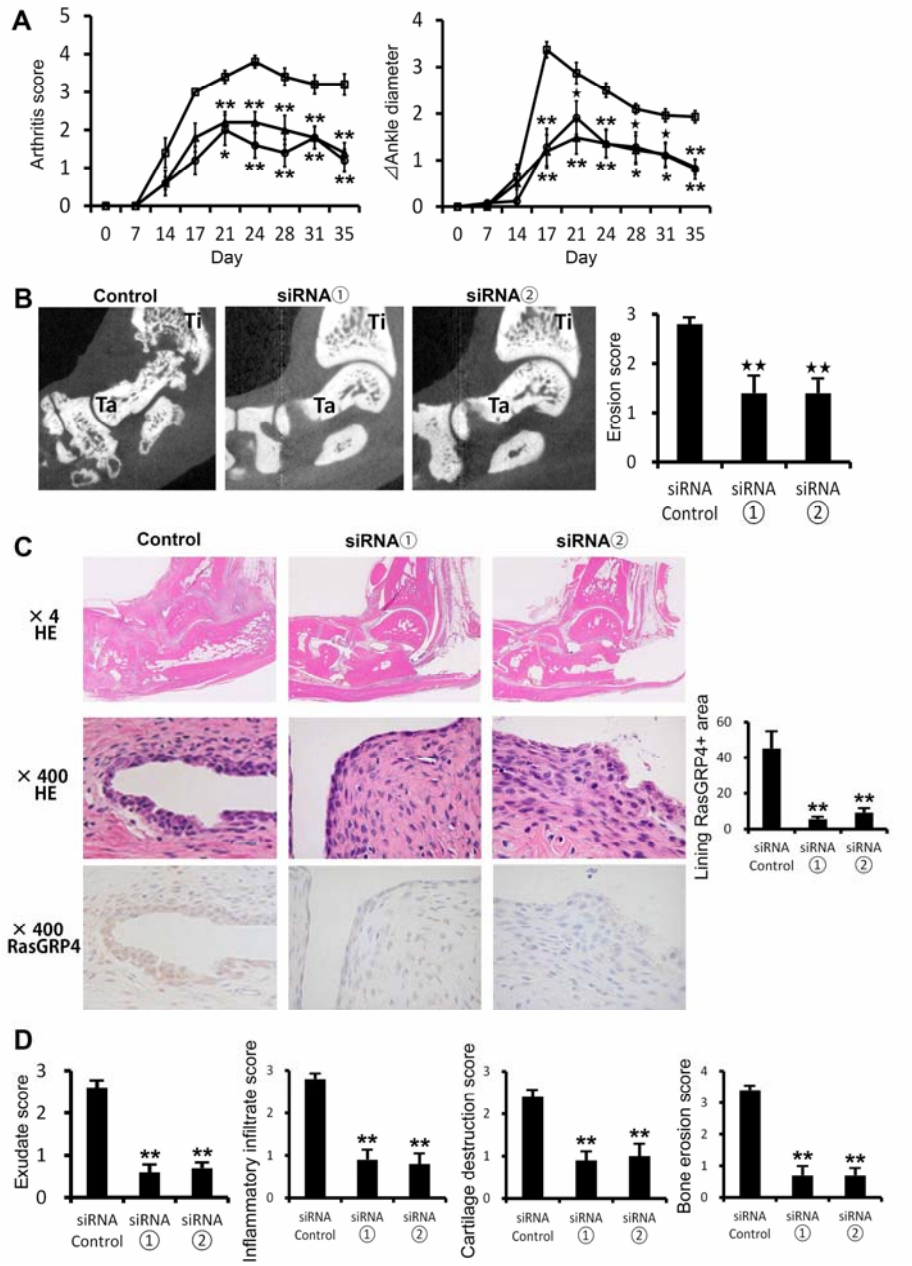

A
RasGRP4 mRNA expression (RQ)
MOCK
siRNA Control
siRNA ①
siRNA ②
B
Absorbance ratio
MOCK
siRNA Control
siRNA s41861
siRNA s41862
C
RANKL mRNA expression (RQ)
IL-6 mRNA expression (RQ)
VEGF mRNA expression (RQ)
MMP-1 mRNA expression (RQ)
MMP-3 mRNA expression (RQ)
miPGES-1 mRNA expression (RQ)

Figure 5.


A
Arthritis score
Day
⊿Ankle diameter
Day
B
Control
siRNA①
siRNA②
Ti
Ta
Erosion score
siRNA Control
siRNA ①
siRNA ②
C
Control
siRNA①
siRNA②
× 4 HE
× 400 HE
× 400 RasGRP4
Lining RasGRP4+ area
D
Exudate score
Inflammatory infiltrate score
Cartilage destruction score
Bone erosion score