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ORIGINAL ARTICLE

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Transforming growth factor β stimulates rheumatoid synovial fibroblasts via the type II receptor

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Abstract Transforming growth factor (TGF)-β regulates the function of fibroblasts, and has been shown to have a role in the pathogenesis of rheumatoid arthritis (RA) because several studies have demonstrated the presence of TGF-β in the synovial tissue and synovial fluids of RA patients. In this study, we examined the expression of TGF-β receptors in synovial fibroblasts of patients with RA and demonstrated the significance in functional responses of synovial fibroblasts to TGF-β in this disorder. Transforming growth factor β1 stimulated the expression of connective tissue growth factor (CTGF) in fibroblasts of patients with RA more than in those of patients with osteoarthritis (OA). Transforming growth factor β1 induced the chemotactic migration of RA synovial fibroblasts and inhibited their proliferation significantly more than OA synovial fibroblasts. Both RA and OA synovial fibroblasts expressed detectable amounts of TGF-β receptor type II mRNA, but the expression was higher in RA patients than in OA patients, as assessed by reverse transcriptase–polymerase chain reaction. There was no significant difference in the expression of TGF-β receptor type I or type III in synovial fibroblasts between RA and OA patients. These results indicate that synovial fibroblasts of RA patients express the increased TGF-β receptor type II, which is associated with altered responses to TGF-β observed in CTGF expression, chemotaxis, and proliferation of RA synovial fibroblasts, and may have an important role in the pathogenesis of RA.

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Introduction

Transforming growth factor (TGF)-β is a large family of multifunctional cytokines that regulates proliferation, differentiation, adhesion, and migration of various cell types including fibroblasts, and favors the production of extracellular matrix proteins.[1] In human tissues, three isoforms, TGF-β1, -β2, and -β3, have been characterized.[2] On the other hand, three classes of cell surface TGF-β binding proteins are known as TGF-β receptors (R)I, -βRII and –βRIII.[3,4] Both TGF-βRI and -βRII contain an intracellular serine/threonine kinase domain that transduces the intracellular signals, while the type III receptor does not have an intrinsic signaling function.[5] Transforming growth factor βRII binds active TGF-β with high affinity, and TGF-βRI is subsequently recruited and has the potential to modulate TGF-β signaling. Transforming growth factor βRIII has a role for stabilizing the interaction between TGF-β and TGF-βRII, and enhances TGF-β signaling through interaction with the cytoplasmic domain of TGF-βRII. Endoglin, an accessory molecule for TGF-β receptor binding, is a member of the TGF-β receptor III family.[6]

Rheumatoid arthritis (RA) is a chronic inflammatory disease that primarily affects synovial tissues. Joint parenchymal lesions in patients with RA are characterized by synovial hyperplasia with an increased number of synovial fibroblasts and a cellular infiltrate comprised predominantly of macrophages and lymphocytes.[7] Of these cells, synovial fibroblasts are believed to have critical roles in RA because they aggressively proliferate to form a pannus which results in destruction of articular bone and cartilage. Transforming growth factor β, which can regulate the function of synovial fibroblasts, has been shown to be expressed at cartilage-pannus junction, in the lining layer, in the endothelial cells, and in the fibroblastic areas in the synovial tissue of RA

joints.[8–10] The cell source of TGF-β is likely to be synovial macrophages and fibroblasts.[10,11] Thus, several studies have shown the overexpression of TGF-β in RA joints, but little is known about the expression of TGF-β receptors.

In this study, we examined the expression of TGF-β receptors in synovial fibroblasts and demonstrated their significance in functional capacities of synovial fibroblasts to TGF-β in RA.

Materials and methods

Isolation and culture of synovial fibroblasts

Human synovial fibroblasts were obtained by enzymatic digestion of synovial tissue of patients with RA and osteoarthritis (OA) undergoing total joint replacement as described previously.[7] Briefly, the synovial tissues were minced and incubated with 2.5% trypsin in phosphate-buffered saline (PBS) for 1 h at 37°C under continuous agitation. The cells were pelleted, resuspended, and cultured in Dulbecco's modified Eagle's medium (DMEM; Nissui Pharmaceutical, Tokyo, Japan) containing 10% fetal bovine serum (FBS; GIBCO, Grand Island, NY, USA). Cells were passaged at 90% confluence and harvested after 4–9 passages. Most cells were considered to be synovial fibroblasts because they were negative for the expression of CD1, CD3, CD14, CD19, and HLA-DR by flow cytometric analysis using fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody (data not shown).

Isolation of messenger RNA (mRNA) and reverse transcriptase–polymerase chain reaction (RT-PCR)

Total RNA was extracted from cultured synovial fibroblasts using Isogen (Nippon Gene, Tokyo, Japan).[7] First-strand cDNA was synthesized from 1 μg of total RNA using a First Strand Synthesis Kit (Pharmacia, Piscataway, NJ, USA) according to the manufacturer's instructions. The synthesized first-strand cDNA (1 μl) was amplified by polymerase chain reaction (PCR) in a final volume of 50 μl. The specific primer pairs used for PCR amplification were 5′-CGTGC TGACATCTATGCAAT-3′ and 5′-AGCTGCTCCATTG GCATAC-3′ for TGF-βRI, 5′-TCCACCTGTGACAAC CCAGAAA-3′ and 5′-TATGACTAGCAACAAGTCA GG-3′ for TGF-βRII, 5′-GGTGTTCCTGGTCCTCGTTT-3′ and 5′-CAAAGGAGGTGACAATGCTGG-3′ for TGF-βRIII, 5′-ACGGCGAGGTCATGAAGAAGAA CA-3′ and 5′-TGGGGCTACAGGCAGGTCAGTG-3′ for CTGF, and 5′-AAGAGAGGCATCCTCACCCT-3′ and 5′-TACATGGCTGGGGTGTTGAA-3′ for β-actin. Polymerase chain reaction amplification of the cDNA was performed under the following conditions: TGF-βRI, -βRII and -βRIII: for 30 s at 94°C, for 2 min at 55°–59°C, and for 1 min at 72°C; β-actin: for 30 s at 94°C, for 30 s at 52°C, and for 30 s at 72°C. To quantitate expression levels of the transcripts, PCRs were conducted in the linear exponential phase of amplification throughout 25–35 cycles and samples loading was monitored by a β-actin transcript that was subjected to the same treatment. Polymerase chain reaction products were electrophoresed in 1.5% agarose gel, and visualized and photographed after ethidium bromide staining.

Chemotaxis assay

Chemotactic migration of synovial fibroblasts was assessed by 48-well microchemotaxis chamber as described previously.[12] The number of migrated cells in the three high-power fields (400 × magnification) was counted by light microscopy after coding the samples. The migration was expressed as chemotaxis index (CI) calculated as follows: CI = migration to stimuli/migration to medium.

Cell proliferation assay

Fibroblast growth-stimulating and -inhibitory activities were determined by methyl thiazol tetrazolium (MTT) assay as previously described.[13] Briefly, synovial fibroblasts were seeded at a density of 7.5×10^3 cells/well in 100 μl in DMEM containing 10% FBS with various concentrations of TGF-β1 in 96-well plates, and incubated for 72 h at 37°C in humidified air with 5% CO_2. After the end of incubation, 10 μl of stock MTT solution (5 mg/ml) was added to each well of the plate, and the plates incubated for 4 h at 37°C in a humidified atmosphere of 5% CO_2 in air. Acid–isopropanol (100 μl of 0.04 NHCl in isopropanol) was added to all wells and mixed thoroughly to dissolve the dark-blue crystals. After a few minutes at room temperature to ensure that all crystals were dissolved, the plates were measured with an MTP-32 Microplate Photometer (Corona Electric, Tokyo, Japan), using a test wavelength of 550 nm and a reference wavelength of 630 nm.

Statistical analysis

All results are expressed as the mean ± SEM. Statistical analysis was performed using Student's unpaired two-tailed *t*-test for comparisons between two groups. Differences were considered significant if P values were less than or equal to 0.05. Data were analyzed using StatView software.

Results

Expression of TGF-β receptors in synovial fibroblasts

Fibroblasts principally express TGF-βRI, -βRII, and -βRIII.[1] Therefore, to determine the expression of TGF-β receptors in fibroblasts of RA synovial lesions, the levels of TGF-βRI, -βRII, and -βRIII gene transcripts in cultured synovial fibroblast samples obtained from three RA and three OA patients were examined by RT-PCR. Since endoglin is a member of TGF-βRIII, the expression of endoglin was considered as that of TGF-βRIII. The inten-

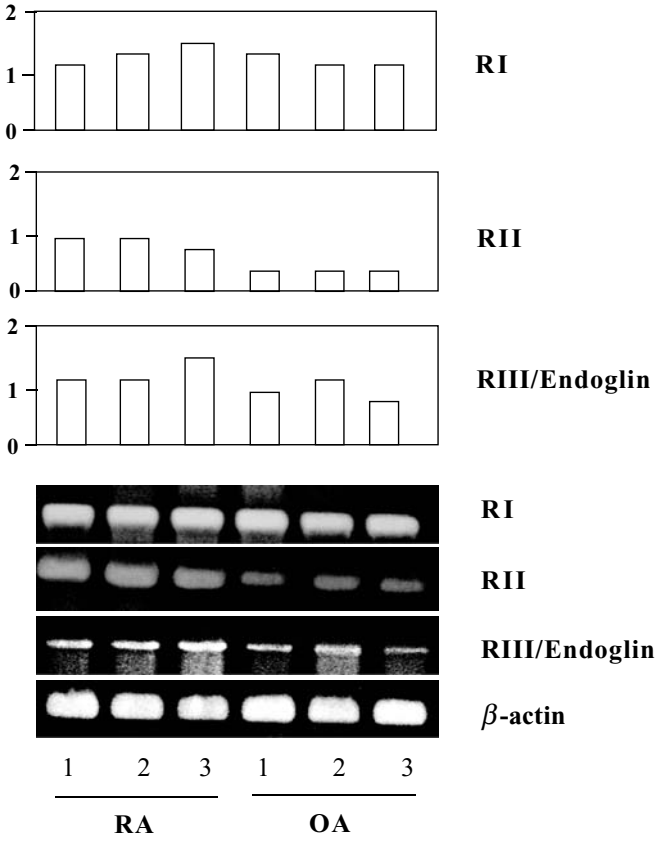

Fig. 1. Expression of transforming growth factor-β (TGF-β) receptor I (*RI*), receptor II (*RII*), and receptor III (*RIII*) mRNA in synovial fibroblasts from three rheumatoid arthritis (*RA*) patients and three osteoarthritis (*OA*) patients by reverse transcription–polymerase chain reaction (RT-PCR). Polymerase chain reaction products representing TGF-β receptors and β-actin were stained with ethidium bromide. The β-actin level was used as a standard for each sample. Data in the *upper panels* show the relative intensity of bands of TGF-β receptors to β-actin using an NIH imaging program

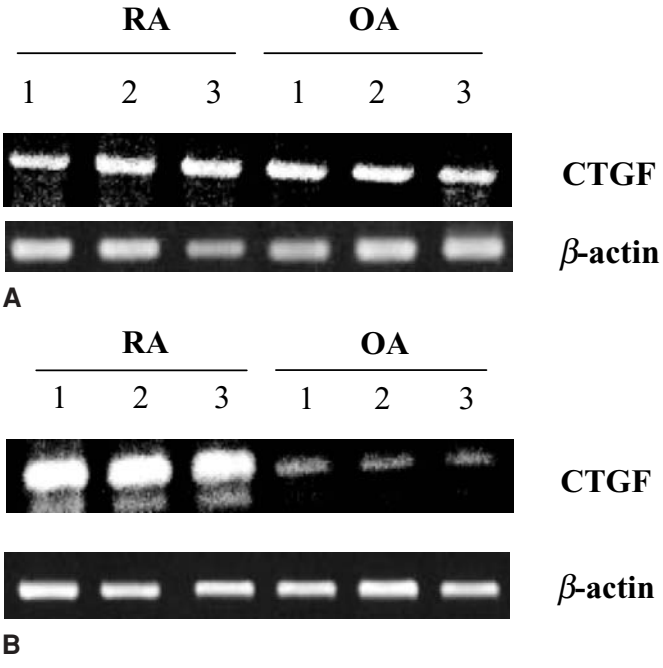

Fig. 2. Expression of connective tissue growth factor (*CTGF*) mRNA in synovial fibroblasts from three RA patients and three OA patients cultured in medium alone (**A**) or stimulated with 10 ng/ml of TGF-β1 (**B**), as detected by RT-PCR. Polymerase chain reaction products representing CTGF and β-actin and were stained with ethidium bromide. The β-actin level was used as a standard for each sample

sity of the bands was quantified using the public domain NIH image program (W. Rasband, Research Service Branch, National Institutes of Health, Bethesda, MD, USA). Data in the upper panels of Fig. 1 show the relative intensity of bands of TGF-β receptors to β-actin.

Both RA and OA synovial fibroblasts expressed detectable amounts of TGF-βRII mRNA, but the expression in synovial fibroblasts of RA patients was significantly higher than that of OA patients (RA: 0.90 ± 0.07; OA: 0.41 ± 0.02, $P = 0.0027$) (Fig. 1). On the other hand, there was no difference in the expression of TGF-βRI (RA: 1.35 ± 0.15; OA: 1.09 ± 0.11) or -βRIII (RA: 1.21 ± 0.11; OA: 0.88 ± 0.08) between RA and OA fibroblasts.

Expression of CTGF in synovial fibroblasts by TGF-β1

In human tissues including synovial tissue, three homodimeric isoforms of TGF-β, TGF-β1, -β2, and -β3, have been characterized.[2] Since TGF-β1 was shown to play the most prominent role in RA,[9,10] in this study we used TGF-β1 to determine the response of synovial fibroblasts to TGF-β. After synovial fibroblasts from RA and OA patients were cultured in the absence of TGF-β1 or with 10 ng/ml of TGF-β1 for 4 h, CTGF mRNA expression in synovial fibroblasts was examined by RT-PCR. mRNA of CTGF was detected in RA fibroblasts under control conditions without TGF-β1 stimulation at the extent similar to that in OA synovial fibroblasts (Fig. 2A). On the other hand, TGF-β1 augmented CTGF mRNA expression in synovial fibroblasts of RA patients which was much greater than that in OA patients (Fig. 2B).

Chemotactic migration of synovial fibroblasts by TGF-β1

Transforming growth factor β has been shown to be a potent chemoattractant for fibroblasts.[14] Therefore, to examine the heterogeneity in synovial fibroblasts obtained from RA patients, the chemotactic response of RA synovial fibroblasts to TGF-β1 was measured and the result compared with that of OA synovial fibroblasts. Fifty nanograms per milligram of platelet-derived growth factor (PDGF)-BB was included as a positive control. The dotted line represents the chemotactic activity of PDGF-BB (Fig. 3). Both RA and OA fibroblasts responded chemotactically to TGF-β1 in a dose-dependent manner at the concentration from 1 to 100 ng/ml and 50 to 100 ng/ml, respectively, but the response of RA fibroblasts was significantly higher than that of OA fibroblasts (Fig. 3).

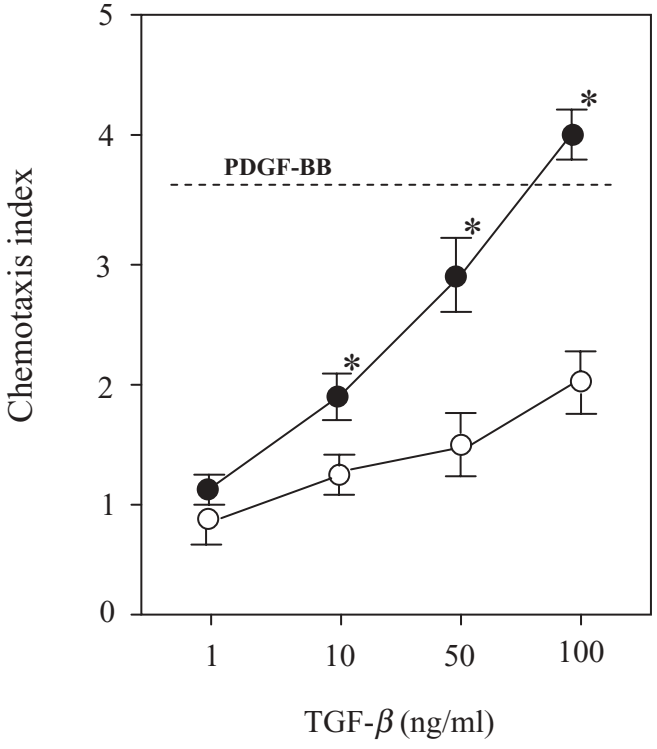

Fig. 3. Chemotactic response of synovial fibroblasts induced by TGF-β1. Chemotaxis assay was performed as described in Materials and methods. The chemotactic activities for RA fibroblasts (*closed circles*) and OA fibroblasts (*open circles*) are expressed as the chemotaxis index, which represents the ratio of the number of cells per high-power field in the test samples and the control samples (medium control). Results are expressed as mean ± SEM. Experiments were repeated three times for fibroblasts for each patient, and data are representative of three RA and three OA patients. *Significance of differences for comparing the value of OA fibroblasts ($P < 0.05$). *PDGF-BB*, platelet-derived growth factor BB

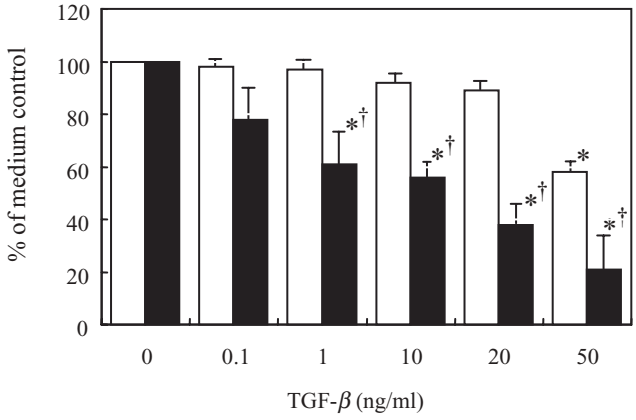

Fig. 4. Effects of TGF-β1 on proliferation of synovial fibroblasts. The fibroblast growth-stimulating and -inhibitory activities for RA fibroblasts (*closed columns*) and OA fibroblasts (*open columns*) were measured by a methyl thiazol tetrazolium (MTT) assay, and expressed as a percentage of the value of medium control. Results are expressed as mean ± SEM. Experiments were repeated three times for fibroblasts for each patient, and data are representative of three RA and three OA patients. *,† Significance of differences for comparing the value of medium control ($P < 0.05$) and for comparing the value of OA fibroblasts ($P < 0.05$), respectively

Proliferation of synovial fibroblasts by TGF-β1

The effect of TGF-β1 on the proliferation of synovial fibroblasts of RA and OA patients was examined. Synovial fibroblasts were treated with various concentrations of TGF-β1 for 72 h, then growth-stimulating and -inhibitory activities were determined by MTT assay. Transforming growth factor β1 inhibited the proliferation of synovial fibroblasts from RA patients in a dose-dependent manner at the concentration from 1 to 50 ng/ml (Fig. 4). Transforming growth factor β1 also inhibited the proliferation of synovial fibroblasts from OA patients, but the inhibitory effect to fibroblasts of OA patients was found only at high concentrations of TGF-β1, and was significantly lower than that to RA fibroblasts.

Discussion

This study shows that synovial fibroblasts of patients with RA express the increased TGF-βRII, which is associated with altered biological responses of RA synovial fibroblasts to TGF-β and may have an important role in the pathogenesis of synovial inflammation and hyperplasia in RA.

In this study, we demonstrated that TGF-β1 stimulated the expression of CTGF in RA synovial fibroblasts more than in OA synovial fibroblasts. Connective tissue growth factor is known to be highly expressed by fibroblasts, and to play roles in the proliferation of fibroblasts and in the stimulation of extracellular matrix production by these cells.[16] Previous reports showed that CTGF secretion and gene expression are directly stimulated by TGF-β, indicating that CTGF may function as a downstream mediator of TGF-β action on fibroblasts.[1,17] Thus, CTGF is thought to mediate many of profibrotic actions of TGF-β in various fibrotic disorders. Although the interaction between CTGF and TGF-β has been shown to play an important role as profibrogenic cytokines in various fibrotic disorders such as scleroderma,[18] pulmonary fibrosis,[19] glomerulosclerosis,[20] and inflammatory bowel disease,[21] this is the first study which showed TGF-β-stimulated expression of CTGF in RA synovial fibroblasts. We showed here that altered biological responses of RA fibroblasts to TGF-β stimulation were also found in chemotaxis and proliferation of RA synovial fibroblasts. Transforming growth factor β1 induced chemotactic migration of RA and OA synovial fibroblasts, but the chemotactic response of RA fibroblasts was significantly higher than that of OA fibroblasts. In fibroblast growth, TGF-β1 suppressed the growth of synovial fibroblasts dose-dependently, and the inhibition was significantly higher in RA fibroblasts than in OA fibroblasts. Transforming growth factor β is a strong chemoattractant for fibroblasts.[14,22,23] On the other hand, TGF-β has been shown to act as a potent growth inhibitor for fibroblasts.[24–27] Accordingly, this study indicates that up-regulation of

functional capacities to TGF-β1 are observed in RA fibroblasts when compared with OA fibroblasts.

A critical mechanism for regulating the cellular response to cytokines resides at the level of their receptor expression. Therefore, to clarify the mechanism of the altered biological responses of RA synovial fibroblasts to TGF-β, we next examined the expression of TGF-β receptors. Three classes of cell surface TGF-β binding proteins have been characterized and are known as types I, II, and III receptor proteins. This study showed that increased expression of TGF-βRII mRNA was detected in RA synovial fibroblasts when compared with OA synovial fibroblasts. There was no significant difference in TGF-βRI or RIII expression between RA and OA fibroblasts. Of the three receptors, the type II receptor appears to be the most important signaling receptor.[1,28] Taken together, these results suggest that increased functional responses of RA synovial fibroblasts to TGF-β observed in CTGF production, chemotactic migration. and proliferation were mainly due to the upregulated expression of TGF-βRII. However, a variety of cytokines besides TGF-β can induce the growth and chemotaxis of synovial fibroblasts. Therefore, the difference in the production of these cytokines between RA and OA synovial fibroblasts is possibly responsible for the increased biological responses in RA synovial fibroblasts.

There are a few reports that have demonstrated the expression of TGF-β receptors in the synovial tissue of RA. Taketazu et al. showed the expression of TGF-βRII in active inflammatory lesions of RA synovial tissues immunohistochemically, but the expression of TGF-βRI or RIII was not examined.[9] Increased expression of endoglin was found immunohistochemically in the synovial lining layer and subsynovial macrophages.[29] Regarding the expression of TGF-β receptors in fibroblasts, there are a few reports only in skin fibroblasts of patients with scleroderma. Increased expression of both TGF-βRI and -RII mRNA was found in fibroblasts of systemic sclerosis,[30,31] and fibroblasts from patients with systemic sclerosis possessed elevated levels of mRNA of endoglin, as compared with fibroblasts from healthy subjects.[32] However, this is the first study which demonstrates selectively increased expression of TGF-βRII in RA synovial fibroblasts when compared with OA synovial fibroblasts by examining the expression of all three TGF-β receptors.

Taken together, results showing that the upregulation of TGF-βRII expression is associated with altered responses of synovial fibroblasts to TGF-β1 in RA suggest that TGF-β has an important role in the pathogenesis of synovial lesions in this disorder. However, the precise role of TGF-β in synovial lesions in RA remains a subject of controversy. Several lines of evidence suggest that TGF-β has a profound suppressive effect on immune cells, and the immunosuppressive role has been shown in RA. Transforming growth factor β selectively inhibits the proliferation of $CD4^+$ cells and B cells,[33] and the production of interleukin (IL)-1, interferon-γ, and tumor necrosis factor by monocytes and macrophages.[34,35] Transforming growth factor β1 has been shown to suppress acute and chronic arthritis in experimental animal models.[36–38] In contrast, TGF-β1 has been reported to have proinflammatory actions. For example, TGF-β1 increased mRNA expression of proinflammatory cytokines such as IL-1, IL-8, and tumor necrosis factor in RA synovial fibroblasts.[39] Allen el al. showed that the injection of TGF-β1 into the joint cavity of rats induced synovial erythema, swelling, and leukocyte infiltration,[23] thereby exerting diverse and even opposite effects in joint inflammations depending on the cell types and conditions. Thus, whether TGF-β promotes rheumatoid inflammation remains unclear. Further studies are necessary to clarify the pathogenic role of the upregulation of TGF-βRII expression and increased response to TGF-β observed in rheumatoid synovial fibroblasts in inflammatory and fibrotic processes of RA.

In conclusion, we have shown that the expression of TGF-βRII is upregulated in RA synovial fibroblasts, and might play a role in the pathogenesis of synovial inflammation and hyperplasia in RA. Although anti-TGF-β strategies seem to be effective at reducing joint inflammation in RA, further studies are necessary to elucidate the critical role of the interaction between TGF-β and its receptors in the pathogenesis of RA.

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