Post-transcriptional regulation of IL-6 production by Zc3h12a in fibroblast-like synovial cells

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Abstract

Objective

Zc3h12a is an RNA binding protein with a CCCH-type finger motif and is known to regulate mRNA metabolism. Previous reports suggest that Zc3h12a acts as a negative regulator of inflammatory processes because it is involved in the degradation of IL-6 mRNA. We investigate the effect of Zc3h12a on IL-6 production in fibroblast-like synovial cells (FLS) from rheumatoid arthritis (RA) patients.

Methods

The expression of Zc3h12a in FLS was determined by polymerase chain reaction. To knock down Zc3h12a expression in FLS, siRNA for Zc3h12a was transfected by the lipofection method. The supernatants were collected after siRNA transfection for the quantification of IL-6 production. The phosphorylation of the signal transducer and activator of transcription 3 (STAT3) was examined by Western blotting. Cell proliferation was analysed by the Cell Counting Kit-8 assay after Zc3h12a knockdown.

Results

mRNA for Zc3h12a were demonstrated in FLS from RA patients. Zc3h12a transcripts were induced by LPS or IL-1β in FLS. The production of IL-6 as well as its mRNA expression was significantly increased by the Zc3h12a knockdown. The Zc3h12a knockdown also induced the activation of STAT3, which the anti IL-6 receptor antibody inhibited. Proliferation of Zc3h12a-knockdown FLS increased significantly in the presence of recombinant soluble IL-6 receptor (sIL-6R).

Conclusion

Our data suggest that Zc3h12a is a novel IL-6 regulator in FLS, which may be involved in the progression of RA.

Key words

rheumatoid arthritis, IL-6, Zc3h12a, synoviocyte

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Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterised by synovial inflammation and subsequent bone destruction (1). Although the causes of RA are not fully understood, constitutive overproduction of IL-6, a multifunctional cytokine that regulates the immune response, inflammatory reaction and bone metabolism, is thought to play a major pathological role in RA (2, 3). Tocilizumab, a humanised monoclonal antibody that recognises the IL-6 binding site of human IL-6 receptor, has proved to be beneficial for the treatment of RA (4-6). Tocilizumab seems to inhibit osteoclast formation in synovia by downregulating RANKL expression on synovial cells through IL-6 trans-signalling. Consistent with those hypotheses, Hashizume *et al*. have reported that IL-6/sIL-6R directly induced RANKL expression in FLS and is essential for RANKL induction by TNF-α and IL-17 (7).

Recently, post-transcriptional control of cytokines has been regarded as a new mechanism that regulates cytokine production in immune responses (8). Matushita *et al*. have clearly demonstrated that Zc3h12a, a CCCH zinc finger protein with RNase activity, is essential for inhibiting the development of severe autoimmune responses culminating in the lethality of mice. Importantly, the production of IL-6 and that of IL-12p40 were increased in *Zc3h12a*$^{-/-}$ macrophages due to mRNA decay failure of those transcripts (9).

Zc3h12a encodes MCP-1-induced protein 1 (MCPIP) in humans and was recently identified not only in monocytes treated with monocyte chemotactic protein 1 (MCP-1) but also in human monocyte-derived macrophages stimulated with IL-1β. Expression of the *Zc3h12a* gene was induced by inflammation-related factors, such as MCP-1, TNF-α, LPS, and IL-1 in monocytes or macrophages (10, 11). Those data suggest the important role of Zc3h12a in chronic inflammatory disease.

Here, we investigated the effects of Zc3h12a on fibroblast-like synovial cells (FLS) from RA patients, and found that Zc3h12a is a potent IL-6 regulator in FLS.

Materials and methods

Cell cultures and reagents

Synovial tissue samples were obtained during synovectomy from 3 patients with RA, who met the American College of Rheumatology (formerly the American Rheumatism Association) 1987 revised criteria (12). None of the RA patients had taken biologic disease-modifying antirheumatic drugs (DMARDs) before surgery. Synovial fibroblasts were isolated from the synovial tissues by enzymatic digestion as described previously (13). After overnight culture, nonadherent cells were removed and adherent cells were trypsinised, split at a 1:3 ratio, and cultured. Synoviocytes were used from passages 6 through 8 for all subsequent experiments. LPS was purchased from R & D Systems (Minneapolis, MN, USA). Human recombinant TNF-α, IL-1β, and IL-6 were purchased from R & D Systems. Tocilizumab was kindly provided by Chugai Pharmaceutical Co., Ltd (Tokyo, Japan). This is a humanised anti-human IL-6 receptor monoclonal antibody that is used as a biological disease-modifying antirheumatic drug (DMARD). The whole study was approved by the Ethics Committee of Nagasaki University. Informed consent was obtained from each subject.

Quantitative real-time PCR

Total RNA was extracted from FLS using Trizol reagent (Invitrogen, San Diego, CA, USA), according to the manufacturer's instructions. To generate complementary DNA (cDNA), total RNA (500-700 ng) was subjected to reverse transcription using ReverScript III (Wako Pure Chemical Industry, Osaka, Japan). Quantification of specific messenger RNA (mRNA) was performed by SYBR Green real-time PCR, using Light Cycler (Roche Diagnostics, Mannheim, Germany). The following primer sequences were used: for human Zc3h12a, sense primer 5'-CCATCACAGACCAGCACATC-3'and anti-sense primer 5'-GACTCGTAGGCCAGCTTCAC-3' for human IL-6, sense primer 5'-GAAAGCAGCAAAGAGGCACT-3' and anti-sense primer 5'-TTTCACCAGGCAAGTCTCCT-3' for human β-actin, sense primer 5'-CGTACCACTGGCATCGTGAT-3' and

anti-sense primer 5'-GTGTTGGCGTACAGGTCTTTG-3'. β-actin was used to verify equal loading. To confirm specific amplification by the SYBR Green PCR, a dissociation curve analysis was performed for each primer pair, and both non-RT negative controls and water controls were used for these analyses. Differential gene expression was calculated with the threshold cycle (Ct), and relative quantification was calculated with the comparative Ct method. Only samples with a difference of at least 4 cycles between the signals in cDNA samples and negative controls (corresponding to a 4 (16-fold) difference in expression) were considered for the calculations. All experiments were performed at least in triplicate.

Western blotting for analysis

The conditioned FLS were washed with ice-cold PBS, and proteins were isolated using a lysis buffer (50 mM HEPES, 150 mM NaCl, 0.02% NaN2, 0.1% SDS, 1% NP-40). Protein concentrations of cell extracts were determined with a BCA protein assay kit (Pierce Chemical, Rockford, IL, USA). An identical amount of protein for each lysate (5 μg/well) was subjected to 12% SDS-polyacrylamide gel electrophoresis (PAGE). Proteins were transferred to a nitrocellulose membrane, which was subsequently blocked for 1 h using 5% normal horse serum (NHS) in phosphate-buffered saline (PBS) and incubated at room temperature with anti-STAT3 antibody (Cell Signaling Technology, Danvers, MA, USA), anti phospho-STAT3 (Tyr705) antibody (Cell Signaling Technology) and anti-β-actin antibody (Sigma-Aldrich, St. Louis, MO, USA). The filter was washed with TBS-T and incubated with a 1:5000 dilution of sheep anti-rabbit IgG coupled with horseradish peroxidase (HRP; Jackson Immunoresearch, West Grove, PA, USA). The enhanced chemiluminescence (ECL) system (Amersham, Buckinghamshire, UK) was used for detection.

Small interfering RNA (siRNA) transfection

FLS cells at 70% confluence in 6-well plates were harvested and transfected with siRNA for Zc3h12a or with scrambled negative control SMART pool siRNA (Dharmacon, Lafayette, CO, USA) in each well according to standard protocols for FuGENE HD transfection reagent (Roche Applied Science, Indianapolis, IN, USA), with slight modification. Briefly, DNA solution containing 5 μL (20 nM) siRNA was diluted in 100 μl of Opti-MEM (Invitrogen, Carlsbad, CA, USA). The mixtures were incubated at room temperature for 20 min. In the meantime, the culture medium in the 6-well plates was removed and cells were washed with Opti-MEM. To each well were added 900 μl of fresh serum-free DMEM and 100 μl of transfection mixture. After overnight incubation, the medium was changed to fresh serum-supplemented DMEM and incubated for 48 h. siRNA transfection was performed every 24 h (total three times) during incubation.

Cytokine protein assays

IL-6 production was determined in culture supernatants from FLS at 72 h after siRNA transfection. Culture supernatants were aspirated at 48 h after transfection, replaced with fresh medium containing serum-free DMEM, and cultured for 24 h. The IL-6 protein concentration was examined by enzyme-linked immunosorbent assays (ELISA) (SRL, Tokyo, Japan).

Proliferation assay

24 h prior to assay, FLS (2×10^4cells/0.1 ml/well) in 96-well flat bottom plate were transfected control siRNA or Zc3h12a siRNA. These cells were cultured with or without sIL-6R (250ng/ml) for 3 days. At 72 h after the addition of sIL-6R, the Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) was used to assay cell proliferation according to the manufacturer's instructions.

Statistical analysis

All experiments were conducted at least twice to confirm the reproducibility, and all three FLS were tested in each experiment. Statistical analysis was performed with Student's *t*-test except where indicated otherwise. *P*-values less than 0.05 were considered significant.

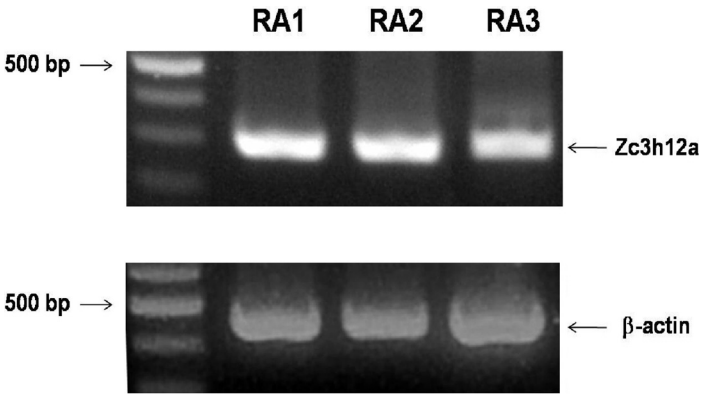

Fig. 1. Zc3h12a mRNA and protein expression in FLS. Total RNA was analysed by RT-PCR using primers specific for Zc3h12a and β-actin in samples from three different patients.

Results

Zc3h12a expression of FLS

To determine whether or not FLS express Zc3h12a, Zc3h12a mRNA in FLS was investigated by RT-PCR. As shown in Figure 1, *Zc3h12a* gene expression was detected by RT-PCR. Previous studies have revealed that Zc3h12a mRNA was rapidly induced in mouse macrophages after LPS stimulation (9, 14). Thus, in order to evaluate whether or not proinflammatory mediators induce Zc3h12a gene expression in FLS, we investigated the level of Zc3h12a and IL-6 mRNA by quantitative real-time PCR. Zc3h12a expression was induced in FLS by 1 h treatment with LPS or IL-1β stimulation, but not by TNF-α or IL-6 (Fig. 2A). Meanwhile, IL-6 expression was induced by treatment with TNF-a, LPS or IL-1β (Fig. 2B).

Knockdown of Zc3h12a increased IL-6 secretion in FLS

In macrophages from Zc3h12a knockout mice, the production of IL-6 and that of IL-12p40 aberrantly increase in response to TLR ligands (9). This suggested that the increased production of IL-6 in FLS is also mediated by gene

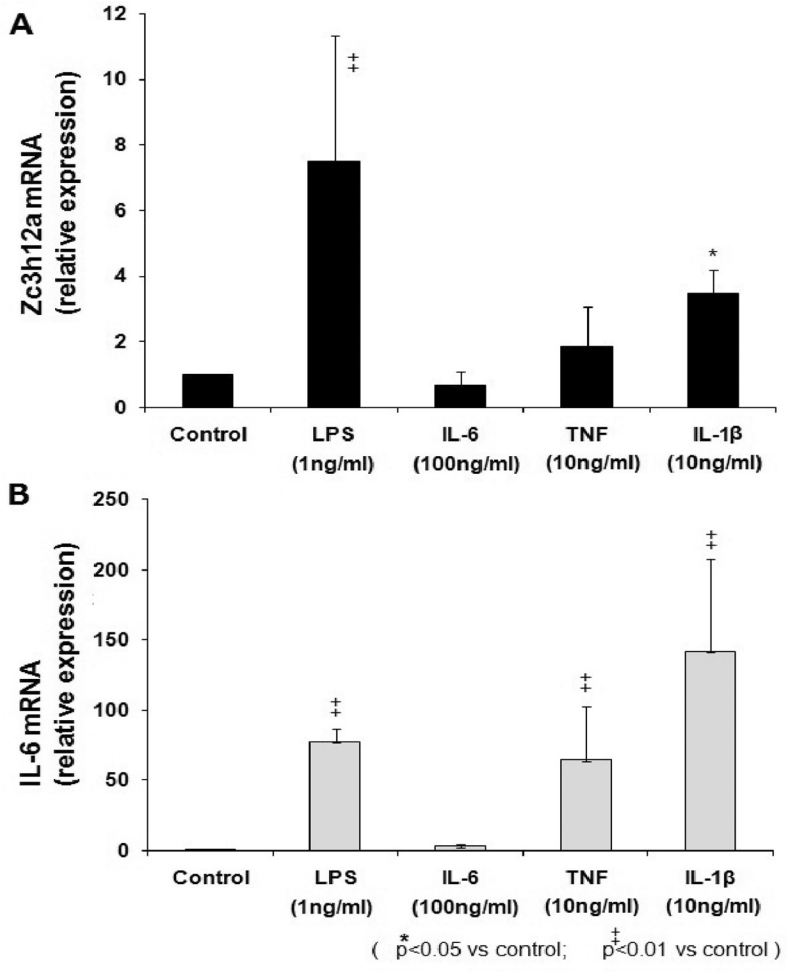

Fig. 2. The change of Zc3h12a and IL-6 gene expression levels by LPS, IL-6, TNF-α and IL-1β in FLS. FLS were stimulated with LPS (1 ng/ml), IL-6 (100 ng/ml), TNF-α (10 ng/ml) or IL1-β (10 ng/ml) for 1 h. The Zc3h12a and IL-6 expression levels were determined by quantitative real-time PCR. Each column and vertical line indicate mean and S.D., respectively. Data represent the means of four independent experiments run in triplicate.

silencing of Zc3h12a. By using an RNA interference technique, we successfully knocked down Zc3h12a mRNA expression (Fig. 3A). Zc3h12a siRNA increased IL-6 mRNA expression compared with control siRNA as determined by quantitative PCR (Fig. 3B). Zc3h12a siRNA also increased IL-6 production as determined by ELISA (Fig. 3C).

The effect of Zc3h12a knockdown on cytokines or LPS induced IL-6 mRNA expression

We further examined the effect of Zc3h12a knockdown on cytokines or LPS induced IL-6 mRNA expression in FLS by quantitative real-time PCR. Consistent with Figure 3, the knockdown of Zc3h12a enhanced the expression of IL-6 mRNA in the absence of stimulation. IL-6 mRNA expressions in the FLS treated with LPS or IL-1β were significantly increased by Zc3h12a ablation compared to those in the control cells. Knockdown of Zc3h12a did not affect IL-6 dependent IL-6 mRNA expression. Zc3h12a ablation clearly suppresses the IL-6 mRNA level in TNF-α treated FLS (Fig. 4). The knockdown was confirmed by quantitative PCR at 72 h after transfection (data not shown).

Role of STAT3 activation in FLS knocked down using Zc3h12a siRNA

To realise the signalling mechanism

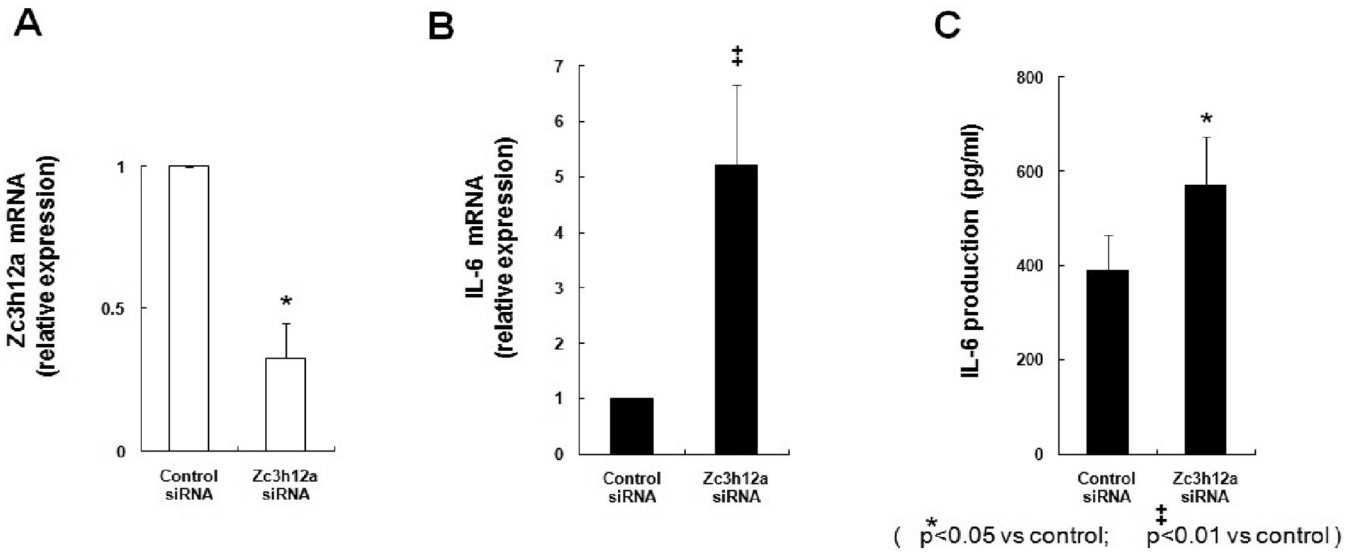

Fig. 3. Zc3h12a knockdown induced IL-6 gene production in FLS. The knockdown was confirmed by quantitative PCR at 72 h after transfection (A). IL-6 mRNA expression levels were determined by quantitative PCR (B). IL-6 production was determined by ELISA (C). Each column and vertical line indicate mean and S.D., respectively. Data represent the means of three independent experiments from three RA patients.

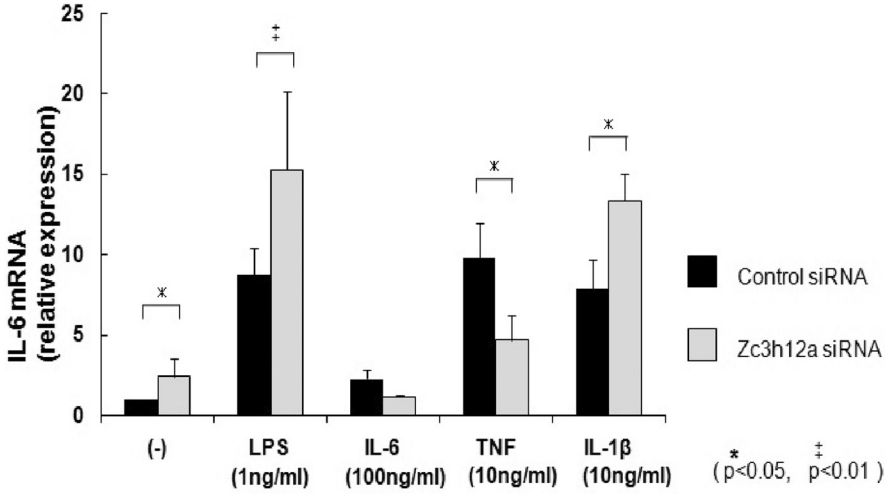

Fig. 4. The effect of Zc3h12a knockdown on cytokines or LPS induced IL-6 mRNA expression. FLS were treated with control siRNA or Zc3h12a siRNA for 72 hours. Total RNA was extracted from FLS cultured with or without indicated factor for 1 hour. Data represent the means of 3 independent quantitative PCR experimants run in triplicate.

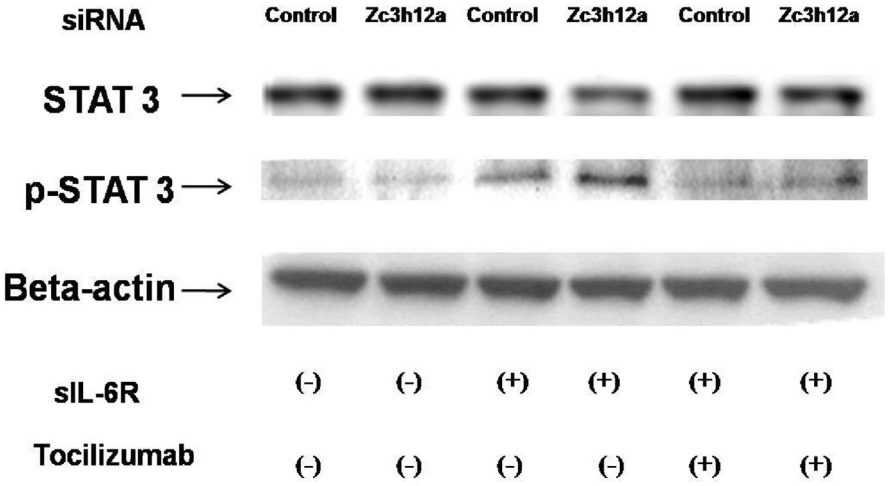

Fig. 5. Phosphorylation of STAT3 in siRNA-treated FLS. Phosphorylation of STAT3 was determined by Western blotting using specific antibodies against STAT3 or phospho-STAT3 stimulated with sIL-6R (250 ng/ml) or sIL-6R plus tocilizumab (50 g/ml) for 30 minutes as indicated.

of knockdown-induced IL-6 production, we determined the activation of STAT3 by Western blotting analysis. Previous study showed that FLS themselves do not express IL-6R (7). Thus, we speculated FLS did not activate IL-6 signalling without sIL-6R and added sIL-6R in the culture. The knockdown of Zc3h12a in FLS cultured without recombinant sIL-6R did not alter the phosphorylation of STAT3. However, the addition of sIL-6R greatly enhanced phosphorylation. The addition of Tocilizumab, a potent IL-6 receptor inhibitor, almost completely blocked the phosphorylation of STAT3 (Fig. 5).

Effect of Zc3h12a on proliferation in FLS

STAT3 regulates the expression of genes that mediate survival (survivin, bclxl, mcl-1, cellular FLICE-like inhibitory protein) and proliferation (c-fos, c-myc, cyclin D1) (15). Mihara M *et al*. have suggested that IL-6 is very likely to be involved in synovial cell proliferation in FLS in co-operation with sIL-6R (7, 16). We investigated the effect of Zc3h12a ablation on FLS proliferation. As shown in Figure 6, the proliferation of FLS treated with Zc3h12a siRNA was promoted significantly more in comparison with that of control siRNA-treated FLS in the presence of sIL6-R.

Discussion

The transcriptional regulation of IL-6 by nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB) is well understood in FLS (17, 18). Matsushita *et al*. (9) suggested that Zc3h12a, as a novel IL-6 regulatory system, may be an important factor regulating the half-lives of mRNA coding for IL-6. Consistent with this, our data suggest that Zc3h12a is a potent IL-6 regulator in FLS. Other cytokines, such as TNF-α, GM-CSF and IL-1β are known to be subject to post-transcriptional control by ARE-binding proteins including the RNA recognition motif found in the TIA-1, TIAR and HuR, the zinc-finger domain found in tristetraprolin (TTP) (19-21). Besides, these ARE-binding proteins are involved in RA progression. Likewise, our data indicate that Zc3h12a is one of the important cytokine regulators in RA.

Zc3h12a is a negative regulator of IL-6 production, as IL-6 production in FLS was clearly induced by the Zc3h12a knockdown. sIL-6R supplementation can induce proliferation in FLS treated with siRNA for Zc3h12a. Therefore, Zc3h12a regulates IL-6 production, which may, in turn, promote FLS overgrowth. Moreover, IL-6 induction by Zc3h12a siRNA is capable of activating the JAK/STAT signalling pathway. These data suggest that Zc3h12a has the potential to play an important role in inflammation and synovial overgrowth in RA.

It is known that IL-6 has a various biological effects on FLS such as inductions of matrix metallo-proteinases (MMPs) (22), RANK ligand (RANKL) (7), and vascular endothelial growth factor (VEGF) (23) in addition to its proliferation. Effect of IL-6 on proliferation in FLS is controversial. Our observation is inconsistent with the previous report that IL-6 inhibits the proliferation of FLS in the presence of sIL-6R (24). Although we have no definite information to explain this discrepancy, siRNA treatment may greatly affect FLS property in our experiments. Namely, unknown substrates of Zc3h12a alter the proliferation of FLS. Our data implies the possibility that IL-6/sIL-6R complex, which is up-regulat-

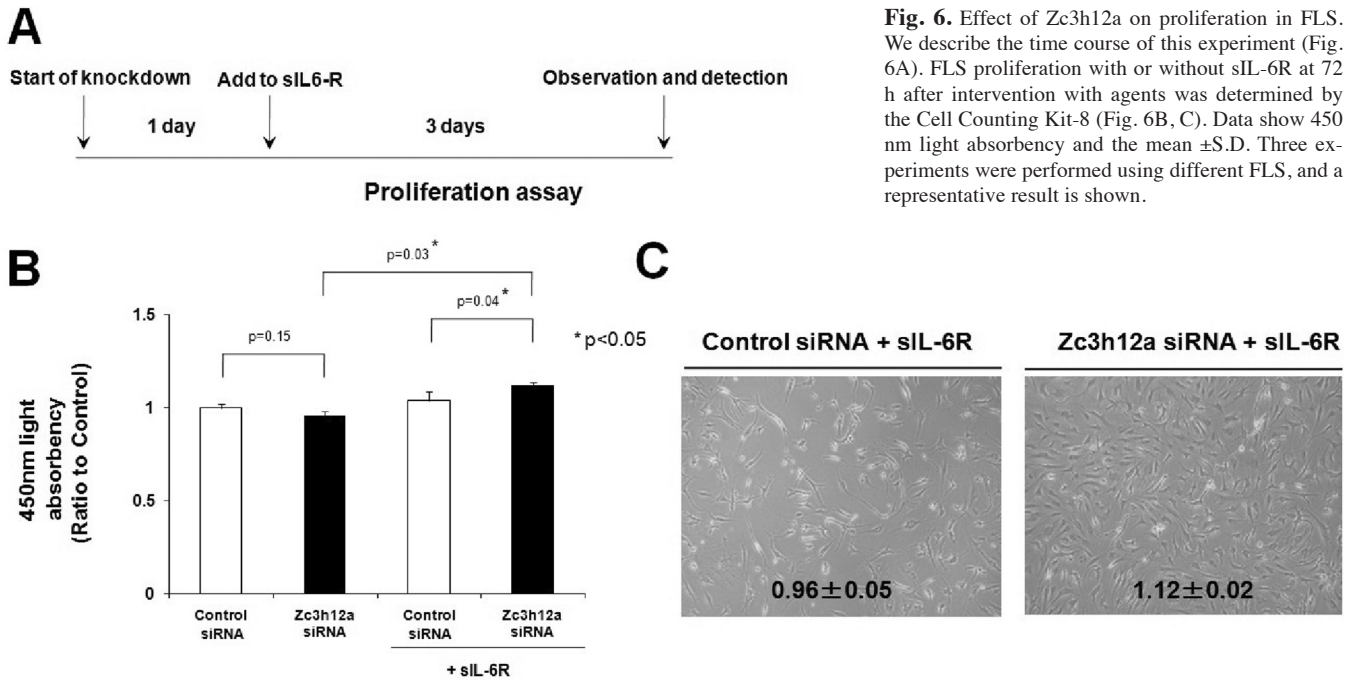

Fig. 6. Effect of Zc3h12a on proliferation in FLS. We describe the time course of this experiment (Fig. 6A). FLS proliferation with or without sIL-6R at 72 h after intervention with agents was determined by the Cell Counting Kit-8 (Fig. 6B, C). Data show 450 nm light absorbency and the mean ±S.D. Three experiments were performed using different FLS, and a representative result is shown.

ed by Zc3h12a ablation, promotes FLS proliferation. However, further studies are required to clarify this point.

FLS express Zc3h12a, which is induced by LPS or IL-1β as previously reported. On the other hand, neither IL-6 nor TNF-α induced Zc3h12a mRNA expression. Both IL-1β and TNF-α are well known IL-6 inducer in FLS (25), however, these cytokines differ in their regulation of Zc3h12a. Namely TNF-α stimulates IL-6 mRNA transcription without affecting its stability. IL-1β promotes the transcription of IL-6 mRNA, while IL-1β promotes its decay. We illustrated the role of Zc3h12a in IL-6 regulation (Fig. 7). As we expected, the knockdown of Zc3h12a enhanced the mRNA expression of IL-6 in the cell treated with IL-1β or LPS. Those results imply that both LPS and IL-1β can inhibit and promote IL-6 expression at the same. Surprisingly, Zc3h12a ablation inhibited TNF-α dependent IL-6 expression. These data suggests that Zc3h12a affects TNF-α signalling by unknown mechanism. These facts lead to the supposition that TNF-α is a purer accelerator of IL-6 than IL-1β. Therefore, our data may explain, at least in part, why the blockade of TNF-α is more effective than that of IL-1β in treating RA.

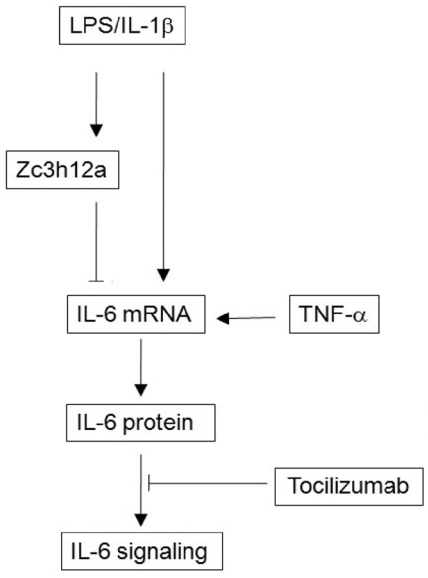

Fig. 7. The role of Zc3h12a in IL-6 regulation.

Recently, Zcchc11 (zinc-finger, CCHC domain-containing protein 11) was introduced as another post-transcriptional IL-6 regulator, which is a ribonucleotidyltransferase with a preference for uridine and is essential for maintaining the poly (A) tail length and stability of transcripts for IL-6 (26). Interestingly, Zcchc11 is highly expressed in the murine macrophage-like cell line RAW 264.7 cell (27). Macrophages appear to play a pivotal role in RA because they are numerous in the inflamed synovial membrane and at the cartilage-pannus junction. Therefore it is also very important to analyse the action of this molecule for a better understanding of the IL-6 regulator in RA.

In conclusion, we obtained evidence of IL-6 overproduction in Zc3h12a siRNA-transfected FLS. Since IL-6 is important in the pathogenesis of RA, modification of Zc3h12a expression in FLS might be a potent strategy in the pharmacological management of rheumatoid synovial cells. Better knowledge of the molecular mechanisms that control IL-6 gene regulation will provide a deeper understanding of the initiation and development of RA triggered by IL-6.

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