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Research article

Exosomes derived from synovial fibroblasts from patients with rheumatoid arthritis promote macrophage migration that can be suppressed by miR-124-3p

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ABSTRACT

Objectives: Exosomes are potent vehicles for intercellular communication. Rheumatoid arthritis (RA) is a chronic systemic disease of unknown etiology. Local administration of miR-124 precursor to rats with adjuvant-induced arthritis suppresses systemic arthritis and bone destruction. Thus, exosomes may be involved in this disease. We aimed to determine the role of exosomes in the pathology of RA.

Methods: Fibroblast-like synoviocytes (FLS) were collected from patients with RA and osteoarthritis (OA). miR-124-3p mimic was transfected into the RA FLS (RA miR-124 FLS). Exosomes were collected from the culture medium by ultracentrifugation. Macrophages were produced from THP-1 cells. MicroRNAs in the exosomes were analyzed using real-time PCR. Proteomics analysis was performed using nanoscale liquid chromatography-tandem mass spectrometry. Macrophage migration was evaluated using a Transwell migration assay. SiRNA was used to knockdown proteins of interest.

Results: MicroRNAs in the RA FLS, RA miR-124 FLS, and OA FLS exosomes were similar. Proteomics analysis revealed that pentraxin 3 (PTX3) levels were higher in RA FLS exosomes than in RA miR-124 FLS and OA FLS exosomes, and proteasome 20S subunit beta 5 (PSMB5) levels were lower in RA FLS exosomes than in RA miR-124 FLS and OA FLS exosomes. The RA FLS exosomes promoted and the RA miR-124 FLS exosomes suppressed macrophage migration. PTX3-silenced RA FLS exosomes suppressed and PSMB5-silenced OA FLS exosomes promoted macrophage migration.

Conclusions: RA FLS exosomes promote macrophage migration via PTX3 and PSMB5, and miR-124-3p suppresses this migration.

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1. Introduction

Extracellular vesicles (EVs) are small membrane-enclosed particles that are released by all cells into the extracellular space. They contain RNA, lipids, proteins, and DNA that can be shuttled to other cells to influence the recipient cell's phenotype. EVs have been recognized as potent vehicles of intercellular communication [1,2]. EVs with diameters ≤100–150 nm are formed inside multivesicular bodies (MVBs) and are defined as exosomes. EVs with diameters ranging up to 1000 nm, which presumably bud from the plasma membrane, are variably called ectosomes, microvesicles, microparticles, or large oncosomes [3].

Exosomes have a double-layered membrane with a lipid composition rich in sphingomyelin, cholesterol, and glycerophospholipids [4]. Exosomes are formed as intraluminal vesicles (ILVs) by budding into early endosomes and multivesicular bodies (MVBs). MVBs can be either fusion with lysosomes or fusion with the plasma membrane (PM), which allows the release of their content to the extracellular milieu. Several RAB proteins have been shown to be involved in the transport of MVBs to the PM and in exosome secretion [5]. Exosmes contain functional proteins, lipids, DNA and various of RNA. Exosomes interact with and regulate the function of target cells. Several types of interactions are proposed, including binding of vesicles to the surface of a recipient cell through exosomal adhesion molecules, or phosphatidylserine (PS)/lysophosphatidylcholine and cellular receptors, direct fusion of vesicles with recipient plasma membrane after adhesion, or internalization of vesicles into endocytic compartments through receptor-mediated endocytosis or phagocytosis [6]. Exosomes can be released from a variety of cells including fibroblasts, intestinal epithelial cells, neurons, adipocytes, and tumor cells, and they are found in many biological fluids, such as synovial fluid, blood, urine, saliva. The biology, function, and heterogeneity of exosomes depend on the cell of origin and the status of the originating tissue or cell at the time of exosome generation [7]. Exosomes have specialized functions and play key roles in intercellular signaling and cellular waste management [8]. Exosomes differ from each other mostly in their intracellular origins [9] and can affect a diverse range of basic physiological processes, such as inflammation, neuronal function, cell proliferation, and immune responses [7]. For example, tumour-derived exosomes uptaken by organ-specific cells. Treatment with exosomes from lung-tropic models redirected the metastasis of bone-tropic tumour cells. Exosome proteomics revealed distinct integrin expression patterns, in which the exosomal integrins α6β4 and α6β1 were associated with lung metastasis, while exosomal integrin αvβ5 was linked to liver metastasis [10].

Rheumatoid arthritis (RA) occurs in 0.5–1.0% of the adult population worldwide, RA is a symmetric polyarticular arthritis that primarily affects the small diarthrodial joints of the hands and feet. In addition to inflammation in the synovium, which is the joint lining, the aggressive front of synovium called pannus invades and destroys local articular structures. $CD4^+$ T cells, B cells and macrophages infiltrate the synovium and sometimes organize into discrete lymphoid aggregates with germinal centres. Hyperplasia of the intimal lining results from a marked increase in macrophage-like and fibroblast-like synoviocytes (FLS). Locally expressed degradative enzymes, including metalloproteinases, serine proteases and aggrecanases, digest the extracellular matrix and destroy the articular structures, which can easily cause disability and seriously affect people's quality of life [11]. Macrophages play an important role in the pathogenesis of RA. Macrophages can be subdivided into two distinct phenotypes, called M1 and M2 macrophages [12]. M1 macrophages are proinflammatory and produce tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1β, IL-6, and IL-23. M2 macrophages are involved in the resolution of inflammation. Inflamed RA synovial tissue and inflammatory vascular reactions present a higher number of macrophages, especially M1 macrophages, than healthy joints [13,14]. When biopsy specimens from synovial membranes of patients with RA are compared with normal synovial membranes, the diseased tissue contain greater numbers of macrophage. These cells make up almost the entire synovial lining, and their numbers are also dramatically increased in the sublinig tissue [15,16]. Treatment of patients with anti-TNF agents has a rapid and pronounced effect on the infiltration of monocyte-derived macrophages into tissues, suggesting an important role for infiltrating monocyte-derived macrophages. Radiological progression of joint destruction is also correlated with synovial macrophage infiltration [16].

There are several reports on the role of exosomes in RA pathogenesis. Ding et al. reported that exosomes derived from synovial fibroblasts under hypoxia could significantly induce T cells differentiation, which Th17 cells increased and Treg cells decreased in RA model mice [17]. However the role of exosomes in RA pathology has not yet been fully investigated.

Previously, we revealed that microRNA-124a (miR-124a) expression is lower in proliferative FLS from patients with RA than in those from patients with osteoarthritis (OA). The transfection of precursor (pre)-miR-124a into RA FLS significantly suppresses FLS proliferation and the secretion of monocyte chemoattractant protein 1 (MCP-1) [18]. Local administration of pre-miR-124 to the right ankle joint of rats with adjuvant-induced arthritis suppresses the infiltration of inflammatory cells (including macrophages) into the synovial membrane, the proliferation of synovial cells, systemic arthritis, and bone destruction [19]. However, the mechanism of suppression of systemic arthritis and bone destruction was not clarified. Exosomes have been strongly suggested to explain this phenomenon.

Therefore, to investigate the role of exosomes in the pathogenesis of RA, we analyzed exosomes derived from FLS from patients with RA using of proteomic analysis.

2. Materials and methods

2.1. Samples and cell culture

Joint tissue specimens were obtained from patients with RA and OA at the time of joint surgery. RA and OA were diagnosed according to the criteria of the American College of Rheumatology [20,21]. FLS were also isolated from synovial joint tissues collected from patients with RA and OA. The samples were obtained in accordance with the Declaration of Helsinki - Ethical Principles for

Medical Research Involving Human Subjects, as approved by the World Medical Association. All patients provided informed consent and the study was approved by the Ethics Committee of Kobe University Hospital (approval number B190178).

Tissue specimens were minced and digested, and the dissociated cells were cultured as described previously [22]. After 7 days of culture, non-adherent cells were removed, and adherent cells were maintained in RPMI-1640 medium with L-glutamine and phenol red (RPMI 1640, Fujifilm Wako Pure Chemical Corporation, Osaka, Japan) supplemented with 10% fetal calf serum (FCS) and penicillin/streptomycin (Lonza, Basel, Switzerland). All experiments were conducted using cells from passages 3 to 7.

Normal healthy (NH) FLS were purchased from Cell Applications (San Diego, CA, USA). The NH FLS were first cultured in synoviocyte growth medium (Cell Applications) for proliferation. The growth medium was then changed to RPMI 1640 containing 10% FCS and penicillin/streptomycin, and the cells were cultured for >1 week before they were used in the experiments.

THP-1 cells were purchased from the National Institutes of Biomedical Innovation, Health, and Nutrition, JCRB Cell Bank (Ibaragi, Japan). The THP-1 cells were maintained in RPMI-1640 medium supplemented with 10% FCS and penicillin/streptomycin. The cells were stimulated for 2 days with 150 nM phorbol 12-myristate 13-acetate (PMA, Fujifilm Wako Pure Chemical Corporation) to induce their differentiation into adherent macrophages [23]. The cells were cultured at 37 °C in 5% CO_2.

2.2. Transfection of miRNA mimic

To establish RA FLS with high expression of miR-124-3p (RA miR-124 FLS), 100 pmol of hsa-miR-124-3p mimic (Thermo Fisher Scientific, Waltham, MA, USA) and 20 μl of Lipofectamine RNAiMAX (Thermo Fisher Scientific) were mixed in 1 ml of Opti-MEM (Thermo Fisher Scientific) and incubated for 20 min at room temperature. Then, the mixture was added to RA FLS in 10 ml RPMI 1640 with 10% FCS and cultured at 37 °C in 5% CO_2 for 24 h.

2.3. Exosome isolation and characterization

The cells were grown to approximately 80% confluence, washed three times with phosphate-buffered saline (PBS), and cultured in AIM V serum-free medium (Thermo Fisher Scientific) for 2 or 3 days. Exosomes were collected from the medium by centrifugation. In brief, the medium was collected and sequentially centrifuged at 300×*g* for 10 min, 2000×*g* for 20 min, and 10,000×*g* for 30 min, and then filtered through a 0.22 μm Millex-GV filter (Merck Millipore, Burlington, MA, USA) to remove cells, cellular debris, and large EVs. The medium was then centrifuged at 210,000×*g* for 70 min using a Beckman L-70K ultracentrifuge (Beckman Instruments, Brea, CA, USA) with an SW 41 Ti swinging-bucket rotor (Beckman Instruments) [24]. The supernatant was discarded, and the pellet was washed twice with PBS. The pellet was resuspended in PBS and stored at −80 °C. Protein content was measured using a micro BCA protein assay (Thermo Fisher Scientific). The exosomes were characterized using tunable resistive pulse sensing with a qNano instrument (Meiwafosis Co., Tokyo, Japan) according to the manufacturer's instructions.

2.4. Effect of exosomes on cytokine secretion from macrophages

Macrophages were cultured with 15 μg/ml exosomes for 24 h and washed with PBS. In addition, to induce the M1 phenotype, macrophages were cultured for 24 h with 20 ng/ml interferon-γ (R&D Systems, Minneapolis, MN, USA) and 10 pg/ml of lipopolysaccharide (LPS) 0111 (Sigma-Aldrich, St. Louis, MO, USA) [25].

RNA was extracted from the macrophages using TRIzol (Thermo Fisher Scientific), and gDNA wipeout and reverse transcription were performed using the QuantiTect Reverse Transcription Kit (QIAGEN, Hilden, Germany). PCR was performed using PikoReal Real-Time PCR System (Thermo Fisher Scientific) with QuantiTect SYBR® Green PCR Kits (QIAGEN) using the following primer pairs: IL-1β (*IL1B*, forward: 5′-CAGAAGTACCTGAGCTCGCC-3′, reverse: 5′-AGATTCGTAGCTGGATGCCG-3′), IL-6 (*IL6*, forward: 5′-TGCAATAACCACCCCTGACC-3′, reverse: 5′-GTGCCCATGCTACATTTGCC-3′), TNF-α (*TNF*, forward: 5′-CTGGGCAGGTCTACTTTGGG-3′, reverse: 5′-CTGGAGGCCCCAGTTTGAAT-3′), and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*, forward: 5′-GTCATCCCTGAGCTGAACGG-3′, reverse: 5′-AAGTGGTCGTTGAGGGCAAT-3′). The PCR cycle consisted 95 °C for 5 min, 40 cycles of 94 °C for 15 s, 60 °C of 30 s, 72 °C of 30 s, respectively. The expression of cytokine mRNAs was normalized to that of *GAPDH* mRNA.

2.5. Transwell migration assay

Macrophages (1×10^5 cells) and 9 μg exosomes in 200 μl RPMI were placed in the upper chambers of Falcon Cell Culture Inserts (8 μm pore size in 24-well plates, Corning Inc., Corning, NY, USA), and 700 μl RPMI without FCS was placed in the lower chambers. The cells were cultured for 24 h. To allow for migration, the RPMI medium in the lower chamber was replaced with RPMI containing 10% FCS. After 24 h, the cells on the upper surface of the insert were wiped off using a cotton swab. Macrophages that had migrated through the filter and appeared on the lower side were fixed with methanol for 3 min, stained with Giemsa stain (Sysmex, Kobe, Japan), rinsed with sterilized distilled water, and counted (five fields per cell culture insert at 100× magnification).

2.6. Identification of proteins by nanoscale liquid chromatography-tandem mass spectrometry (LC-MS/MS)

A mixture of six RA FLS exosomes, a mixture of six RA miR-124 FLS exosomes, and a mixture of five OA FLS exosomes were washed twice with ultrapure water (Fujifilm Wako Pure Chemical Corporation) and then resuspended in ultrapure water. The RA FLS exosomes and RA miR-124 FLS exosomes were obtained from the same patients. Nanoscale LC-MS/MS analysis was performed by Hakarel Co.

(Ibaraki, Japan). Exosomes were dried, dissolved in 100 mM Tris buffer pH 8.5 with 0.5% sodium dodecanoate, and subjected to cleavage of protein disulfide bonds, alkylation of cysteine residues, and peptide fragmentation. The peptides were desalted, dried, dissolved in 3% acetonitrile with 0.1% formic acid, and quantified by bicinchoninic acid (BCA) protein assay. Nanoscale LC-MS/MS analysis was performed using an UltiMate 3000 RSLCnano LC system and Q Exactive HF-X mass spectrometer (both from Thermo Fisher Scientific). The analysis was performed using Scaffold DIA software (Proteome Software, Portland, OR, USA), and functional annotation was performed using DAVID (https://david.ncifcrf.gov/summary.jsp).

2.7. Analysis of miRNAs

Small RNAs were extracted from a mixture of six RA FLS exosomes, a mixture of six RA miR-124 FLS exosomes, and a mixture of OA FLS exosomes using the mirVana™ miRNA Isolation Kit (Thermo Fisher Scientific). Reverse transcription of the small RNAs was performed with the miScript II RT Kit (Qiagen), and 756 types of miRNA were detected with miRNome PCR panels (Qiagen), according to the manufacturer's instructions. The RA FLS exosomes and RA miR-124 FLS exosomes were obtained from the same patients.

2.8. Transfection of siRNA

To knockdown tumor necrosis factor-inducible gene 6 protein (TNFAIP6), proteasome 20S subunit beta 5 (PSMB5), and pentraxin 3 (PTX3), 100 pmol of each FlexiTube siRNA (Hs_TNFAIP6_6, HS_PSMB5_11, and Hs_PTX3_1, respectively, Qiagen) and 20 μl of Lipofectamine RNAiMAX (Thermo Fisher Scientific) were mixed in 1 ml of Opti-MEM (Thermo Fisher Scientific) and incubated for 20 min at room temperature. Each mixture was then incubated with RA FLS or OA FLS at 37 °C in a 5% CO_2 incubator for 48 h.

2.9. Wester blotting

Western blotting was performed according to standard procedures. Anti-Pentraxin 3/PTX3 abtibody [EPR6699] ab125007, Anti-PSMB5/MB1 antibody ab3330, anti-beta action antibody and Goat Anti-Rabbit IgG H&L (HRP) (ab6721) secondary antibody were purchased from abcom (Cambridge, England). Specific bands were detected by chemiluminescenceusing ECL Plus detection reagents (Amersham Japan, Tokyo, Japan).

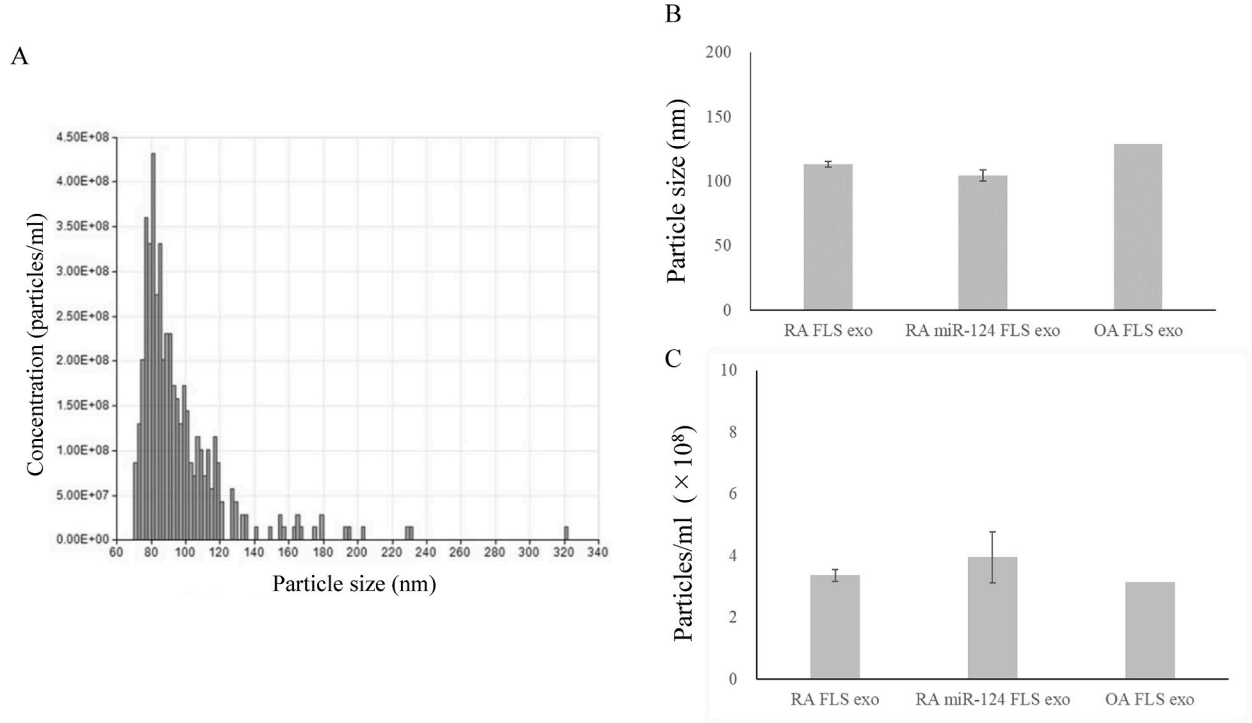

Fig. 1. Characteristics of RA FLS, RA miR-124 FLS, and OA FLS exosomes are similar Exosomes were quantified and characterized using tunable resistive pulse sensing with qNano. (A) Particle size distribution of RA FLS miR-124 exosomes. (B) Particle sizes of RA FLS exosomes (n = 3), RA FLS miR-124 exosomes (n = 3), and OA FLS exosomes (n = 1). (C) Number of exosomes derived from RA FLS (n = 3), RA FLS miR-124 (n = 3), and OA FLS (n = 1). RA FLS exo: exosomes derived from fibroblast-like synoviocytes from patients with rheumatoid arthritis; RA miR-124 FLS exo: exosomes derived from hsa-miR-124-3p-transfected fibroblast-like synoviocytes from patients with rheumatoid arthritis; OA FLS exo: exosomes derived from fibroblast-like synoviocytes from patients with osteoarthritis. Values are plotted as mean ± SE.

2.10. Statistics

The Mann–Whitney *U* test and Wilcoxon signed-rank test were used to evaluate the significance of differences between the two groups. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Characteristics of exosomes derived from RA FLS, RA miR-124 FLS, and OA FLS are similar

Fig. 1A shows the particle size distribution of RA miR-124 FLS exosomes determined using qNano. The mean, mode, the 10% of the cumulative distribution, and the 90% of the cumulative distribution were 96, 81, 77, and 119 nm, respectively. Moreover, we identified 1628 proteins in RA FLS exosomes, RA miR-124 FLS exosomes and OA FLS exosomes by proteomic analysis, and 927 proteins (56.7%) were classified as 'extracellular exosome' by DAVID Functional annotation. These results suggest that exosomes can be collected.

There was no significant difference between the mean particle sizes of RA FLS and RA miR-124 exosomes, and the mean particle size of OA FLS exosomes was similar to that of RA FLS and RA miR-124 FLS exosomes (Fig. 1B).

There was no significant difference in the number of RA FLS and RA miR-124 FLS exosomes, and the number of OA FLS exosomes was similar to that of RA FLS and RA miR-124 FLS exosomes (Fig. 1C).

3.2. MiRNAs contained in RA FLS, RA miR-124 FLS, and OA FLS exosomes are similar

We examined 756 miRNAs in a mixture of six RA FLS exosomes, a mixture of six RA miR-124 FLS exosomes, and a mixture of five OA FLS exosomes by real-time PCR. Seventy-one miRNAs were identified. Only the level of miR-124-3p in the RA miR-124 FLS exosomes was higher than that in the RA FLS and OA FLS exosomes. The other miRNA levels did not differ between the RA FLS, RA miR-124 FLS, and OA FLS exosomes (Fig. 2).

3.3. Differential amounts of proteins contained in RA FLS, RA miR-124 FLS, and OA FLS exosomes

Next, we performed proteomic analysis of a mixture of six RA FLS exosomes, a mixture of six RA miR-124 FLS exosomes, and a mixture of five OA FLS exosomes by nanoscale LC-MS/MS (Fig. 3A and B). A total of 1628 proteins were identified, and 1613 of them (99%) were found in all three types of exosomes: RA FLS, RA miR-124 FLS, and OA FLS exosomes. The amounts of 1291 of the proteins (80%), including common exosome molecules, such as proteins CD9, CD63, and CD81 did not differ among the three exosomes. The levels of 43 proteins (3%) in RA miR-124 FLS exosomes, 99 proteins (6%) in OA FLS exosomes, and 16 proteins (1%) in both RA miR-124 FLS and OA FLS exosomes were two-fold higher than those in the RA FLS exosomes. The levels of 72 proteins (4%) in RA miR-124 FLS exosomes, 108 proteins (7%) in OA FLS exosomes, and 27 proteins (2%) in both RA miR-124 FLS and OA FLS exosomes were two-fold lower than those in the RA FLS exosomes These proteins included TNF-α-stimulated proteins PTX3 and TNFAIP6. However, proinflammatory cytokines such as IL-6 and TNF-α were not detected (Fig. 3C and D). Functional annotation of the proteins that were decreased compared with the RA FLS exosome levels was performed using DAVID, and these proteins were found to be involved in cell migration (Fig. 3E and F). Specifically, we focused on PTX3 and TNFAIP6, (whose levels were much higher in the RA FLS exosomes than in both the RA miR-124 FLS and OA FLS exosomes), as well as PSMB5, (the expression of which was higher in the OA FLS and RA miR-124 FLS exosomes than in the RA FLS exosomes).

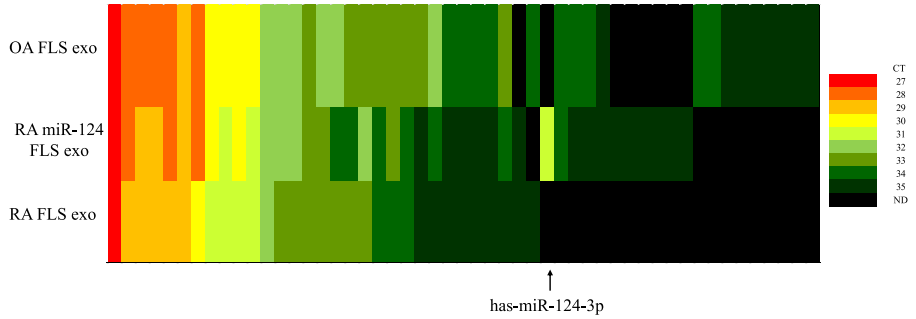

Fig. 2. The level of miR-124-3p in RA miR-124 FLS exosomes was only higher than in RA FLS exosomes and OA FLS exosomes. The other miRNA levels did not differ between the RA FLS exosomes, RA miR-124 FLS exosomes and OA FLS exosomes. Seven hundred and fifty-six microRNAs in a mixture of RA FLS exo from six patients, a mixture of RA miR-124 FLS exo from six patients (the same patients as with RA FLS exo), and a mixture of OA FLS exo from five patients were analyzed by real-time PCR. Seventy-one microRNAs that were detected are shown by a heat map. RA FLS exo: exosomes derived from fibroblast-like synoviocytes from patients with rheumatoid arthritis; RA miR-124 FLS exo: exosomes derived from hsa-miR-124-3p-transfected fibroblast-like synoviocytes from patients with rheumatoid arthritis; OA FLS exo: exosomes derived from fibroblast-like synoviocytes from patients with osteoarthritis; CT: threshold cycle; ND; not detected.

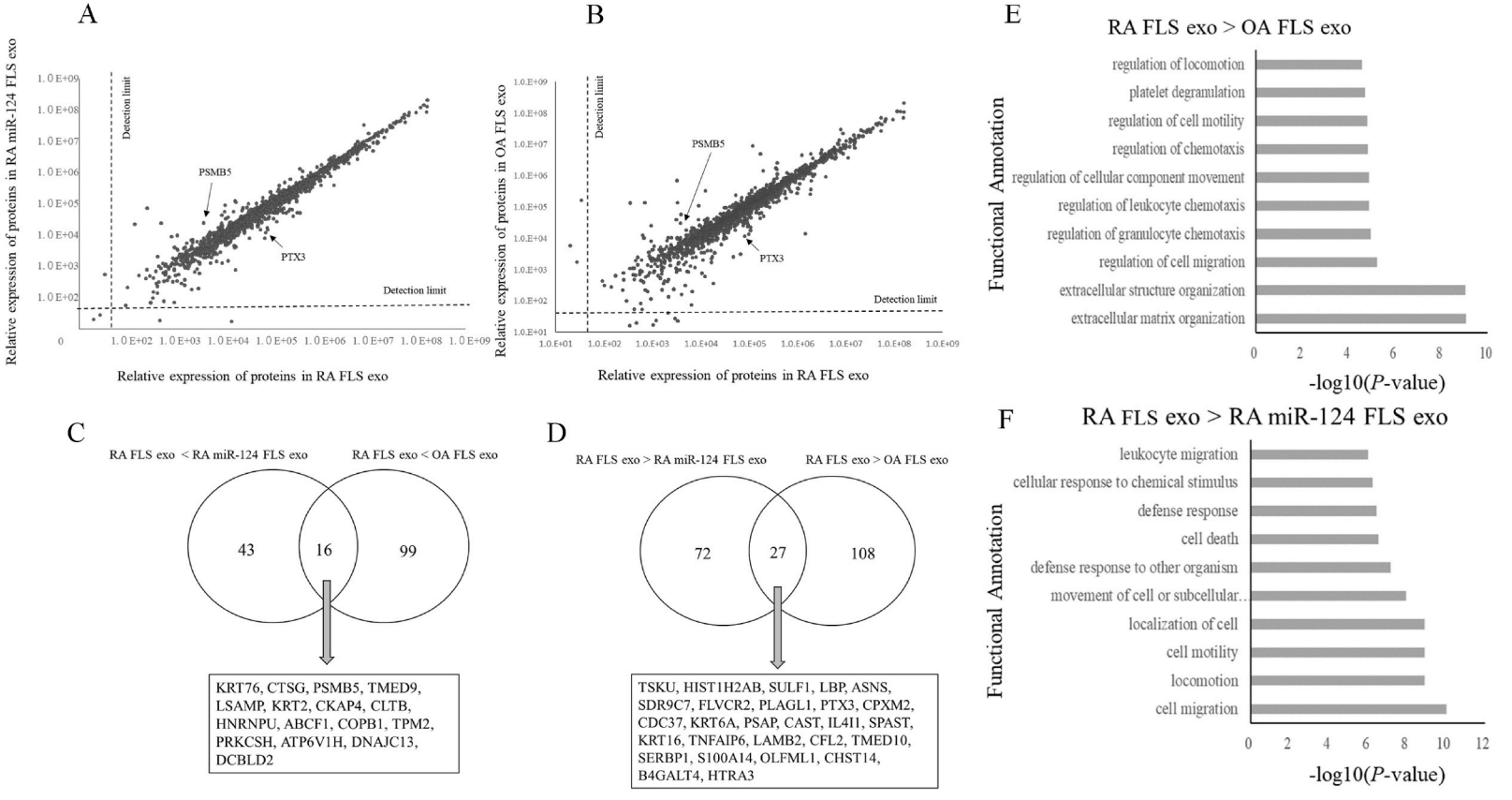

Fig. 3. Proteomic analysis revealed that RA FLS exosomes contained high amounts of proteins involved in cell migration A mixture of RA FLS exosomes from six patients, a mixture of RA miR-124 FLS exosomes from six patients (the same patients as with RA FLS exosomes), and a mixture of OA FLS exosomes from five patients were analyzed by nanoscale liquid chromatography-tandem mass spectrometry (LC-MS/MS). (A) Correlation plot of RA FLS and RA miR-124 FLS exosomes. (B) Correlation plot of RA FLS and OA FLS exosomes. (C) Venn diagram of two times increased proteins in RA miR-124 FLS exosomes and OA FLS exosomes compared to RA FLS exosomes. (D) Venn diagram of two times decreased proteins in RA miR-124 FLS exosomes and OA FLS exosomes relative to RA FLS exosomes. (E) The 2-fold decrease in proteins in OA FLS exosomes relative to RA FLS exosomes was analyzed by DAVID. (F) The 2-fold decrease in proteins in RA miR-124 FLS exosomes relative to RA FLS exosomes was analyzed by David. RA FLS exo: exosomes derived from fibroblast-like synoviocytes from patients with rheumatoid arthritis; RA miR-124 FLS exo: exosomes derived from hsa-miR-124-3p-transfected fibroblast-like synoviocytes from patients with rheumatoid arthritis; OA FLS exo: exosomes derived from fibroblast-like synoviocytes from patients with osteoarthritis.

3.4. RA FLS, RA miR-124 FLS, and OA FLS exosomes do not affect expression of inflammatory cytokines in M1 macrophages

Although exosomes did not contain inflammatory cytokines, we investigated whether exosomes affect the production of pro-inflammatory cytokines in M1 macrophages using real-time PCR. To confirm whether M1 macrophages were induced, mRNA levels of *IL1B*, *IL6* and *TNF* were measured before and after induction. As the results, mRNA expressions of *IL1B*, *IL6* and *TNF* in M1 macrophage was increased compared to before induction, 8-fold, 39-fold and 7-fold respectively.

We investigated whether 15 μg/ml RA FLS, RA miR-124 FLS, and OA FLS exosomes affected the expression of inflammatory cytokines in M1 macrophages. As the results in Fig. 4 show, treating macrophages with each exosome did not affect the mRNA expression of *TNF*, *IL6*, and *IL1B* in M1 macrophages.

3.5. RA FLS exosomes promote macrophage migration and RA miR-124 FLS exosomes suppress macrophage migration

The Transwell migration assay revealed that the RA FLS exosomes enhanced macrophage migration compared with the effect of the OA FLS exosomes. The effects of the OA FLS and NH FLS exosomes on macrophage migration were comparable. In addition, the RA miR-124 FLS exosomes suppressed macrophage migration compared with that of the RA FLS exosomes (Fig. 5A and B).

3.6. Exosomes derived from PTX3-Silenced RA FLS suppress macrophage migration and exosomes derived from PSMB5-Silenced OA FLS promote macrophage migration

We collected exosomes derived from PTX3-silenced RA FLS (RA siPTX3 FLS), exosomes derived from TNFAIP6-silenced RA FLS (RA siTNFAIP6 FLS), and exosomes derived from PSMB5-silenced OA FLS (OA siPSMB5 FLS), which had been generated using siRNAs. The RA siPTX3 FLS exosomes significantly suppressed macrophage migration compared with that of the RA FLS exosomes. The OA siPSMB5 FLS exosomes promoted macrophage migration compared with that of the OA FLS exosomes. It was confirmed by western blotting whether the protein levels were decreased by siRNA. As the result, the protein levels of PTX3 in RA FLS and PSMB5 in OA FLS were decreased by siRNA (Fig. 6A–C).

4. Discussion

Exosomes are widely involved in the maintenance of homeostasis and the regulation of physiological functions, such as in cancer and autoimmune diseases. Several mechanisms for the cellular uptake of exosomes have been considered [26], including energy-dependent receptor-mediated endocytosis [27], micropinocytosis [27], phagocytosis [28], and endosomal-mediated fusion [29]. Various types of cells take up exosomes, among which macrophages are the most well-known [26].

B cell-derived exosomes carry major histocompatibility complex (MHC) I, MHC II, CD45RA, and other adhesion and costimulatory

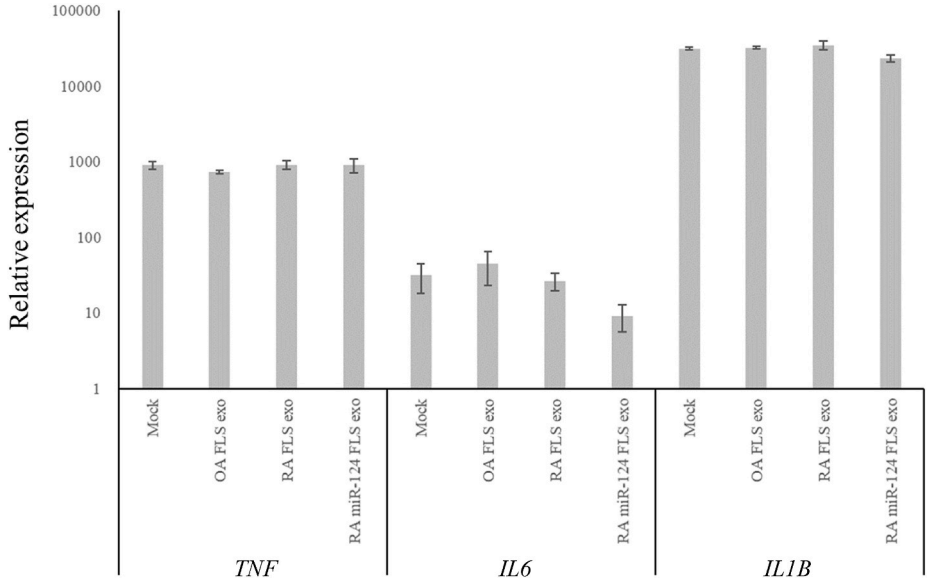

Fig. 4. Exosomes do not affect production of inflammatory cytokines from macrophages The expression of inflammatory cytokines in THP-1 macrophages treated with 15 μg/ml OA FLS, RA miR-124 FLS, and OA FLS exosomes was measured by real-time PCR. Mock: no exosomes; RA FLS exo: exosomes derived from fibroblast-like synoviocytes from patients with rheumatoid arthritis; RA miR-124 FLS exo: exosomes derived from hsa-miR-124-3p-transfected fibroblast-like synoviocytes from patients with rheumatoid arthritis; OA FLS exo: exosomes derived from fibroblast-like synoviocytes from patients with osteoarthritis. Values are plotted as mean ± SE (n = 3).

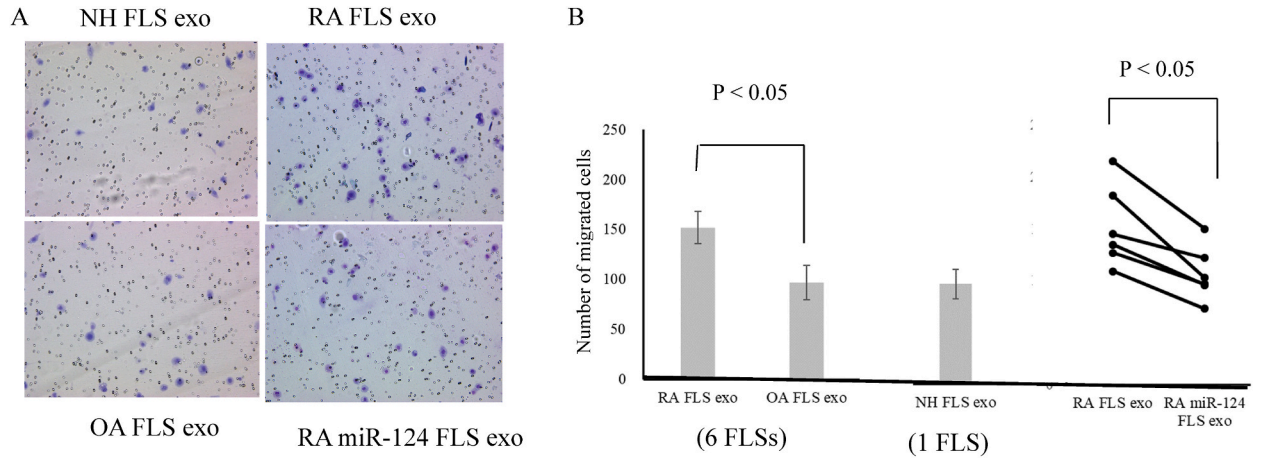

Fig. 5. RA FLS exosomes increase macrophage migration and miR-124 suppresses macrophage migration by RA FLS exosomes The effects of RA FLS, RA miR-124 FLS, OA FLS, and NH FLS exosomes on macrophage migration were evaluated using a Transwell migration assay. (A) Migrated cells were stained with Giemsa stain. (B) Migrated cells were counted in five fields at 100 × magnification. RA FLS exosomes (n = 6) and RA miR-124 FLS exosomes (n = 6) were obtained from the same six patients with RA. OA FLS exosomes (n = 6) were obtained from six patients with OA. NH FLS exosomes (n = 5) were obtained one purchased cell preparation. Values are plotted as mean ± SE. RA FLS exo: exosomes derived from fibroblast-like synoviocytes from patients with rheumatoid arthritis; RA miR-124 FLS exo: exosomes derived from miR-124-3p-transfected fibroblast-like synoviocytes from patients with rheumatoid arthritis; OA FLS exo: exosomes derived from fibroblast-like synoviocytes from patients with osteoarthritis. NH FLS exo: exosomes derived from fibroblast-like synoviocytes from healthy volunteers.

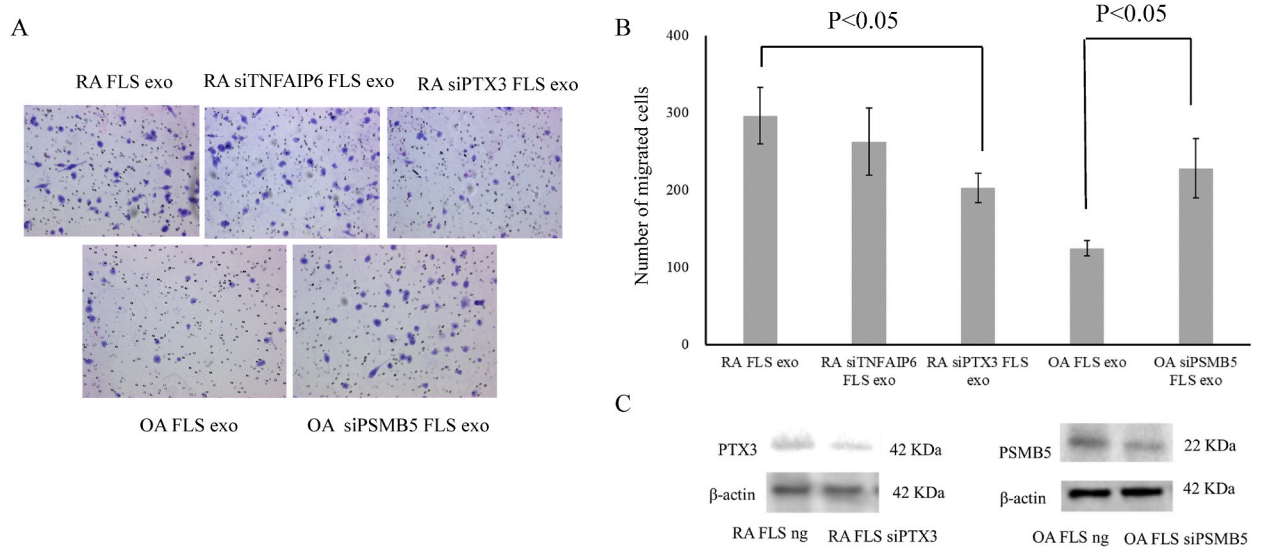

Fig. 6. PTX3 promotes macrophage migration and PSMB5 inhibits macrophage migration The effects of PTX3 and PSMB5 on macrophage migration were evaluated using siRNA and a Transwell migration assays. (A) Migrated cells were stained with Giemsa stain. (B) Migrated cells were counted in five fields at 100 × magnification. Values are plotted as mean ± SE. RA FLS exo (n = 4): exosomes derived from fibroblast-like synoviocytes from patients with rheumatoid arthritis. OA FLS exo (n = 5): exosomes derived from fibroblast-like synoviocytes from patients with osteoarthritis. RA siTNFAIP2 FLS exo (n = 5): exosomes derived from fibroblast-like synoviocytes (in which levels of TNF-α induced protein 2 (TNFAIP2) were reduced by siRNA) from patients with rheumatoid arthritis. RA FLS siPTX3 FLS exo (n = 5): exosomes derived from fibroblast-like synoviocytes (in which levels of PENTRAXIN 3 (PTX3) were reduced by siRNA) from patients with rheumatoid arthritis. OA siPSMB5 FLS exo (n = 4): exosomes derived from fibroblast-like synoviocytes (in which levels of proteasome 20S subunit beta 5 (PSMB5) were reduced by siRNA) from patients with osteoarthritis. Values are plotted as mean ± SE. (C) The protein level of PTX3 in RA FLS and PSMB5 in OA FLS treated with siRNA were examined by western blotting.

molecules, leading to immune responses [30]. Tumor-derived exosomes regulate macrophage polarization [31]. In mice with collagen-induced arthritis, granulocytic myeloid-derived suppressor cell-derived exosomes (G-exos) efficiently reduce the mean arthritis index, leukocyte infiltration, and joint destruction. G-exos also decreased the percentage of Th1 and Th17 cells both *in vivo* and *in vitro* [32]. However, it is unclear how these findings relate to the pathology of RA. Therefore, we collected exosomes from the culture

medium of FLS by ultracentrifugation and investigated the effects of RA FLS and RA miR-124 FLS exosomes on macrophages. We found that RA FLS and RA miR-124 FLS exosomes did not affect the expression of inflammatory cytokines in macrophages. However, RA FLS exosomes promoted macrophage migration, more than that promoted by RA miR-124 FLS exosomes.

The total plasma EV number was higher in RA patients than in healthy controls, and the total number of EVs was higher in RA synovial fluid (SF) than in plasma and OA SF [33]. Therefore, we investigated the number of exosomes derived from RA FLS, RA miR-124 FLS, and OA FLS. Our results showed no difference between RA FLS, RA miR-124 FLS, and OA FLS exosome size and number.

Exosomes contain proteins and various forms of RNA such as miRNAs, long noncoding RNAs (lncRNAs), and mRNAs. We investigated miRNAs and proteins in exosomes to determine which molecules are involved in macrophage migration. Several reports have shown that exosomal miRNAs play important roles in immune responses, tumor progression, and neurodegenerative disorders [34]. We studied 756 miRNAs using real-time PCR. However, the miRNAs contained in RA FLS, RA miR-124 FLS, and OA FLS exosomes were not different, except for miR-124-3p in RA miR-124 FLS.

RA SF EVs contained more citrullinated peptides and IgG than RA plasma and healthy control plasma [35]. Exosomes containing TLR3 were higher in RA plasma than in OA and healthy control plasma [36]. Therefore, we performed proteomics analysis and revealed no difference between the proteins in RA FLS, RA miR-124, and OA FLS exosomes. Zhang et al. detected TNF-α-binding membranes in RA FLS exosomes. Exosomes containing TNF-α activate protein kinase B (PKB or AKT) and nuclear factor-kappa B (NF-κB), rendering activated T cells resistant to apoptosis [37]. In our study, the RA FLS exosomes contained TNF-α-induced proteins such as PTX3 and TNFAIP6, but they did not contain TNF-α. This discrepancy was considered to be due to differences in the recovery of exosomes and cells. We collected exosomes by centrifugation at 210,000×*g*, while Zhang et al. collected exosomes at 70,000×*g* [37]. The composition of proteins contained in EVs is significantly different depending on the type of EV [38]. The composition of proteins contained in two exosome subpopulations Exo-h (90–120 nm), and Exo-S (60–80 nm), and non-membranous nanoparticles (~50 nm, exomers) were previously shown to be different [39]. In addition, we used FLS from passages 3 to 7, whereas Zhang et al. used primary FLS. Primary RA FLS are composed of 15% $CD14^+$ cells (probably macrophages), while fourth-passage RA FLS are composed of <1% $CD14^+$ cells [40].

Functional annotation using DAVID revealed that the proteins that were more abundant in RA FLS exosomes than in RA miR-124 FLS and OA FLS exosomes were involved in cell migration. Therefore, we focused on PTX3, TNFAIP6, and PSMB5 in this study.

We collected RA siPTX3 FLS, RA siTNFAIP6 FLS, and OA siPSMB5 FLS exosomes. The RA siPTX3 FLS exosomes suppressed the migration of macrophages compared to that of the RA FLS and RA siTNFAIP6 FLS exosomes.

RA FLS constitutively express high levels of PTX3 [41]. PTX3 is an inflammatory modulator of innate immunity that is synthesized by inflammatory factors including LPS, IL-1β, and TNF-α [41]. PTX3 has been implicated in angiogenesis, atherosclerosis, cellular proliferation, and tumor escape from immune surveillance. Choi et al. reported that PTX3 promotes tumor cell migratory potential in gastric cancer, and that gastric cancer-derived PTX3 promotes macrophage recruitment [42]. Kim et al. reported that PTX3 is involved in osteogenic/odontogenic differentiation and migration of human dental pulp stem cells [43]. Therefore, RA FLS exosomes, which contain abundant PTX3, were considered to promote macrophage migration. The promoter of PTX3 has NF-κB and activator protein 1 (AP1) binding sites [44]. NF-κB signaling plays important roles in multiple biological processes, including immune response, differentiation, cell survival, proliferation, and migration. MiR-124 was previously shown to downregulate the expression of tumor necrosis factor receptor-associated factor 6 (TRAF6) and NF-κB p65, leading to suppression of the NF-κB pathway [45,46]. Therefore, it was considered that the level of PTX3 was lower in RA miR-124 FLS exosomes than in RA FLS exosomes.

The OA siPSMB5 FLS exosomes promoted the migration of macrophages more than OA FLS exosomes. PSMB5 is an important component of the 20S proteasome and plays an important role in the hydrolysis of proteasome substrates [47]. The ubiquitin proteasome system (UPS) is one of the main routes responsible for the degradation of most intracellular proteins that are misfolded, damaged, or unneeded [48]. Proteasome function or activity is essential for a wide variety of cellular processes such as transcriptional regulation, cell cycle progression, apoptosis, cell growth, immune responses, trafficking, and signaling [48]. Silencing PSMB5 expression with shRNA in MDA-MB-231 breast cancer cells suppressed their growth and migration [49]. Overexpression of miR-127-3p inhibits prostate cancer cell invasion and migration *in vitro* by targeting PSMB5 [50]. Our findings show that OA siPSMB5 FLS exosomes promote macrophage migration. We believe that the OA siPSMB5 FLS exosomes contained more abundant proteins related to cell migration than the OA FLS exosomes due to the loss of UPS function.

MiR-124 inhibits lung cancer cell migration and invasion by directly suppressing the expression of zinc finger E-box binding homeobox 1 (ZEB1) [51] and ZEB1 has been shown to promote macrophage migration [52]. The RA miR-124 FLS exosomes contained miR-124. It was considered that miR-124 in the RA miR-124 FLS bound to ZEB1 after uptake by macrophages and suppressed the macrophage migration.

Collectively, these findings suggest that the enhanced macrophage migration promoted by RA FLS exosomes involves PTX3 and PSMB5, and that RA miR-124 FLS exosomes suppress macrophage migration, both of which are significantly involved in the etiology of RA. These findings should be considered in future strategies for diagnosing and treating RA.

Author contribution statement

Yuji Nakamachi: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Kenichi Uto: Performed the experiments; Analyzed and interpreted the data.

Shinya Hayashi, Akio Morinobu, Ryosuke Kuroda: Contributed reagents, materials, analysis tools or data.

Takaichi Okano: Analyzed and interpreted the data.

Seiji Kawano, Jun Saegusa: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

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Data availability statement

The data that has been used is confidential.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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