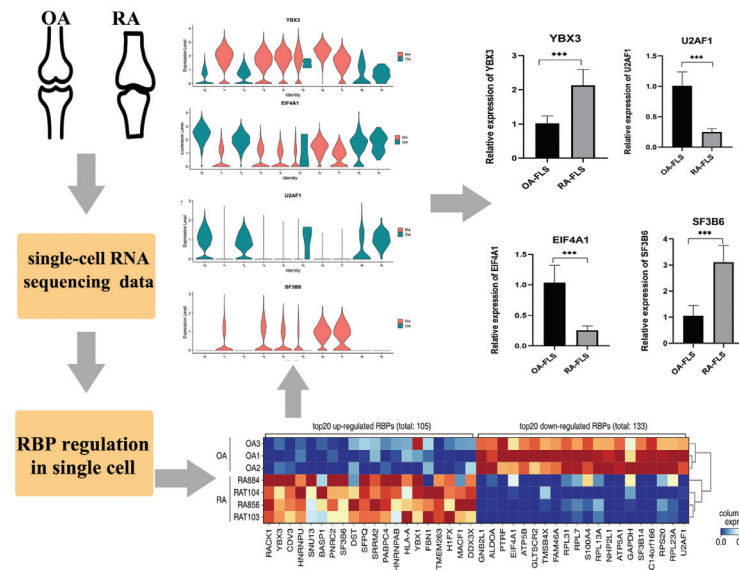


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Deciphering the regulatory programs of RNA binding proteins in rheumatoid arthritis through single-cell transcriptome analysis

Graphical abstract



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In brief

Dysregulation of RBPs may contribute to the development of RA and provide potential targets for therapeutic interventions.

Highlights

- RNA-binding proteins (RBPs) could serve as potential therapeutic targets for the development of drugs to treat rheumatoid arthritis (RA).
- The upregulation of YBX3 and SF3B6 and downregulation of EIF4A1 and U2AF1 may contribute to the development of RA through single-cell transcriptome analysis.
- YBX3 and SF3B6 mRNA were confirmed to be upregulated in RA-FLS and CIA mice, while EIF4A1 and U2AF1 mRNA were downregulated in RA-FLS.

Deciphering the regulatory programs of RNA binding proteins in rheumatoid arthritis through single-cell transcriptome analysis

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ABSTRACT

Single-cell RNA sequencing (scRNA-seq) data from published datasets were obtained to investigate the expression and dysregulation of RNA-binding proteins (RBPs), which are critical for alternative mRNA splicing and translational control in rheumatoid arthritis (RA). How RBP regulation differs between RA and osteoarthritis (OA) was examined using RBP for single-cell sub-clustering. Quantitative polymerase chain reactions (PCRs) were performed to confirm differentially expressed RBPs in RA fibroblast-like synoviocytes (FLSs) and OA-FLSs, as well as in mice with collagen-induced arthritis (CIA) and control mice. Additionally, bulk RNA-seq data were collected and RBP-alternative splicing event (ASE) co-expression analyses were performed to reveal the potential regulatory role of RA-related RBPs on ASEs. Significant variations in relative proportions of cell subtypes were demonstrated between RA and OA with downregulated RBPs outnumbering upregulated RBPs in each cell type and showing high specificity for particular subsets. One hundred five upregulated and 133 downregulated RBPs were identified in fibroblasts. Y-Box binding protein 3 (YBX3) and splicing factor 3b subunit 6 (SF3B6) were confirmed to be upregulated in RA-FLS and CIA mice, while eukaryotic translation initiation factor 4A1 (EIF4A1) and U2 small nuclear RNA auxiliary factor 1 (U2AF1) were downregulated in RA-FLS. The RA group displayed stronger cell type interactions compared to the OA group with enhanced signaling pathways, such as fibronectin 1-cluster of differentiation 44 (FN1-CD44) and C-X-C motif chemokine ligand 12-C-X-C motif chemokine receptor 4 (CXCL12-CXCR4). Furthermore, three upregulated genes (spectrin repeat containing nuclear envelope protein 2 [SYNE2], S100 calcium binding protein A9 [S100A9], and interferon induced protein with tetratricopeptide repeats 3 [IFIT3]) and four downregulated genes (ribonuclease 1 [RNASE1], granulysin [GRN], FN1, and sorbin and SH3 domain containing 2 [SORBS2]) were co-expressed in RA-associated RBPs and ASEs. These findings suggest that dysregulation of RBPs may contribute to the development of RA and provide potential targets for therapeutic interventions.

Keywords: scRNA-seq, RNA binding proteins, rheumatoid arthritis, cell-to-cell communication, fibroblasts

1. INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune disorder characterized by persistent joint inflammation and deformity. RA significantly impacts daily activities, work performance, and quality of life [1]. RA has a global prevalence ratio of approximately 1% and is more prevalent in women than men [2]. Unlike osteoarthritis (OA), which is characterized by cartilage wear

on the inner bone surface of joints, RA is an inflammatory arthritis in which the immune system attacks the joints starting from the synovial membrane. The management of RA has significantly advanced over the past few decades, aiming to control symptoms, prevent joint damage, and improve overall patient outcomes. Despite these advances, a significant proportion of patients may not respond adequately to current therapies, justifying a need for further research into the

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mechanisms underlying RA and novel therapeutic targets.

Endothelial cells, T and B cells, monocytes, and fibroblasts are among the cell types that contribute to the inflammatory response in synovial tissues affected by RA. Endothelial cell activation and proliferation result in angiogenesis and leukocyte infiltration, actively recruiting immune cells into the tissue and leading to an expanding synovial pannus [3]. The pannus is characterized by intimal lining layer hyperplasia and often invades nearby bone, causing erosion [4]. Fibroblast-like synoviocytes (FLSs) are considered the primary invasive cells in RA [5]. Understanding the role of these cell types and the underlying mechanisms is crucial for developing effective treatments and identifying new targets for RA therapy.

RNA-binding proteins (RBPs) are a group of proteins that bind to RNA. RBPs are essential in regulating gene expression through various post-transcriptional processes, such as mRNA splicing, maturation, degradation, subcellular localization, RNA editing, and translation [6]. However, RBP dysregulation has been linked to diseases, including immune-based disorders and RA [7-11]. Various cellular events and disease processes are influenced by RBPs. For example, PUM1 interacts with the 3'-untranslated region (UTR) of Toll-like receptor 4 (TLR4) mRNA to control translation and regulate nuclear factor- κ B (NF- κ B) and helps maintain the chondrogenic phenotype in human chondrocytes, thereby mitigating cellular OA [12]. The loss of functional muscleblind-like protein 1 (MBNL-1), which is due to sequestration by mutant transcripts with expanded CUG repeats, results in myotonic dystrophy type 1 [13]. Furthermore, mitochondrial RBP tumor necrosis factor receptor-associated protein 1 (TRAP1)-linked chaperone-mediated autophagy can induce ferroptosis [14]. RBPs have also been highlighted as prognostic markers and therapeutic targets in cancer. For example, elevated levels of RNA-binding motif protein 38 (RBM38) are correlated with improved overall survival in patients with ovarian cancer, breast cancer, and gliomas [15] but predict poorer outcomes in patients with breast cancer [16]. Dysregulated expression of several RBPs has been reported in RA and studies have suggested the critical role in RA pathogenesis through modulation of key regulatory mechanisms [17]. However, comprehensive evaluations of the characteristics and patterns of RBP expression across the various cell types involved in RA are limited.

Single-cell RNA sequencing (scRNA-seq) is a powerful tool for accurately evaluating gene expression in specific cell types. scRNA-seq facilitates the exploration of molecular mechanisms underlying the initiation and development of RA [18]. scRNA-seq allows for identification of individual cell gene expression levels, highlighting intercellular heterogeneity [18, 19]. Recent studies have utilized scRNA-seq techniques to identify cell-specific gene expression during the onset and progression of RA [20-22]. Given the limited research on the role of RBPs in RA using single-cell sequencing data, the

objective in this study was to leverage scRNA-seq datasets to uncover intrinsic characteristics and aberrant patterns of RBP expression across diverse cell types involved in RA. Such an analysis may reveal the pivotal role of RBPs in the pathogenesis and treatment of RA.

2. MATERIALS AND METHODS

2.1 scRNA-seq data retrieval and process

A count matrix of unique molecular identifiers (UMIs) was obtained for four RA and three OA samples from GSE200815 and GSE152805, respectively, to process scRNA-seq data. This matrix was transformed into a Seurat object using the R package Seurat (version 4.0.4) [23]. Low-quality cells were removed from further analysis based on UMI counts <1000, gene detection <500 cells, and UMI counts derived from mitochondrial genes >15%. Additionally, genes detected in less than five cells were excluded from downstream analyses.

2.2 scRNA-seq data pre-processing and quality control

Log normalization was applied to the data after performing quality control on the UMI count matrix. Using the Find Integration Anchors function of Seurat, the top 2000 variable genes were used to create potential anchors. Principal component analysis (PCA) was used on the integrated data matrix to further reduce the dimensionality of the scRNA-Seq dataset. The top 50 PCs were selected for downstream analyses based on the Elbow plot function of Seurat. Clusters of main cell types were identified using the Find Clusters function of Seurat with the default resolution setting (res = 0.6). Cell types were annotated using ScType tools [24], which utilized gene markers for each cell cluster identified by the Find Markers function in the Seurat package (v4.0.4). OA marker genes were also used for cell type annotation [25]. Finally, t-distributed stochastic neighbor embedding (tSNE) or Uniform Manifold Approximation and Projection (UMAP) plots were used to visualize major cell types.

2.3 scRNA-seq differential gene expression analysis

The Seurat package FindMarkers/FindAllMarkers function was used with a one-tailed Wilcoxon rank-sum test and adjusted *P*-values using Bonferroni correction for multiple testing to identify differentially expressed genes (DEGs) [25]. Genes were considered for DEG computation if the expression on a natural log scale differed by at least 0.5 and the percentage of detected cells differed by at least 0.15. Furthermore, adjusted *P*-values < 0.05 were required to ensure statistical significance of the results.

2.4 RBP clustering analysis

A comprehensive catalog of 2141 RBPs (Supplementary Table 1) was compiled from previous studies [6, 26].

The UMI count matrix of RBPs was utilized as input for Seurat to perform cell clustering [27]. Differentially activated RBPs were identified using the Find All Markers function in the Seurat package. Co-expression associations between differential RBP and targeted genes were identified using the “grn” algorithm from the SCENIC Python workflow (version 0.11.2) with default parameters [6]. Cytoscape (v3.9.1) was used to visualize networks of modules consisting of differential RBPs and the target genes. Co-expression analysis was also performed for differential RBPs and the targeted genes in fibroblasts to identify co-expression associations. The AUCell algorithm, an innovative computational method used for single-cell RNA-Seq data analysis that emphasizes gene expression rankings over absolute expression levels was applied to score individual cells based on a predefined set of genes [28].

2.5 Functional enrichment analysis

KOBAS2.0 [29] was used to identify Gene Ontology (GO) terms and KEGG pathways to assess the functional roles of the studied genes. Enriched categories were determined using the hypergeometric test and the significance of each term was assessed using the Benjamini-Hochberg false discovery rate (FDR) controlling procedure. This approach ensured that the enriched GO terms and KEGG pathways were statistically significant and provided meaningful insight into the function of the target genes.

2.6 Cell-to-cell communication

Cell Chat [30] (v1.0.0) was used to identify cell-to-cell interactions in various cell types based on the expression of known ligand-receptor pairs. Standard parameters were used for pre-processing operations, including finding overexpressed genes, placing overexpressed interactions, and projecting data. The human protein-protein interactions database was used as an *a priori* network information. Core functions, such as compute CommunProb, compute CommunProb Pathway, and aggregate Net were applied with standard parameters and fixed randomization seeds for principal analysis. The function net Analysis signaling Role was then applied to the netP data slot to identify the senders and receivers in the network.

2.7 Bulk RNA-seq data retrieval and process

Sequence data files were obtained for five RA tissue and five OA tissue samples from the Sequence Read Archive (SRA) and downloaded from www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE89408. The SRA run files were converted to the fastq format using the NCBI SRA Tool (fastq-dump). Low-quality bases were trimmed from the raw reads using the FASTX-Toolkit (v.0.0.13) and quality evaluation of the resulting clean reads was performed using FastQC.

2.8 Reads alignment and DEG analysis

Clean reads were aligned to the human genome using HISAT2 (v.2.2.1) for RNA-seq data sequencing analysis [31]. Uniquely mapped reads were then selected for further analysis and gene expression levels were estimated using fragments per kilobase of transcript per million (FPKM) [32]. DESeq2 software (version 1.30.1) was used to perform differential gene expression analysis on the reads count file [31]. Fold-change (FC) and the FDR were determined using DESeq2 to identify DEGs with the following significant differential expression criteria: FC values ≥ 2 or ≤ 0.5 with an FDR ≥ 0.05 .

2.9 Alternative splicing analysis

The SUVA (v2.0) pipeline was utilized to quantify and define regulatory alternative splicing (RAS) events [33]. The different splicing patterns for each group of alternative splicing events (ASEs) were analyzed, followed by calculating the proportion of reads supporting each ASE (pSAR) for each SUV ASEs.

2.10 RBP-RAS Co-expression analysis

A comprehensive catalog of 2141 RBPs was compiled from four previous reports [6, 25-27]. These RBPs were overlapped with the set of RAS events (pSAR $\geq 50\%$) and co-expression analysis of RBP-RAS was performed. Pearson correlation analysis was used to evaluate the correlation between each RBP and RAS. RBP-RAS pairs with an absolute correlation coefficient ≥ 0.6 and a *P*-value ≤ 0.01 were identified and selected for downstream analyses.

2.11 Cell isolation, culture, and identification

RA-FLSs and OA-FLSs were isolated and cultured from synovial tissues obtained during knee joint replacement surgeries of four patients each with RA and OA, respectively. Synovial tissue was extracted from the knee joint and cartilage and fat were removed under sterile conditions. The tissue was placed in pre-cooled PBS containing antibiotics for 15 min. The synovial tissue was then cut into small pieces and digested with trypsin and collagenase. The remaining cells were suspended in complete culture medium for human synovial fibroblasts, seeded in a culture dish, and cultured under appropriate conditions. When the cells reached 90% confluence, the cells were passaged and used for subsequent experiments. Vimentin immunofluorescence staining was performed to identify the synovial fibroblasts. This research was approved by the Research Ethics Board of the First Affiliated Hospital of Fujian Medical University (Approval No. MRCTA, ECFAH of FMU [2022]597).

2.12 Quantitative real-time PCR

Total RNA was isolated from RA-FLSs and normal FLSs using TRIzol buffer [5]. The RNA was extracted and reverse-transcribed with a PrimeScript™ RT reagent kit (Takara, Shiga, Japan) and a gDNA eraser (Takara). The

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Table 1 | Primer sequences.

Gene name	Primer sequence (5'-3')
YBX3 (homo)	F: AAGTTCTCGCCACCAAGTC R: CCTTCTCTCCTCAACCACATC
EIF4A1(homo)	F: ACTGGGAAAACGGCCACATT R: GATACAGGCGTGACAGGAGG
U2AF1 (homo)	F: AGTAGAGGAGATGAACGTCTGTG R: GGATCGGCTGTCCATTAAACC
SF3B6 (homo)	F: GGCGAACATTCGACTTCCAC R: TTCAGGTGTGTTCCCCACTC
GAPDH (homo)	F: GGTGTGAACCATGAG AAGTATGA R: GAGTCCTTCCACGAT ACCAAAG

F, forward, R, reverse.

resulting cDNA was analyzed through quantitative PCR (qPCR) utilizing TB Green™ Premix Ex Taq™ II (Takara). The relative mRNA levels were determined using the $2^{-\Delta\Delta CT}$ method with GAPDH as the normalization control.

Table 1 contains a list of primers that were utilized during this process.

2.13 Other statistical analysis

PCA was performed with the R package, factoextra, to showcase sample clustering using the first two components. Clustering was based on the PCA results using Euclidean distance and performed with the R package, pheatmap. The R package, speckle (version: 0.0.3) [34], was also utilized to compare two groups of replicates and providing valuable insight into sample clustering and comparisons between replicate groups. These analyses contribute to a better understanding of the underlying factors in the experiment.

2.14 Animals

Twenty healthy male DBA1/J mice (6–7 weeks old; body weight, 16–18 g) were used in the current study (Guangdong Yaokang Biotechnology Co., Ltd., Guangdong, China). The experimental animal feeding room was kept at a room temperature of 20–26°C and 40%–70% relative humidity with a 12 h day/night cycle. Mice had access to food and water *ad libitum*, bedding was changed daily, and food was provided by the Experimental Animal Center. Animal experiments were carried out in accordance with the *Guidelines for the Care and Use of Laboratory Animals of the Laboratory Animal Center of Fujian Medical University* (Certificate No. SYXK [Fujian] 2022-0003) and the protocol was approved by the Animal Experiment Ethics Review Committee of Fujian Medical University (No. FJMU IACUC 2023-Y-0077).

2.15 Induction of collagen-induced arthritis (CIA)

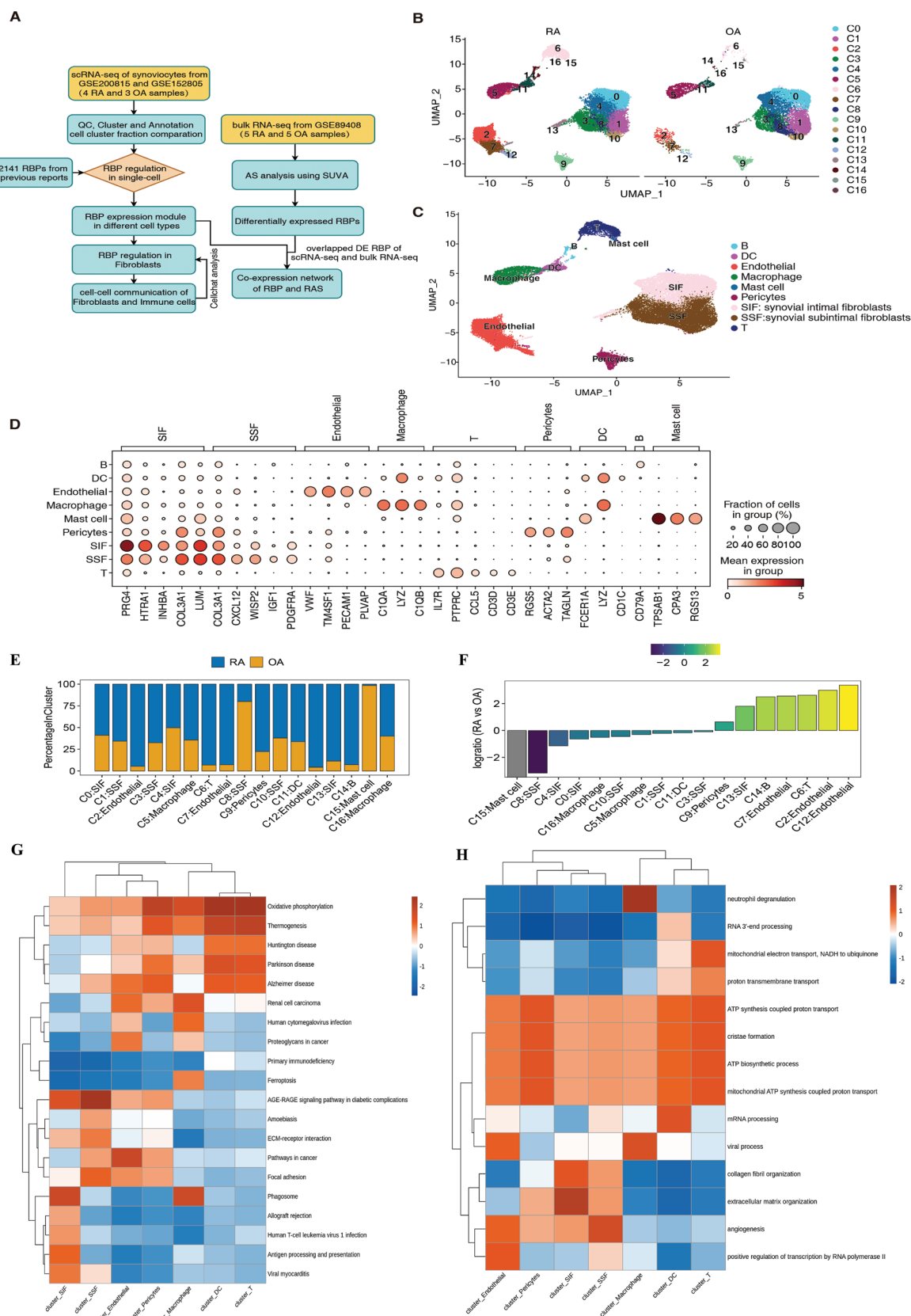
The mice were randomly divided into 2 groups (n = 10 each): control; and model. An emulsion of bovine type II collagen (catalog #L22511C125306; Yuanye, Shanghai, China) and complete Freund's adjuvant [CFA] (catalog #F5881-10ML; Sigma-Aldrich, St. Louis, MO, USA) at a terminal viscosity of 0.5 mg/mL was injected subcutaneously into the caudal root on day 0 and an emulsion of CFA was injected on day 21 in the CIA group. Mice were sacrificed on day 42. The ankle joints were collected, fixed in 4% paraformaldehyde for 48 h, decalcified in 10% ethylenediaminetetraacetic acid solution, and embedded in paraffin. The tissue sections were stained with hematoxylin and eosin (HE), Safranin-O-Fast Green (SaO-FG), and Toluidine blue solution for histopathologic analysis. Arthritis severity was assessed using a 0–4 scoring criterion scale: 0 represents normal; one represents measurable arthritis as well as erythema; two represents significant swelling and redness; three represents severe swelling of the joints and fingers, as well as redness; and four represents the most severe swelling and deformity with ankylosis. The cumulative value of the four paw disease scores is the total arthritis score up to a maximum of 16 points [35].

3. RESULTS

3.1 ScRNA-seq analysis identified significant alterations in cell subtypes between synovial samples from OA and RA patients

The research strategy of the current study is shown in **Figure 1A**. Synovial tissue scRNA-seq data were obtained from two publicly available datasets (GSE200815 and GSE152805) containing samples from four RA and three OA patients, respectively. A total of 33,206 single cells in the transcriptome profile analysis (**Supplementary Table 2**) were included after rigorous quality control. The transcriptomic expression of these cells was normalized and principal component downsizing analysis was performed. The top 50 principal components were selected for UMAP downsizing and visualization and unbiased cluster analysis identified 17 cell subgroups (**Figure 1B**). Differences in the predominant subtypes between OA and RA samples were noted, with C2, C7, and C12 showing significantly higher expression in RA than OA, while C6, C15, and C16 exhibited the opposite trend.

Nine distinct cell types were identified among 17 subgroups of cells, including SIF, SSF, endothelial cells, B cells, T cells, DC, macrophages, mast cells, and pericytes (**Figure 1C**, **Supplementary Table 3**) using characteristic expressed genes of clusters. The top marker genes for each cluster are depicted in **Figure 1D**. The relative number of cell subtypes in the RA and OA groups was compared and significant alterations were found (**Figure 1E, F**). The proportions of various cell types, such as endothelial cells (C2, C12, C7), T cells (C6), B cells (C14), small intensely fluorescent [SIF] (C13), and pericytes (C9),



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Figure 1 | ScRNA-seq analysis identified significant alterations in cell subtypes between synovial samples from OA and RA patients.

A. Schematic illustration of scRNA-seq and bulk RNA-seq data processing. B, C. UMAP plot of composite single-cell transcriptomic profiles from all 7 samples from RA and OA. Colors indicate cell clusters along with annotations. D. Dot plots show the expression of representative genes annotated by cell types in each cell type. E. Stacked bar plot shows the relative proportions of cell populations from RA or OA in each cell type. F. Rank order based on decreasing values of the relative frequency ratio between RA and OA groups. G. KEGG enrichment analysis of biological processes of dysregulated genes of each cell type in two comparison groups. H. Gene ontology enrichment analysis of biological processes of dysregulated genes of each cell type in two comparison groups.

were markedly increased in the RA group compared to the OA group. Conversely, the proportion of cells, such as mast cells (C15), and soluble suppressor factor [SSF] (C8), were decreased in the RA group.

KEGG and GO enrichment analyses were performed (Figure 1G, H) to identify enriched biological processes and pathways in dysregulated genes of each cell type. Enrichment analysis revealed that the endothelial subgroup was significantly enriched in phagosomes and antigen processing and presentation pathways, as well as transcription by RNA polymerase II and angiogenesis. The T cell subgroup was significantly enriched in oxidative phosphorylation and thermogenesis pathways, as well as ATP synthesis and cristae formation.

3.2 ScRNA-seq analysis identified cellular heterogeneity and regulatory modules of specific RBP expression in synovial samples from OA and RA patients

Seurat was used to perform unsupervised clustering analysis and investigate changes in RBP expression patterns between the OA and RA groups. The analysis demonstrated that downregulated RBPs outnumbered upregulated RBPs in all types of cells in RA (Figure 2A). Moreover, the overall area under the receiver operating characteristic curve (AUC) of RBPs were significantly lower in the RA group than the OA group (Figure 2B). Specifically, considerably lower levels of RBP expression in B cells, T cells, and dendritic cells (DCs) and the SSF subgroup were noted in the RA group.

All cells were categorized into 16 RBP subgroups (R0–R15) and the resulting clusters of RBP expression were highly specific to different cell types and disease states (Figure 2C–E). The pattern of RBP expression was highly specific to particular subsets for most cell types with an overwhelmingly dominant RBP-expressing cellular taxon. Some RBP subgroups (R0, R8, R13, and R14) were significantly decreased in RA but increased in OA, while others (R1, R2, R11, and R12) were significantly upregulated in RA but downregulated in OA. GO enrichment analysis demonstrated that different RBP clusters were enriched in various biological processes, including ribosome and protein processing in the endoplasmic reticulum (Figure 2F).

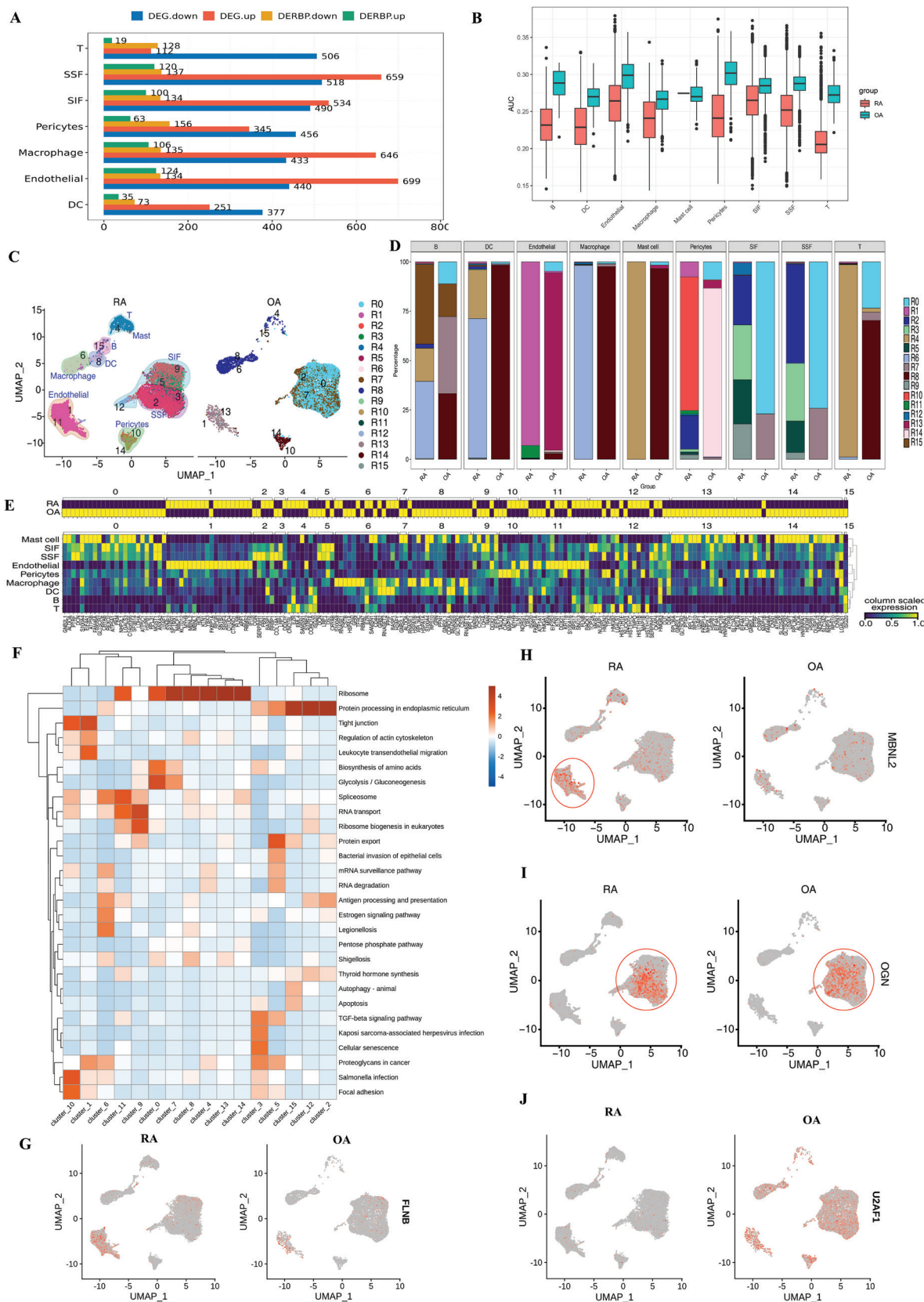
In addition, the expression of specific RBP genes differed across different sample subgroups and cell types. For example, osteoglycin (OGN), a marker for RBP

subgroup R2, and muscleblind-like splicing regulator 2 (MBNL2), a marker for R1, were highly expressed in fibroblasts in the RA group (Figure 2G, H). In contrast, filamin B (FLNB), a marker for RBP subgroup R2, was highly expressed in endothelial cells of the RA group (Figure 2I). U2AF1, a marker for RBP subgroups R0, R13, and R14, was downregulated in each subtype of cells in RA (Figure 2J).

3.3 Functional RBPs are largely regulated in fibroblasts between RA and OA samples

Fibroblast classes were isolated and secondary clustering and analysis were performed using RBP-genes to gain a better understanding of the potential functions of RBPs in fibroblasts in RA and OA. This approach facilitated that classification of fibroblasts into 10 distinct subgroups (FR0–FR9) based on the RBP expression profiles. The RA group primarily expressed FR1, FR3, FR4, FR5, FR6, and FR7, while the OA group mainly expressed FR0, FR2, FR8, and FR9 (Figure 3A, B). The top three marker genes for each cluster are shown in Figure 3C. One hundred five upregulated RBPs and 133 downregulated RBPs were identified by comparing fibroblasts from RA and OA patients (Figure 3D), which were co-expressed with genes enriched in various functional pathways related to extracellular matrix organization, cell adhesion, collagen fibril organization, and cytokine signaling (Figure 3E–G). These pathways suggest potential roles for RBPs in regulating fibroblast proliferation, antigen presentation, and pro-inflammatory responses.

Moreover, differential expression of specific RBPs associated with important cellular pathways in FLSs of RA was shown compared to OA. For example, YBX3 was significantly upregulated, while EIF4A1 was downregulated in the RA group of fibroblasts (Figure 4A, B). Significant differences also existed in the expression of critical splicing factors (U2AF1 and SF3B6) between the RA and OA groups. Specifically, U2AF1 was significantly downregulated (Figure 4A, B), while SF3B6 was significantly upregulated (Figure 4G, H) in the RA group of fibroblasts. To validate these findings, RA-FLSs and OA-FLSs from synovial tissues obtained during knee joint replacement surgeries of four patients each with RA and OA, respectively, were isolated and cultured. The clinical characteristics of the included patients are shown in Table 2. Immunofluorescence staining showed positive expression of vimentin in RA-FLSs and OA-FLSs



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Figure 2 | ScRNA-seq analysis identified cellular heterogeneity and regulatory modules of specific RBP expression in synovial samples from OA and RA patients.

A. A bar plot shows the number of upregulated and downregulated DEG and RBP in each cell type comparing RA and OA samples. B. The box plot depicts activation of RBPs in each cell type inferred by SCENIC-calculated AUCell scores. C. UMAP plot of scRNA-seq profile. Cells are colored according to cell clusters based on RBP-gene expression module. D. Stacked bar plot comparing the proportions of cell populations of each RBP expression module within each sample group for different cell types. E. Unsupervised clustering heatmap showing relative expression (z score, column scaled) levels of RBP markers of each RBP expression module in single-cell dataset according to different clinical variables containing cell types, and sample groups. F. Gene ontology enrichment analysis of biological processes of top 50 markers in each RBP cluster. Top 3 terms were selected for each cluster and heatmap shows the enrichment q-value of these terms (scaled by column). G. Level of *FLNB* gene expression represented in the UMAP plot split by different sample groups. H. Level of *MBNL2* gene expression represented in the UMAP plot split by different sample groups. I. Level of *OGN* gene expression represented in the UMAP plot split by different sample groups. J. Level of *U2AF1* gene expression represented in the UMAP plot split by different sample groups.

(Figure 4I, J). Finally, PCR results confirmed upregulation of YBX3 and SF3B6, as well as downregulation of EIF4A1 and U2AF1 in RA-FLSs compared to OA-FLSs (Figure 4K-N).

3.4 RBP-mediated fibroblast subpopulations contributed to the aberrant activation of signaling pathways with immune cells during RA

A cellular communication analysis of RBP-expressing cell populations was performed to determine the potential role of RBPs in mediating interactions between fibroblasts and immune cells. Communication between fibroblasts and immune cells was increased with respect to total connections and strength in the RA group relative to the OA group (Figure 5A). Moreover, among different immune cell types, communication between fibroblasts and macrophages had the highest strength (Figure 5B).

Cell Chat was used to examine the potential role of ligand-receptor pairs in interactions between different cell types in RA and OA by analyzing ligand-receptor signaling pathways. Several signaling pathways, including FN1-CD44 and CXCL12-CXCR4, were enhanced in RA and may be implicated in disease development (Figure 5C). Moreover, the ligand-receptor network analysis demonstrated that CXCL12 interacts with RBP FN1 as a receptor (Figure 5D). The interaction level and strength of the FN1 signaling pathway between fibroblasts and immune cells were significantly higher in the RA group than the OA group (Figures 5E-G), with the FN1 signaling pathway more frequently involved in interactions between fibroblasts and macrophages, DCs, and T cells in the RA group.

3.5 Identification of highly conserved RA-associated ASEs co-regulated with differentially expressed RBPs in RA and OA patients

Bulk RNA-seq data from the GSE89408 dataset were analyzed to determine the role of RBPs associated with RA in alternative splicing regulation. Seven hundred fifteen differential RAS events were identified using the SUVA software tool (mainly alt5p and alt3p; Figure 6A) and mapped to classical splicing events. A5SS, cassette exon, ES, and A3SS were the most frequently detected

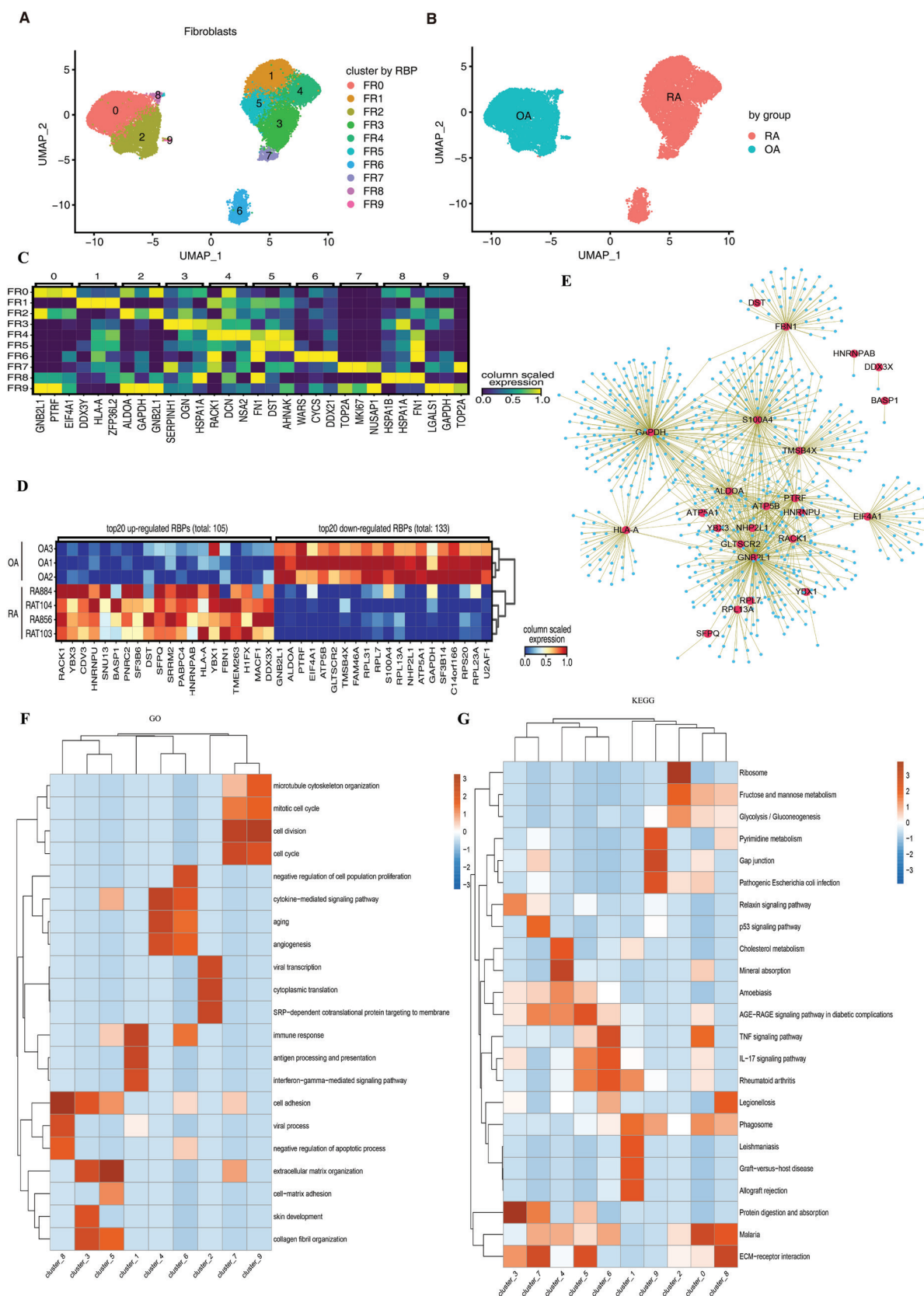
RAS events (Figure 6B). Five hundred fifty-six RAS events in which the proportion of reads supporting each ASE was $\geq 50\%$ were filtered for further analysis. PCA analysis revealed that the ASEs with a pSAR $\geq 50\%$ were strongly associated with disease status between the two groups (Figure 6D).

The ASE-associated genes were enriched in multiple functional pathways, including cytoskeleton organization, GTPase activation, and damage repair (Figure 6E). Co-expression analysis using co-regulated RBPs and ASEs showed significant correlations ($|\text{correlation}| \geq 0.6$; $P\text{-value} \leq 0.01$) with the co-regulated genes significantly enriched in functional pathways, such as cytoskeleton organization, GTPase activation, and damage repair (Figure 6J).

Significant differences were identified in gene expression between the RA and OA control groups with 2183 genes upregulated and 823 genes downregulated (Figure 5F). Among the genes, 209 RBPs were upregulated and 232 RBPs were downregulated (Figure 6G, H). An overlap analysis was then performed between the differentially expressed RBPs in scRNA-seq and the differentially expressed RBPs detected in bulk RNA-seq. Three genes (*SYNE2*, *S100A9*, and *IFIT3*) were co-upregulated, while four genes (*RNASE1*, *GRN*, *FN1*, and *SORBS2*) were co-downregulated (Figure 6G-I). Notably, *SYNE2*, *FN1*, and *SORBS2* had the most significant expression differences between the two groups (Figure 6I). *SYNE2* is a marker gene for endothelial, T, and SIF cells, and pericytes, *S100A9* is a marker gene for macrophages, *IFIT3* is a marker gene for SIF cells, *RNASE1* and *GRN* are marker genes for T cells, *FN1* is a marker gene for endothelial and T cells, and *SORBS2* is a marker gene for pericytes.

3.6 Levels of YBX3 and SF3B6 expression are significantly increased in CIA mice

DBA/1J mice had increased paw swelling and arthritic scores after immunization with type II collagen (Figure 7A(a, b) and B). Histologic examination by hematoxylin and eosin (H&E), Safo-FG, and Toluidine blue staining showed that the DBA/1J mice had attenuated bone erosion, synovial inflammation, and cartilage erosion (Figure 7A(c-h)). Finally, PCR results showed that the levels of YBX3 and SF3B6 expression were



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Figure 3 | Functional RBPs are largely regulated in fibroblasts between RA and OA samples.

A. UMAP plot of scRNA-seq profile. Fibroblasts are colored according to cell clusters based on RBP-gene expression module. B. UMAP displays the distribution of fibroblasts. The color indicates the RA and OA group. C. Unsupervised clustering heatmap showing relative expression (z score, row scaled) levels of top 3 marker RBPs in each RBP-gene expression module of fibroblasts. D. Unsupervised clustering heatmap showing relative expression (z score, row scaled) levels of top 20 up- and down-regulated RBP between RA and OA. E. Cytoscape shows the co-expression networks comprising up- and down-regulated DERBP in edges connect DERBP-target gene pairs while nodes represent genes. DERBP are displayed in larger font size and red color. Co-expression associations of DERBP and target genes in fibroblasts were built by the “grn” algorithm from SCENIC. F. Gene ontology enrichment analysis of biological processes of differential RBP co-expressed fibroblast genes. Top 3 terms were selected for each cluster and heatmap shows the enrichment q-value of these terms (scaled by column). G. KEGG enrichment analysis of biological processes of differential RBP co-expressed fibroblast genes. Top 3 terms were selected for each cluster and heatmap shows the enrichment q-value of these terms (scaled by column).

significantly increased in CIA mice compared to control mice (Figure 7C, D). Although the level of *EIF4A1* gene expression was lower in CIA rats compared to the control group, the difference was not statistically significant (Figure 7E).

4. DISCUSSION

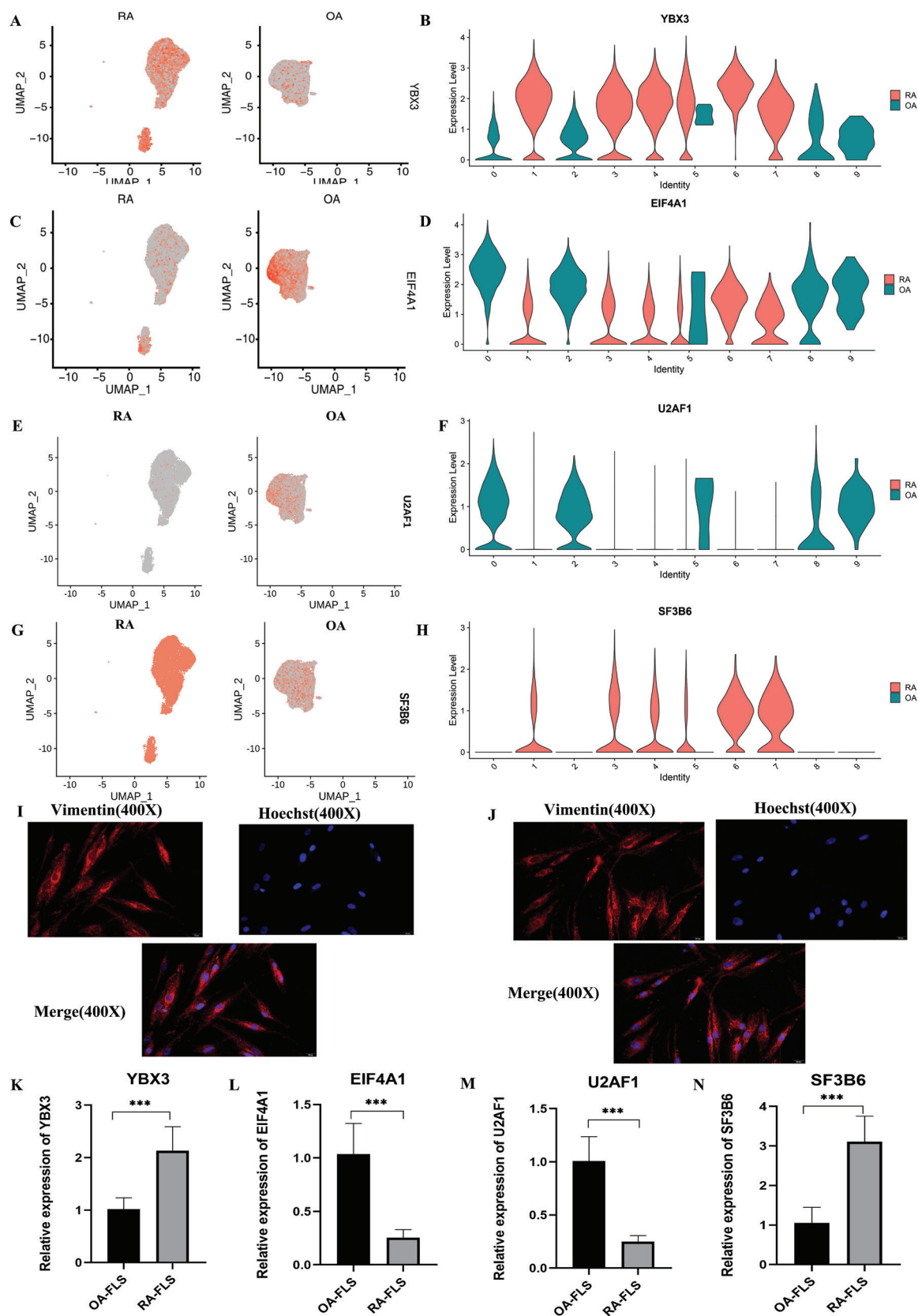
We hypothesized that RBPs have cell type-specific expression patterns in synovial samples from patients with RA and the dysregulation may be closely associated with disease initiation and progression. Therefore, RBPs were used for single-cell sub-clustering to investigate how RBP regulation differs between RA and OA at the single-cell level. Our analysis revealed cellular-specific RBP expression heterogeneity and regulatory modules that differed between RA and OA patients. Some cell-specific RBPs in RA were linked to regulating inflammatory signaling pathways and splicing. These findings suggest that RBP dysregulation may contribute to the development of RA and provide potential targets for therapeutic interventions.

A total of 2141 RBP genes were initially collected from previously published reports. Subsequently, cell populations were identified and annotated in synovial samples from GSE200815 (4 cases with RA) and GSE152805 (3 cases with OA). The 17 subgroups of cells were clustered into 9 distinct cell types and differences in RBP expression were noted between the OA and RA groups. YBX3 was significantly upregulated, while *EIF4A1* was down-regulated in the RA group of fibroblasts. Additionally, we observed significant differences in the expression of critical splicing factors (*U2AF1* and *SF3B6*) between the RA and OA groups. Furthermore, communication analysis between fibroblasts and different immune cells showed an increase in total connections and strength in the RA group compared to the OA group. Bulk RNA-seq samples from the GSE89408 dataset were used to determine the regulatory role of RA-associated RBPs in alternative splicing. Our analysis revealed that the RBPs (*SYNE2*, *FN1*, and *SORBS2*) were co-expressed in both single-cell data and bulk RNA-seq data, and were associated with alternative splicing events in RA. Finally, the levels of *SF3B6* and *YBX3* gene expression were significantly elevated in CIA rats compared to normal control

rats, suggesting that these two RBPs may serve as potential targets for drug development to treat RA.

Emerging evidence suggests that dysregulated RBPs have a crucial role in the pathogenesis of RA by affecting key regulatory mechanisms [36–38]. However, comprehensive evaluations of the characteristics and patterns of RBP expression in different cell types of RA are limited. In the current study the global landscape of RBPs in different cell types of RA were demonstrated. Our analysis showed that downregulated RBPs were more prevalent than upregulated RBPs in each cell type. The levels of RBP expression in B cells, T cells, and DCs, and SSF subgroups were significantly decreased in the RA group. The results herein are consistent with previous reports. Specifically, TTP, a well-characterized RBP, has been shown to suppress ICAM-1 production [37], which promotes inflammation and the mRNA is reduced in peripheral blood mononuclear cells (PBMCs) from RA patients [36]. Notably, patients with RA exhibit activation of pro-inflammatory cytokine genes across several cell types, including endothelial cells, T and B cells, monocytes, and fibroblasts, which contribute to the inflammatory response in synovial tissues. Expression of these cytokine genes is subject to strict post-transcriptional regulation mediated by a group of immune-related RBPs. Reduced activity levels of RBPs suggest that post-transcriptional regulation of pro-inflammatory mediator genes may be hindered in RA.

The current study revealed a significant association between specific RBP expression profiles and disease states. Notable differences in the levels of specific RBP subgroup expression were observed between RA and OA. These findings suggest that RBP subgroups may have crucial roles in the pathogenesis of these diseases. For example, OGN is a member of the small leucine-rich proteoglycan (SLRP) family and actively controls biological processes, such as cell growth, differentiation, and adhesion [39–41]. OGN has been implicated in multiple physiologic and pathologic processes, including skeletal development [42], cardiovascular disease [43], inflammation, and cancer [44, 45]. Additionally, OGN modulates collagen fibril organization and maturation, influencing connective tissue integrity [46]. Our analysis showed that OGN is predominantly expressed and elevated in fibroblasts, suggesting that increased OGN levels may be



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Figure 4 | Specific RBPs were associated with important cellular pathways in RA.

A. Level of *YBX3* gene expression were represented in the UMAP plot split by different sample groups. B. Violin plot of *YBX3* in each cell type split by different sample groups. C. Level of *EIF4A1* gene expression were represented in the UMAP plot split by different sample groups. D. Violin plot of *EIF4A1* in each cell type split by different sample groups. E. Level of *U2AF1* gene expression were represented in the UMAP plot split by different sample groups. F. Violin plot of *U2AF1* in each cell type split by different sample groups. G. Level of *SF3B6* gene expression were represented in the UMAP plot split by different sample groups. H. Violin plot of *SF3B6* in each cell type split by different sample groups. I. Immunofluorescence staining of vimentin in OA-FLS (×400). J. Immunofluorescence staining of vimentin in RA-FLS (×400). K-N. PCR validation of the relative level of *YBX3*, *EIF4A1*, *U2AF1*, and *SF3B6* expression in RA-FLSs compared to OA-FLSs.

Table 2 | Clinical characteristics of the participants.

Variable	RA (n = 4)	OA (n = 4)
Age, years (range)	54.6 ± 6.13 (49–61)	72 ± 3.48 (65–75)
Gender, female/male	4/0	4/0
Disease duration, months (range)	73 ± 38.62 (45–137)	92 ± 65.38 (18–141)
CRP, mg/L (range)	17.69 ± 5.17 (17.3–23.9)	7.49 ± 4.34 (2.91–10.36)
RF, IU/mL (range)	66.45 ± 37.43 (21.1–136)	Neg.
Anti-CCP, RU/mL (range)	89.13 ± 46.35 (39.6–133.5)	Neg.
ESR, mm/h (range)	56.42 ± 19.14 (38–74)	17.83 ± 11.58 (9–33)

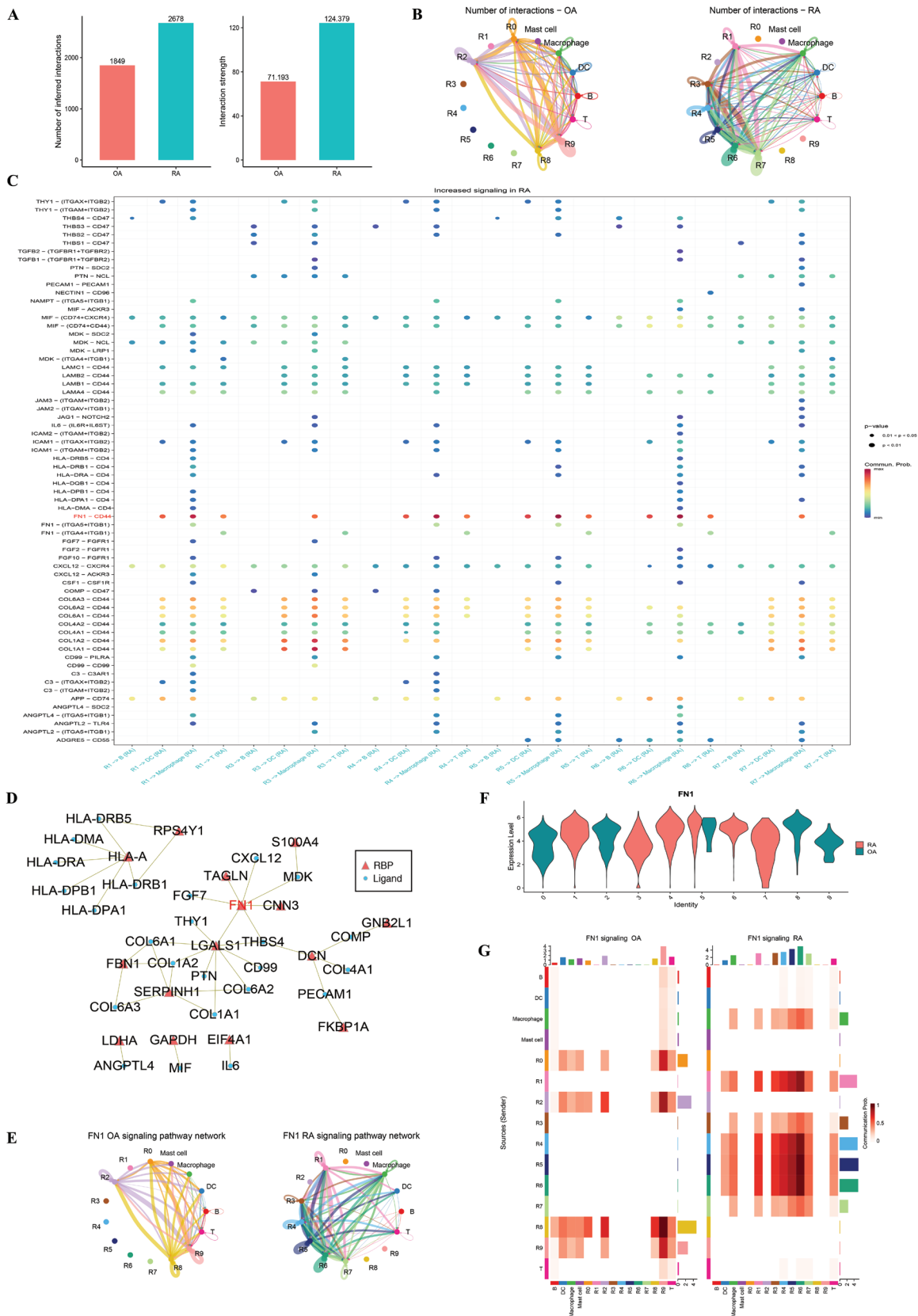
Abbreviations: Anti-CCP, anticyclic citrullinated protein antibodies; CRP, C-reactive protein; RF, rheumatoid factor; ESR, erythrocyte sedimentation rate; Neg., negative.

linked to the abnormal proliferation of synovial fibroblasts in RA. Furthermore, the current study showed that *U2AF1*, an RNA-binding protein crucial for recognizing the 3' splice site during pre-mRNA splicing and initiating recruitment of the U2 small nuclear ribonucleoprotein particle (snRNP) to the splice site [47], was significantly decreased in all cell types within the RA group. In addition, *U2AF1* was significantly downregulated in RA-FLSs. These findings suggest that dysregulated *U2AF1* expression may contribute to the pathogenesis of RA through its crucial role in regulating splicing.

Fibroblasts are a crucial cell type in the synovium of RA patients and have a pivotal role in driving disease development. One hundred five upregulated RBPs and 133 downregulated RBPs were identified based on our analysis of fibroblasts from RA and OA patients with significant differences in RBP-expressing cellular subsets between the two groups. Our study revealed that some RBPs were significantly enriched in RA patients, including *YBX3* and *SF3B6*, while *EIF4A1* and *U2AF1* were significantly downregulated. *YBX3* is essential in the post-transcriptional regulation of mRNA stability and translation [48], influencing various biological processes, such as spermatogenesis, development, differentiation, and proliferation [49]. Upregulation of *YBX3* may promote cellular differentiation and proliferation in RA fibroblasts and contribute to the pathogenesis of RA. Conversely, *EIF4A1*, which is involved in translation initiation, is crucial in loading mRNA onto pre-initiation complexes and unwinding RNA secondary structures

[50]. Downregulation of *EIF4A1* may lead to decreased apoptosis in RA fibroblasts. These results suggest that upregulation of *YBX3* and downregulation of *EIF4A1* in RA fibroblasts may contribute to the development of RA and serve as potential therapeutic targets for this debilitating disease.

RBPs are crucial for alternative splicing, where RBPs bind to pre-mRNA transcripts and facilitate splicing as activators or repressors. Dysregulated expression or function of RBPs can lead to aberrant alternative splicing patterns, which contributes to the development and progression of diseases, including cancer [51] and neurodegenerative disorders [52]. The current study showed that *MBNL2*, an essential regulator of alternative splicing linked to the regulation of endothelial cell differentiation [53–55], is predominantly expressed in endothelial cells of the RA group, indicating its potential role in the pathogenesis of RA through splicing regulation. Furthermore, significant differences were observed in the expression of key splicing factors (*U2AF1* and *SF3B6*) in fibroblast cells between the RA and OA groups. Downregulation of *U2AF1* has been linked to changes in alternative splicing patterns and may contribute to diseases, such as myelodysplastic syndromes [56, 57] and lung cancer [58]. *SF3B6* is a distinctive feature of the human *SF3b* complex that has a crucial role in recognizing the branch-point sequence and preventing untimely transesterification [59–62]. These findings underscore the critical role of RBPs in regulating alternative splicing and highlight the potential as therapeutic targets for RA.



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Figure 5 | RBP-mediated fibroblast subpopulations contributed to the aberrant activation of signaling pathways with immune cells during RA.

A. Number and strength of interactions in RA and OA. B. Comparing the interaction number among RBP-gene expression module of fibroblasts and immune cells in RA and OA samples. C. Scatter plot displaying upregulated ligand-receptor pairs in RA sample group from fibroblasts to immune cells compared with OA samples. D. Cytoscape shows the co-expression networks comprising RBP. Edges connect RBP-ligand gene pairs, while nodes represent genes. RBP are displayed in larger font size and red color. Co-expression associations of RBP and ligand genes were built by the “grn” algorithm from SCENIC. E. Comparing the interaction number among FN1 signaling pathway network of fibroblasts and immune cells in RA and OA samples. F. Violin plot of FN1 in each cluster of fibroblasts in RA and OA samples. G. Signal contribution to FN1 signaling of fibroblasts and immune cell sub-clusters in RA and OA.

Table 3 | Approved rheumatoid arthritis drugs.

Drug name	Target(s)	Mechanism of action
Methotrexate	Dihydrofolate reductase (DHFR)	Inhibits DHFR, reducing folate availability and DNA synthesis [69]
Adalimumab	Tumor necrosis factor- α (TNF- α)	Binds and blocks TNF- α , reducing inflammation [70]
Etanercept	TNF receptor	Acts as a decoy receptor for TNF- α , preventing inflammation [71, 72]
Infliximab	TNF- α	Neutralizes TNF- α , reducing inflammatory response [73]
Tocilizumab	Interleukin-6 receptor (IL-6R)	Inhibits IL-6 signaling, reducing inflammation [74]
Abatacept	CTLA-4	Inhibits T-cell activation, decreasing immune response [75]
Rituximab	CD20 on B cells	Targets B cells for destruction, reducing autoimmune activity [76]
Anakinra	Interleukin-1 receptor antagonist	Blocks IL-1, decreasing inflammation [77]
Tofacitinib	Janus kinase (JAK) 1/3	Inhibits JAKs, interfering with cytokine signaling pathways [78]
Baricitinib	JAK 1/2	Inhibits JAKs, reducing inflammatory responses [79]
Upadacitinib	JAK 1	Specifically inhibits JAK1, modulating immune function [80]
Sarilumab	IL-6R	Blocks IL-6 receptor, inhibiting pro-inflammatory signal cascade [81]

FN-1 is an extracellular matrix protein that is primarily produced by fibroblasts and has been implicated in pathologic changes in various tissues [63, 64]. Moreover, recent studies have highlighted the close association of FN-1 with the initiation and development of RA [65, 66]. Our analysis indicated significant expression differences of FN1 in endothelial and T cells in the RA group. FN1 was shown to regulate alternative splicing and interact with other immune cells during RA. Frequent involvement of the FN1 signaling pathway was noted in interactions between fibroblasts and macrophages, DCs, and T cells in RA through the cell-cell communication analysis. In addition, further analysis identified CXCL12 as a receptor that interacts with RBP FN1 in RA. Consistent with this finding, studies suggest that CXCL12 and CXCR4 are highly expressed in RA and positively correlated with disease activity [67, 68]. This finding suggests that a decrease in FN1 expression may lead to activation of the CXCL12-CXCR4 signaling pathway in RA, emphasizing the possible significance of this pathway in the development of RA.

Finally, although there are many drugs currently available for treating RA (Table 3), the current therapeutic options do not fully meet patient needs. Consequently,

this field has garnered significant attention and concentrated research efforts from scientists and pharmaceutical companies. Our study suggests that some RBPs can serve as potential therapeutic targets for the development of drugs to treat RA. In the current study the levels of *SF3B6* and *YBX3* gene expression were significantly elevated in CIA rats compared to normal control rats, suggesting that these two RBPs may serve as potential targets for drug development to treat rheumatoid arthritis.

In conclusion, we analyzed scRNA-seq datasets to identify the inherent characteristics and abnormal expression patterns of RBPs in different cell types of patients with RA. Our findings revealed that certain cell-specific RBPs were associated with inflammatory signaling pathways and splicing regulation, emphasizing the potential role in causing RA. These results suggest that the dysregulation of RBPs may contribute to RA and provide a basis for identifying possible pathways for therapeutic intervention in the treatment of RA. Furthermore, it is essential to acknowledge certain limitations of the current study. Although we primarily relied on data from public databases and validated our findings with some cellular results, additional animal experimental results

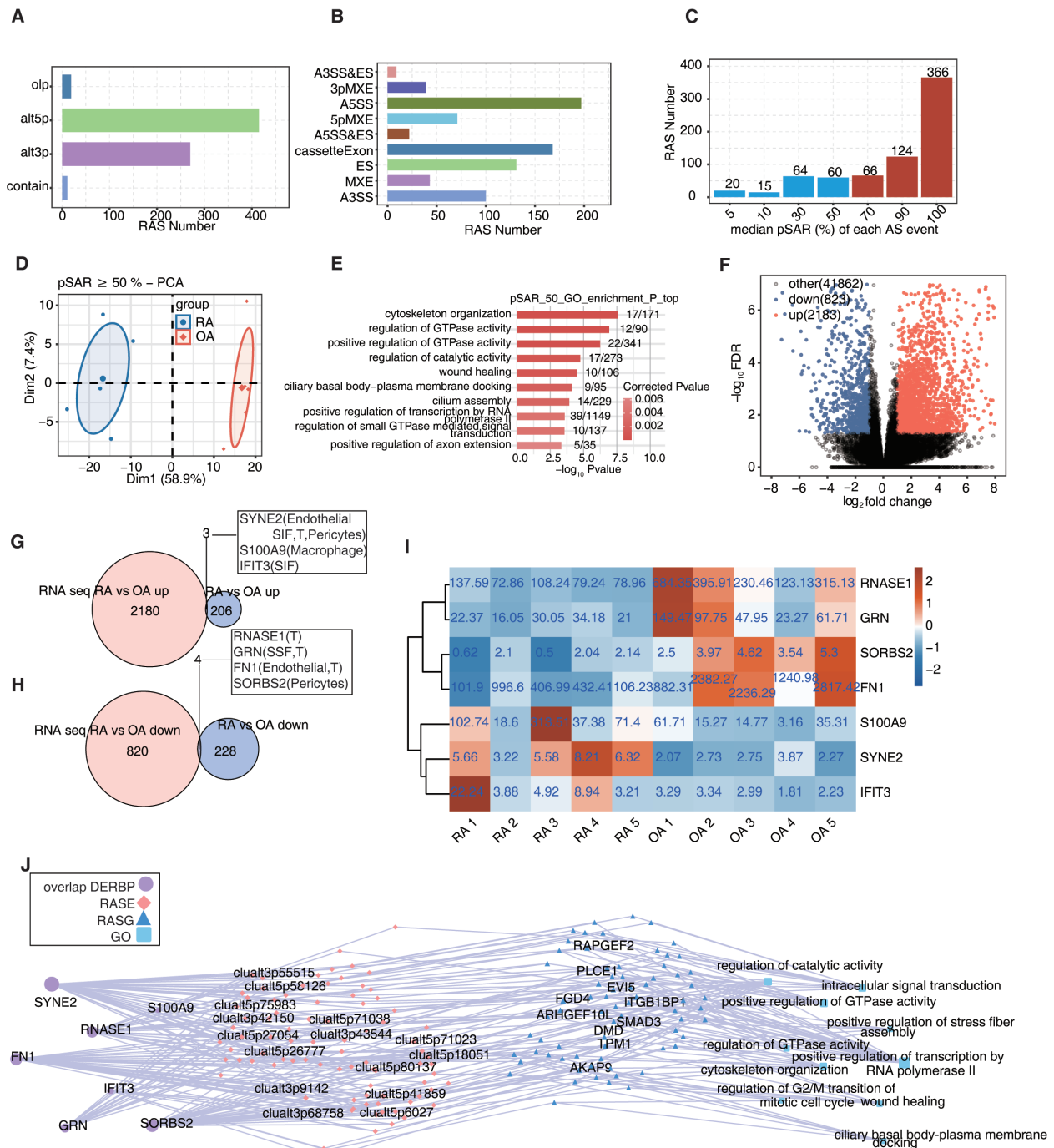


Figure 6 | Identification of highly conserved RA-associated ASEs co-regulated with differentially expressed RBPs in RA and OA patients.

A. Bar plot shows number of regulated AS (RAS) detected by SUVA by comparing RA to OA samples. B. Splice junction constituting RAS events detected by SUVA was annotated to classical AS event types. The number of each classical AS event types is shown with the bar plot. C. Bar plot shows RAS with different pSAR. RAS in which reads proportion of SUVA AS event (pSAR) $\geq 50\%$ were labeled. D. Principal component analysis (PCA) based on splicing ratio of RAS with pSAR $\geq 50\%$. The ellipse for each group is the confidence ellipse. E. Bar plot shows the most enriched GO biological process results of RAS (PSAR $\geq 50\%$). F. Volcano plots presenting all DEGs between RA and OA samples with DESeq2. FDR ≤ 0.05 and fold-change (FC) ≥ 2 or $\leq 1/2$. G, H. Venn diagram shows the overlap of DE RBPs from bulk RNA-seq and ScRNA-seq datasets. I. The heatmap diagram shows the FPKM of 7 overlapped DERBPs. J. The co-disturbed network among expression of co-regulated RBPs shown in C and splicing ratio of RAS events (pSAR $\geq 50\%$) was constructed. [Pearson's correlation] ≥ 0.6 and P -value ≤ 0.01 were retained for RBP and RAS correlation. Ellipses represent RBP. Squares indicate RAS.

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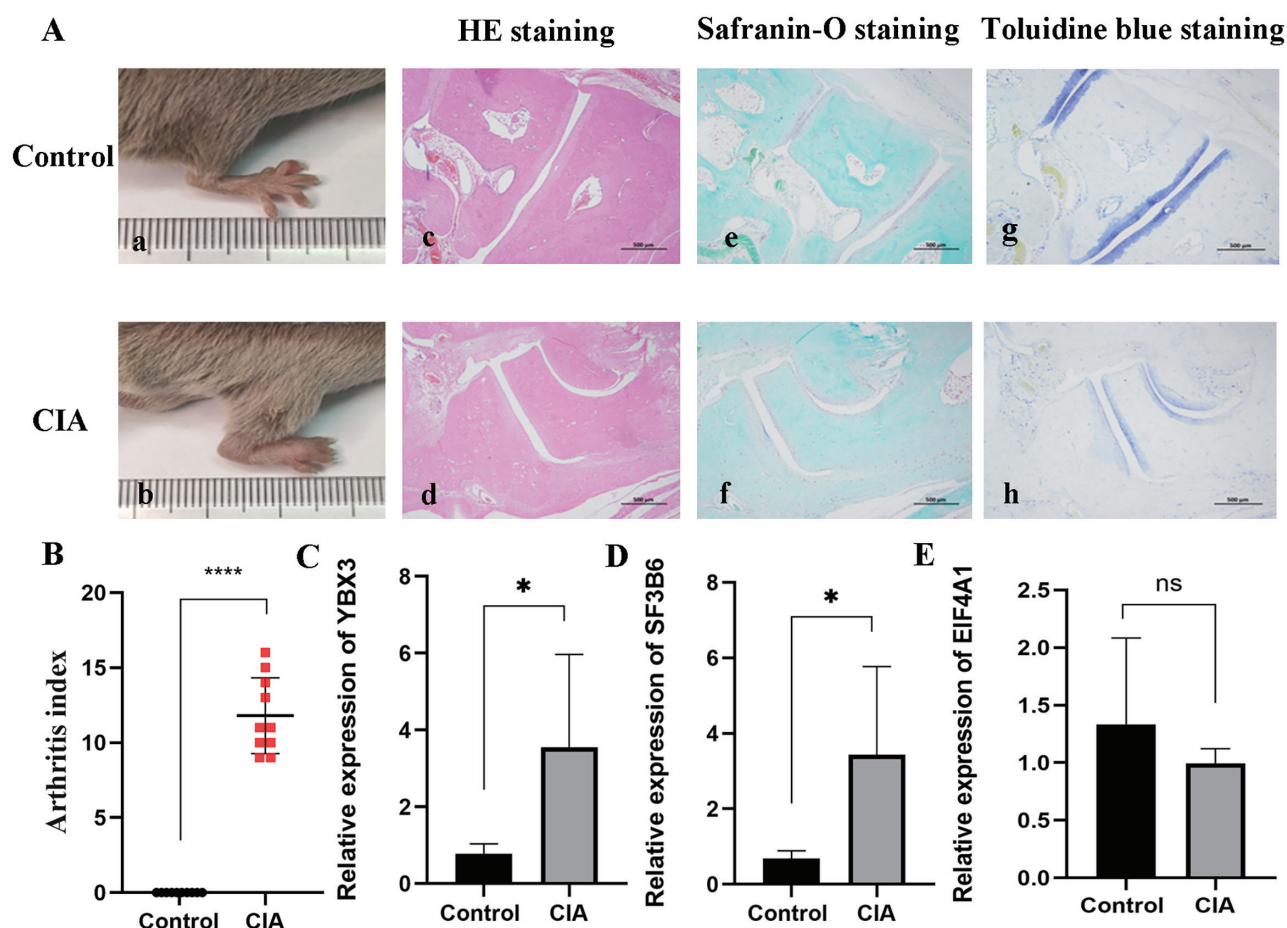


Figure 7 | Effect of CIA model preparation on arthritis development in DBA/1J mice.

A. (a, b) Representative images of the mouse hind paw on day 42. (c, d) Images of H&E of the paw sections. (e, f) Images of Safo-FG staining of the paw sections. (g, h) Images of Toluidine blue staining of the paw sections. B. Evaluation of arthritis scores for the mice ($*P < 0.05$, $****P < 0.0001$, compared to the control group). C-E. PCR validation of the relative level of YBX3, SF3B6, and EIF4A1 expression in CIA mice compared to controls.

are needed to verify the findings. Therefore, further research is required to fully comprehend the specific role and mechanism of certain RPBs in the development and progression of RA.

ABBREVIATIONS

ASEs, Alternative splicing events; BPS, Branch-point sequence; CD44, Cluster of differentiation 44; CXCL12, C-X-C motif chemokine ligand 12; CXCR4, C-X-C motif chemokine receptor 4; DEG, Differentially expressed gene; EIF4A1, Eukaryotic translation initiation factor 4A1; FC, Fold change; FDR, False discovery rate; FLS, Fibroblast-like synoviocyte; FN1, Fibronectin 1; GO, Gene Ontology; GRN, Granulin; IFIT3, Interferon induced protein with tetratricopeptide repeats 3; KEGG, Kyoto Encyclopedia of Genes and Genomes; OA, Osteoarthritis; OGN, Osteoglycin; PCA, Principal component analysis; pSAR, Proportion of each SUVA AS event; RA, Rheumatoid arthritis; RAS, Regulatory alternative splicing; RPBs, RNA binding proteins; RNASE1, Ribonuclease 1; RRM, RNA recognition motif; S100A9, S100 calcium binding protein A9; scRNA-seq, Single-cell RNA sequencing; SF3B6, Splicing factor 3b subunit 6; snRNP, Small nuclear ribonucleoprotein particle;

SORBS2, Sorbin and SH3 domain containing 2; SRA, Sequence Read Archive; SYNE2, Spectrin repeat containing nuclear envelope protein 2; U2AF1, U2 small nuclear RNA auxiliary factor 1; UMI, Unique molecular identifier; UTRs, Untranslated regions; YBX3, Y-Box binding protein 3.

DECLARATIONS

ETHICS APPROVAL FOR CONSENT TO PARTICIPATE

Not applicable.

CONSENT FOR PUBLICATION

Not applicable.

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AVAILABILITY OF DATA AND MATERIAL

The scRNA-seq data from three OA and four RA samples were downloaded from GSE152805 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE152805>) and GSE200815 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE200815>). The bulkRNA-seq data were downloaded from GSE89408 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE89408>).

CONFLICTS OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

SUPPLEMENTARY MATERIAL

Supplementary material for this paper is available at the following link https://amm-journal.org/wp-content/uploads/2025/02/amm20240034_Suppl.zip.

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