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ORIGINAL RESEARCH

Integrated Multi-Level Investigation of Friend Leukemia Integration 1 Transcription Factor as a Novel Immune-Inflammatory Biomarker in Rheumatoid Arthritis: Bridging Bioinformatics, Clinical Cohorts, and Mechanistic Validation

Mengyu Zhang[1], Lei Wan[2], Haoxiang Fang[3], Xiaojun Zhang[4], Siyu Wang[1], Feng Li[1], Dawei Yan[1]

[1]Anhui University of Traditional Chinese Medicine First Clinical Medical College, Anhui, Hefei, People's Republic of China; [2]First Affiliated Hospital of Anhui University of Traditional Chinese Medicine, Anhui, Hefei, People's Republic of China; [3]Anhui University of Traditional Chinese Medicine, Anhui, Hefei, People's Republic of China; [4]Anhui University of Traditional Chinese Medicine Academic Affairs Office, Anhui, Hefei, People's Republic of China

Correspondence: Lei Wan; Xiaojun Zhang, Email yxwanlei@163.com; zhecho223@sohu.com

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Introduction: Friend Leukemia Integration 1 Transcription Factor (FLI1) has attracted attention due to its involvement in rheumatoid arthritis (RA). Nevertheless, the precise mechanism through which FLI1 contributes to RA remains elusive. We investigated the potential role of FLI1 in RA through integrated bioinformatics, clinical experiments, and cellular experiments.

Methods: Based on the GSE1919 and GSE12021 datasets, the Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) analyses identified *FLI1* as a differential gene in RA. Clinical validation was performed by measuring the FLI1 expression in the serum collected from RA patients. Correlational analysis between FLI1 and immune-inflammatory markers confirmed its association with RA inflammation. WGCNA analysis, along with the KnockTF, JASPAR, and ENCODE databases, was employed to predict the potential target genes of FLI1. Receiver operating characteristic analysis and gene-set enrichment analysis-KEGG were conducted to elucidate the biological functions of these target genes. Finally, cellular experiments were performed to validate FLI1's regulatory effects on its target genes and its impact on synovial cell viability and apoptosis in RA.

Results: FLI1 was upregulated in RA. FLI1 exhibited positive correlations with CRP, ESR, CCP, RF, IL-6, IL-10, IL-8, and TNF-α. The combined detection of FLI1 with CRP, ESR, CCP, and RF demonstrated the highest efficacy in evaluating RA disease activity. The target genes most strongly associated with FLI1 were AGA, DCK, LRRC15, MAN2A1, and TES, all of which exhibited positive correlations with FLI1. The suppression of FLI1 expression led to a decreased expression of AGA, DCK, LRRC15, MAN2A1, and TES. Furthermore, the inhibition of FLI1 reduced the viability of RA synovial cells and promoted their apoptosis.

Discussion: FLI 1 is upregulated in RA and can promote inflammation, increase RA synovial cell viability, or inhibit synovial cell apoptosis. This finding suggests that FLI 1 and its target genes may serve as novel therapeutic targets in RA. The present findings integrate bioinformatics and experimental approaches to advance our current understanding of RA and open new avenues for targeted therapies.

Keywords: ETS family, FLI1, rheumatoid arthritis, immunoinflammation, apoptosis

Introduction

Rheumatoid arthritis (RA) is a multifaceted autoimmune disorder that results in chronic inflammation and joint destruction, thereby substantially diminishing the quality of life of patients.[1–3] Notwithstanding substantial development regarding RA prognosis and treatment during the previous years, its pathophysiology is not completely understood. Moreover, the currently available diagnostic and therapeutic options have limitations. In recent years, the advancements in molecular biology and bioinformatics techniques have enabled researchers to explore new biomarkers and therapeutic targets for improved diagnostic accuracy of RA and the development of more effective treatment strategies.

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Friend leukemia integration 1 transcription factor (FLI1), an ETS transcription factor, regulates vascular maturation and stabilization, immune cell differentiation, and inflammation, which makes it vital in inducing immunological response.[4–6] In recent years, researchers have been paying increasing attention to the fact that FLI1 is strongly linked to rheumatic immune diseases, such as systemic lupus erythematosus and systemic sclerosis.[7,8] There are race-specific single nucleotide polymorphisms (SNPs) in the enhancer region of FLI1; these SNPs are associated with elevated levels of FLI1 expression, which, in turn, modulate the downstream inflammation secretion.[9] The FLI1 expression is more strongly correlated with the RA activity (DAS28 score) in Caucasian populations, and the frequency of the coactivation of FLI1 and NF-κB pathways is higher in Asian patients with RA than in European ones. The ETS cluster, in which FLI1 resides, contributed significantly more to RA risk in the East Asian population (3.8%) than in the European population (1.2%).[10,11] Presently, the role of FLI1 in RA is being extensively explored,[12,13] and some researchers have discovered a substantial increase in the FLI1 expression, which is positively correlated with the disease activity index.[14–16] In addition, FLI1 and its downstream genes are involved in the production of a variety of inflammatory mediators, which can exacerbate joint injury.[17–20] Consequently, FLI1 may emerge as a novel therapeutic target for RA. Nevertheless, the precise action mechanism of FLI1 in RA has not yet been completely comprehended, warranting additional research.

The objective of the present study was to conduct a comprehensive examination of FLI1's therapeutic potential and its function in RA through clinical validation, bioinformatics, and cellular experiments. Moreover, differentially expressed genes (DEGs) related to RA were screened using public databases. FLI1 was identified to be a DEG using Venn diagram analysis, and its expression was verified in the clinical samples of patients with RA. The downstream target genes of FLI1 were predicted, and their roles in the disease process of RA were preliminarily verified experimentally. FLI1 and its target genes may affect the development of RA inflammation by regulating apoptosis, thereby providing new therapeutic ideas.

Materials and Methods

GEO Database and Metascape Database

The GEO database (https://www.ncbi.nlm.nih.gov/geo/info/datasets.html) is a gene expression database created and maintained by NCBI. We acquired the publicly available microarray datasets GSE1919 and GSE12021, from which the RA and control samples were obtained.

Metascape and Network Analyst Analysis

The Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) pathway enrichment analysis of differential target genes was performed using DAVID and Metscape. PPI network was analyzed by Metscape. In Metscape, the minimum overlap was set to 3, and the minimal enrichment factor was 1.5. The p-value threshold was 0.01. The critical value was set to FDR <0.05. Network Analyst (http://www.networkanalyst.ca) was used to identify the differential expression of FLI1 relative to normal samples.

Venn Diagrams

We used the Venn Diagram Plotter v1.5.5228 (https://omics.pnl.gov/software/venn-diagram-plotter) to generate the Venn diagrams.

WGCNA Analysis

We used the R WGCNA software package (https://cran.r-project.org/package=WGCNA). The samples were clustered using Pearson's correlation coefficient, and scale-free networks were generated using a gentle threshold of 16 (R-based scale-free topology criterion 2 = 0.85). The adjacency matrix was transformed into a TOM matrix, which was employed to quantify node similarity by using weighted correlations.

KnockTF, JASPAR, and ENCODE Database Analysis

We used the KnockTF online website (http://www.licpathway.net/KnockTF/), the JASPAR database (http://jaspar.genereg.net), and Encode (http://genome.ucsc.edu/ENCODE) to predict the target genes of transcription factor FLI1.

ROC Curve

We utilized the area under the curve (AUC) of the receiver characteristic curve (ROC) to determine the validity of the potential biomarkers for RA on the GSE12021 dataset using the pROC R package.

Gene Set Enrichment Analysis (GSEA) and Molecular Signatures

We acquired the GSEA software (version 3.0) from the GSEA website to evaluate the related molecular mechanisms and pathways. Concordance scores were applied to prioritize significantly enriched gene sets ($P < 0.05$). The GSEA-KEGG analysis served as a tool to uncover the potential biological functions of our key target genes.

Immune-Infiltration Analysis

RNA-seq data were collected from a variety of groups of patients with RA. The "CIBERPORT" program in R software was employed to analyze these data. The relative proportions of 22 distinct categories of immune-infiltrating cells were estimated. In addition, Spearman correlation analysis was conducted to examine the relationship between immune cell content and gene expression. The threshold for statistical significance was set at $p < 0.05$.

RA Specimen Sources and RA-FLS Cell Lines

To verify the differential expression of FLI1 and the expressions of AGA, DCK, LRRC15, MAN2A1, and TES in RA, we collected normal serum samples from 14 healthy individuals and 14 RA patients at the First Affiliated Hospital of Anhui University of Chinese Medicine to validate the FLI1 expression. Each patient provided their written informed consent. This investigation was approved by the First Affiliated Hospital Ethics Committee of Anhui University of Traditional Chinese Medicine. We purchased the RA-FLSs (Shanghai Fuhang Biotechnology Co, FH0699) to evaluate the proliferation and apoptosis of RA cells after silencing FLI1.

Grouping

Healthy individuals were assigned to the NC group and RA patients to the RA group. The FLS group encompassed synoviocytes from healthy people, and the RA-FLS group included synoviocytes from patients with RA. si-NC served as the control group for the silencing of FLI1; si-FLI1 served as the model group for the silencing of FLI1; si-1, si-2, and si-3 were all differentially silenced FLI1 silencing groups. NC-50pmol, NC-100pmol, and NC-150pmol were included in the RA-FLS control group at different concentrations; si-50pmol, si-100pmol, and si-150pmol were the RA-FLS model groups in which FLI1 was silenced at different concentrations. NC-24h, NC-48h, and the NC-72h group were RA-FLS control groups at 24 h, 48 h, and 72 h after treatment; si-24h, si-48h, and si-72h served as RA-FLS model groups at 24 h, 48 h, and 72 h after treatment for silencing FLI1.

Laboratory Index Testing

Laboratory indicators, such as erythrocyte sedimentation rate (ESR), anti-cyclic citrullinated peptide antibody (CCP), ultrasensitive C-reactive protein (Hs-CRP), rheumatoid factor (RF), interleukin-6 (IL-6), IL-8, IL-10, and tumor necrosis factor-alpha (TNF-α), in patients with RA in the clinic, were measured in the laboratory of Anhui University of Traditional Chinese Medicine. DAS28-ESR was calculated using ESR to assess the disease activity of patients, with DAS28-ESR ≤ 5.1 indicating a low disease activity in RA and DAS28-ESR >5.1 denoting high disease activity in RA.

qRT-PCR

Total RNA was isolated from synovial tissues or chondrocytes using the TRIzol kit (Life Technologies). cDNA was generated with 1 μg of RNA. The cDNA was quantified using the SYBR premix. The relative quantification study was employed to analyze the relative levels of each gene, with GAPDH serving as an internal reference, and the $2^{-\Delta\Delta}$Ct approach. Each response was replicated thrice. The primer sequences are shown in Table 1.

Table 1 The Primer Sequences

Gene	Amplicon Size (bp)	Forward Primer (5'→3')	Reverse Primer (5'→3')
β-actin	96	CCCTGGAGAAGAGCTACGAG	GGAAGGAAGGCTGGAAGAGT
FLI1	176	CAAAGAATAAGCGCCCTGCT	GGACTTCCCAGGTCTCACTC
AGA	181	AGCCACTGGGAATGGTGATA	AGCACCGTAACTTCCAGTCA
DCK	112	TGTCTTCCTCAGCAGGTTGG	ACACAGGACACACTACCATT
MAN2A1	192	TCCTGGCTCCACTAGGAGAT	GGCCCTTGTCTCTCTGAGTT
TES	86	CAATGCCATCGACCCAGAAG	AGCAAGAGCACAGAAAGCAC
LRRC15	132	CTAACCAGCCCTGTGGAAGA	CAGGGCGACAATGCCAATTA
FLI1-(NC)		UUCUCCGAACGUGUCACGUTT	ACGUGACACGUUCGGAGAATT
FLI1-HOMO-540(Si1)		CAGUAAGAAUACAGAGCAATT	UUGCUCUGUAUUCUUACUGTT
FLI1-HOMO-833(Si2)		UGACCAAAGUGCACGGCAATT	UUGCCGUGCACUUUGGUCATT
FLI1-HOMO-1116(Si3)		CCACGUGCCUUCACACUUATT	UAAGUGUGAAGGCACGUGGTT

Cell Proliferation Assay

CCK8 and colony formation analyses were performed to determine whether the cells in each group could multiply after FLI1 silencing. The medium was added, the cells were resuspended, and the inoculated cell-culture plates were placed in the incubator to be cultured overnight after the FLS and RA-FLS cells had completed their normal digestion. The RA-FLS cells were cultured for 24, 48, and 72 h after inoculating into a 96-well plate. Subsequently, 10 μL of the CCK-8 solution was added. Following a 2-h incubation period at 37°C, the absorbance of each well was measured. The cells were cultured on a 6-well plate for the cell proliferation assay after injection, and the medium was replaced every 4 days. The cells were stained with 0.5% crystal violet following formaldehyde fixation. The EdU assay was performed using the EdU reagent (Beyotime, Biotechnology) as per the manufacturer's instructions.

Flow Cytometry Analysis

The Annexin V-FITC/PI Apoptosis reagent (Multi Sciences) was used to stain the cells, which were precooled. The apoptosis rate was determined by flow cytometry (Beckman Coulter, USA). Images of flow cytometric apoptosis were analyzed using the NovoExpress software.

Statistical Analysis

Statistical analysis was performed using R software version 4.0.0 and GraphPad Prism 9.0 (GraphPad Software, USA). The Student's *t*-test was performed to analyze variables that were normally distributed. The Kruskal—Wallis test was employed to analyze variables that were not normally distributed. $P < 0.05$ was considered to indicate statistical significance.

Results

Screening and Enrichment Analysis of DEGs in RA

To screen for genes that played important roles in the synoviocytes in RA, differential expression gene analysis was performed on the GSE1919 and GSE12021 datasets. The GSE1919 dataset identified 723 downregulated and 950 upregulated DEGs (Figure 1A), whereas the GSE12021 dataset identified 1478 downregulated and 952 upregulated DEGs (Figure 1B). By ascertaining which DEGs intersected, RA identified 194 downregulated and 255 upregulated DEGs (Figure 1C). In addition, the GO analysis demonstrated that the biological processes most closely associated with the intersection of the ETS family of factors and the DEGs in RA synoviocytes were hemopoiesis, cellular response to cytokine stimulus, and positive regulation of cell–cell adhesion (Figure 1D). The cellular components were mostly located outside the immunological synapse and plasma membrane (Figure 1E). Immune receptor activity and kinase binding were the most frequently observed molecular functions (Figure 1F). KEGG analysis indicated that DEGs were mainly linked to the toll-like receptor, AMPK, and MAPK-signaling pathways (Figure 1G).

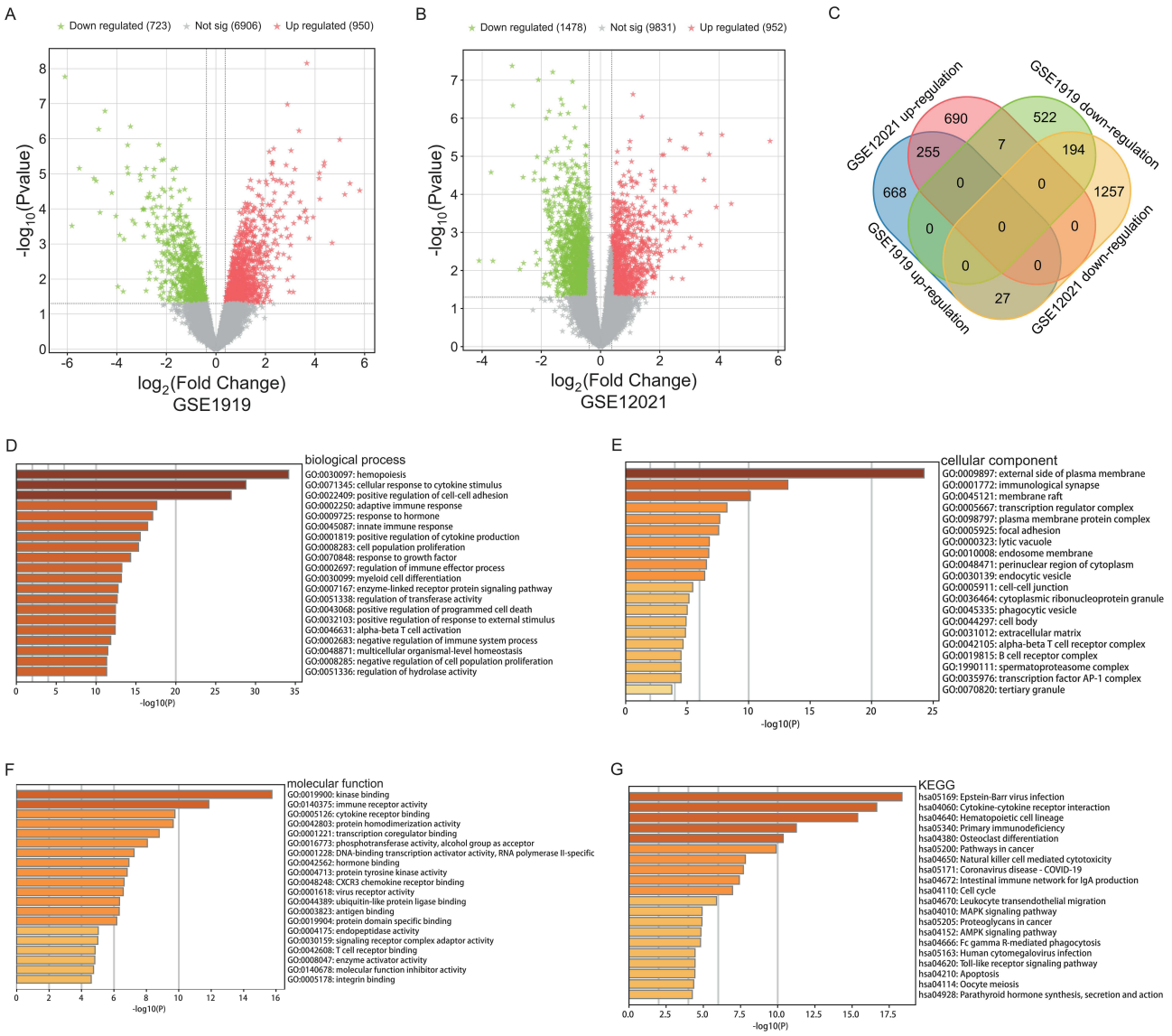

Figure 1 Genes with variable expression in rheumatoid arthritis identified and analyzed for enrichment. (**A**) Volcano plot of GSE1919, (**B**) Volcano plot of GSE12021, (**C**) Wayne plots of differentially expressed genes in the two datasets, (**D**) GO-bp analysis results for intersecting differentially expressed genes, (**E**) GO-cc analysis results for intersecting differentially expressed genes, (**F**) GO-mf analysis results for crossing genes with variable expression, (**G**) KEGG analysis results for crossing genes with differential expression.

The Significant Upregulation of FLI1 in RA

To analyze the role of the ETS family in RA, 27 ETS family genes were identified from the literature.[21] Among them, 15 ETS family genes, including ELF1, ELF2, ELF4, ELK3, ELK4, ERF, ERG, ETS1, ETS2, ETV3, ETV6, FEV, FLI1, GABPA, and SPI1, were detected in both GSE1919 and GSE12021 datasets (Figure 2A and B). The intersection analysis of these genes with DEGs revealed that FLI1 was significantly differentially expressed in RA (Figure 2C). Based on the GSE1919 and GSE12021 datasets, in comparison to the HC group, FLI1 was discovered to be significantly higher in RA (Figure 2D and E).

FLI1 Expression in RA

To observe the role of FLI1, 14 RA patients and 14 hC were selected from the clinic. The serum FLI1 levels of the RA group were significantly higher than that of the NC group ($P < 0.01$), indicating gene upregulation in RA (Figure 3A). Cellular validation revealed that the fibroblast-like synoviocytes from patients with RA (RA-FLS) group had significantly higher FLI1 expression compared to the FLS-only group ($P < 0.01$) (Figure 3B), which further validated our hypothesis.

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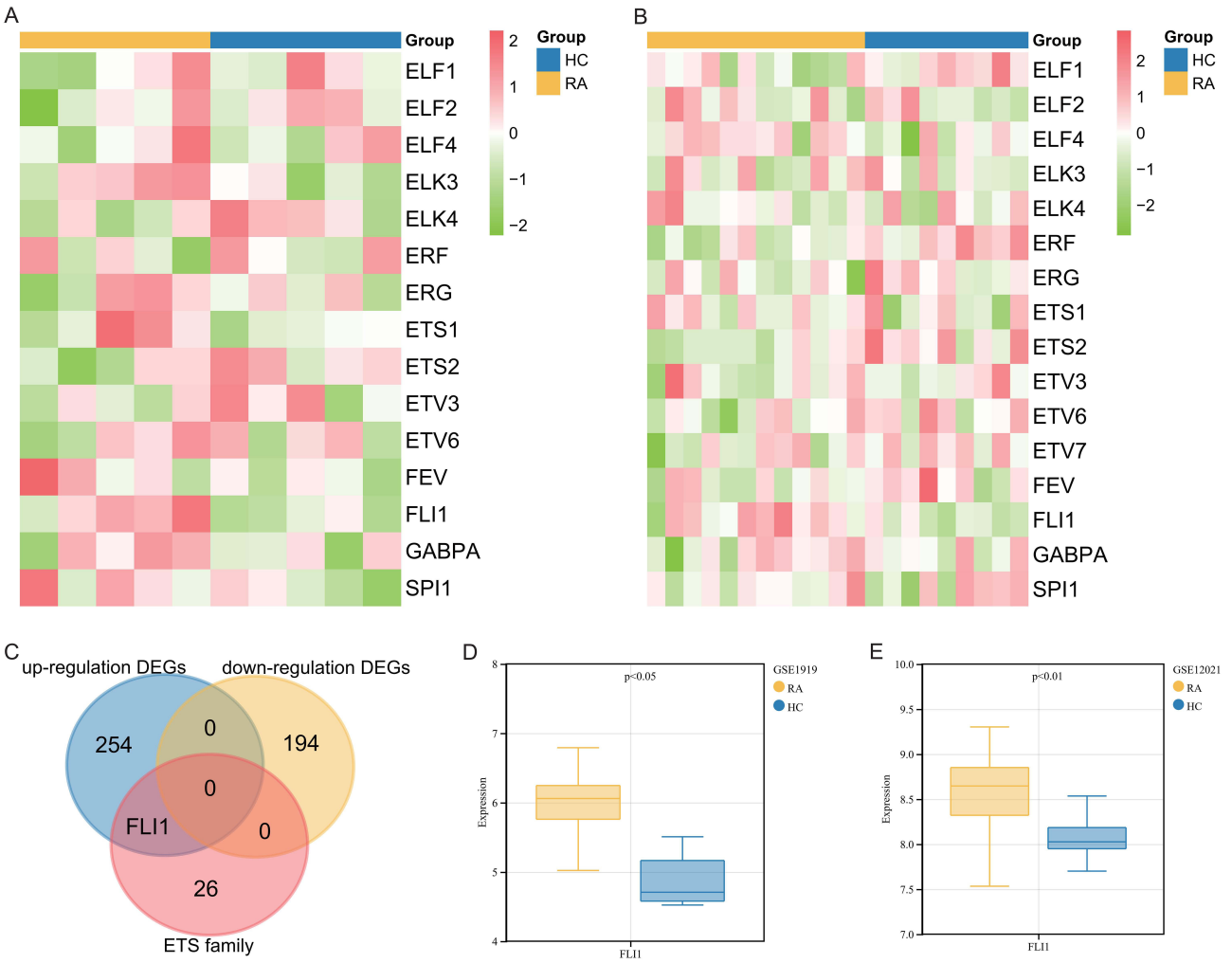

Figure 2 Expression analysis of ETS family in rheumatoid arthritis. (**A**) Heatmap of the ETS family genes in GSE1919, (**B**) Heatmap of the ETS family genes in GSE12021 (**C**) Wayne plots of differentially expressed genes versus ETS family, (**D**) FLI1 expression analysis in GSE1919, (**E**). FLI1 expression analysis in GSE12021.

Clinical Correlational Analysis of FLI1 and RA-Related Immuno-Inflammatory Indicators

To forecast FLI1 functions among the clinic's RA patients, 14 patients were selected. The expressions of FLI1 and immune inflammation-related indicators that included ESR, CCP, Hs-CRP, RF, IL-6, IL-10, IL-8, and TNF-α were measured, followed by correlation analysis (Figure 4A and B). These findings suggested that ESR ($r = 0.568$, $p = 0.034$) CRP ($r = 0.645$, $p = 0.012$), CCP ($r = 0.741$, $p = 0.002$), RF ($r = 0.749$, $p = 0.002$), IL-6 ($r = 0.770$, $p = 0.001$), IL-8 ($r = 0.702$, $p = 0.005$), IL-10 ($r = 0.742$, $p = 0.002$), and TNF-α ($r = 0.538$, $p = 0.046$) were positively correlated with FLI1. The RA patients were categorized into two groups: those with a high disease activity and those with modest disease activity.[22,23] The functional characteristic curves of the subjects were plotted, and the correlation between FLI1 and RA disease activity was examined (Figure 4C–G). The results indicated that FLI1's AUC was 0.750, and the CRP, ESR, CCP, and RF combination was 0.925 (Figure 4H). This finding indicated a basis for FLI1 to be a potential biomarker for RA. The combination of CRP, ESR, CCP, and RF with the routine laboratory indicators of RA showed high diagnostic efficacy in evaluating RA disease activity.

Identification of RA Synoviocyte-Associated Gene Modules and Key Genes

WGCNA coexpression modules comprise a set of genes with a high coexpression level and topological overlap similarity. In order to identify the coexpression modules and essential genes associated with RA and FLI1, WGCNA was applied to 449 DEGs in GSE 12021. The sample clustering tree depicted in Figure 5A was produced by clustering

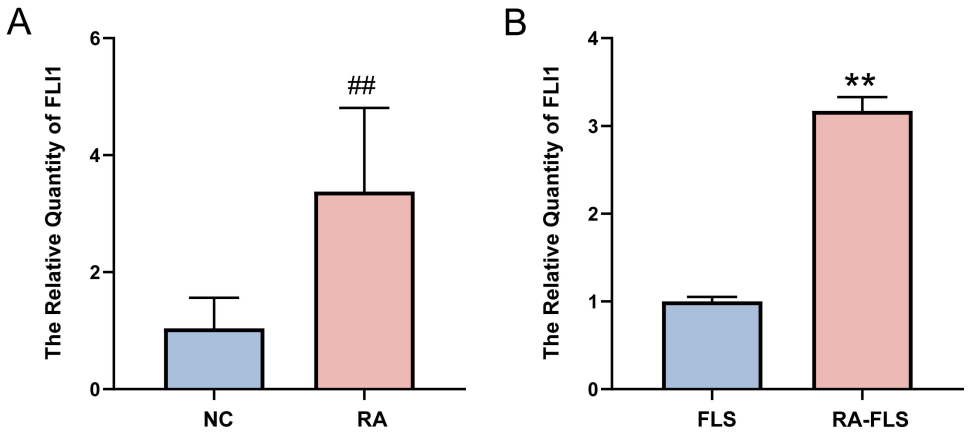

Figure 3 FLI1 expression in RA patients and synoviocytes. (**A**) FLI1 expression in healthy individuals and RA patients, (**B**) FLI1 expression in FLS and RA-FLS.$^{\#\#}P < 0.01$, compared to NC group (Student's *t*-test); $^{**}P < 0.01$, compared to the FLS group (Student's *t*-test).

the data using Pearson's correlational coefficient. A scale-free network was generated by selecting a gentle threshold of 16 (R = 0.85) (Figure 5B and C). In order to illustrate node similarity through weighted correlations, the neighbor matrix was converted into a Topology Overlap Matrix. Ultimately, the tree was reduced using dynamic tree cropping and average hierarchical clustering (Figure 5D). Of the six modules, the brown module was correlated with RA (cor = 0.761 and P = 6.3e-05) and also positively correlated with FLI1 (cor = 0.511 and P = 0.018) (Figure 5E). This led to the identification of the brown module as a necessity for additional investigation. The target genes of the transcription factor FLI1 were predicted using the KnockTF, and a total of 13,022 target genes were obtained after deleting the duplicates (Figure 6A); 44 of these genes belonged to the brown module. By using the R software to analyze the coexpression coefficients of FLI1 with 44 genes, 20 genes were finally selected (R > 0.4, $P < 0.05$) to construct the FLI1–target network (Figure 6B). Of these, the TOP5 displaying a high correlation with FLI1 were AGA, DCK, LRRC15, MAN2A1, and TES, and AGA, DCK, LRRC15, MAN2A1, and TES were positively correlated with FLI1 (Figure 6C–G), which suggested that AGA, DCK, LRRC15, MAN2A1, and TES showed a positive feedback with FLI1.

Receiver-Operating Characteristic (ROC) Curve Analysis of AGA, DCK, LRRC15, MAN2A1, and TES and GSEA-KEGG Analysis

The clinical diagnostic value of FLI1 and the five target genes in the GSE12021 database was determined by calculating the area under the ROC curve (Figure 7A–F). These results indicated that the AUC values were 0.917 for AGA, 0.843 for FLI1, 0.880 for DCK, 0.928 for LRRC15, 0.861 for MAN2A1, and 0.917 for TES, implying the effectiveness of these indicators in diagnosing RA. In order to gain a more comprehensive understanding of the biological functions of these target genes, GSEA-KEGG analysis was implemented to reveal that the pathways most closely related to AGA were calcium and ERBB-signaling pathways, those most closely related to DCK were MAPK and calcium-signaling pathways and oxidative phosphorylation, that most relevant to LRRC15 was the p53-signaling pathway, those most relevant to MAN2A1 were TGF-beta, PPAR-signaling pathway, and those most relevant to TES were the adipocytokine-signaling pathways (Figure 7G–K). These pathways were mainly associated with cell proliferation, cell differentiation, apoptosis, regulation of enzyme activity, cell adhesion and cell migration, angiogenesis, and the regulation of immune inflammation.

Immune Infiltration Analysis of RA and AGA, DCK, LRRC15, MAN2A1, and TES

The immune cell compositions of RA and HCs differed substantially (Figure 8A) as per the CIBERSORT algorithm. Memory B cells, plasma cells, follicular T-helper cells, gamma delta T-cells, and macrophages were significantly elevated in patients with RA. Conversely, healthy controls exhibited significantly fewer activated mast cells (Figure 8B). The correlation among immune cells is illustrated in Figure 8C. The relationship between immune cell infiltration in RA and

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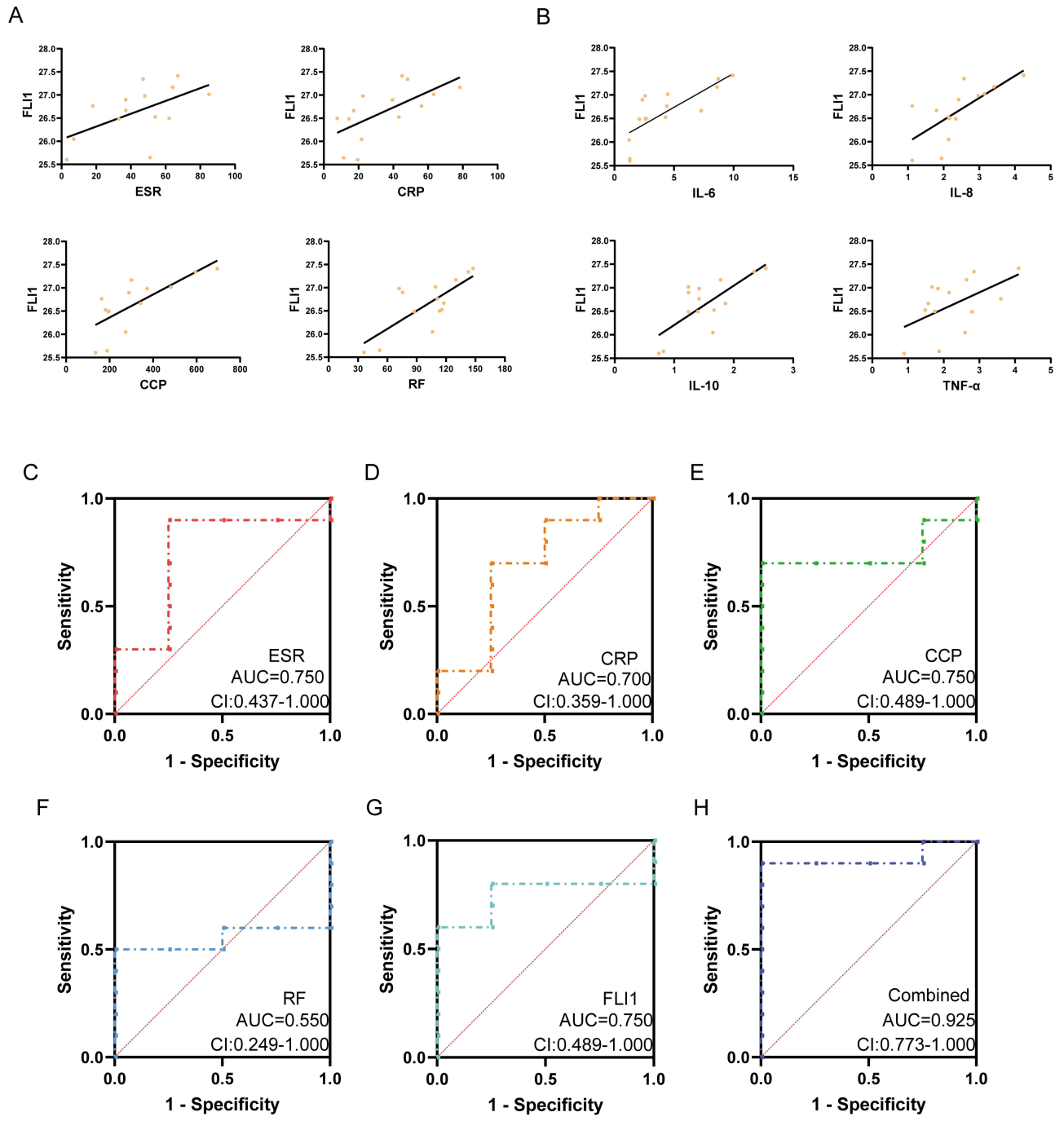

Figure 4 Correlational analysis of FLI1 with RA-related immunoinflammatory indicators. (**A**) Correlational analysis of FLI1 with CRP, ESR, CCP, and RF, (**B**) Correlational analysis of FLI1 with IL-6, IL-8, IL-10, and TNF-α, (**C-G**). ROC analysis of FLI1 as well as CRP, ESR, CCP, and RF, (**H**) ROC analysis of FLI1, CRP, ESR, CCP, and RF in combined assays.

FLI1's main target genes was investigated using Pearson's analysis (Figure 8D). The findings indicated that LRRC15 exhibited a statistically significant negative correlation with eosinophils, monocytes, and quiescent CD4 memory T-cells. Plasma and gamma delta T-cells exhibited a positive correlation with MAN2A1, while monocytes exhibited a significantly negative correlation. Memory B-cells and follicular memory T-cells were significantly correlated with TES. Memory B-cells, follicular helper T-cells, and M1 macrophages demonstrated a highly significant positive correlation with TES, while activated dendritic cells, quiescent natural killer (NK) cells, monocytes, and neutrophils demonstrated a highly significant negative correlation. The correlation between AGA and B cells was substantially

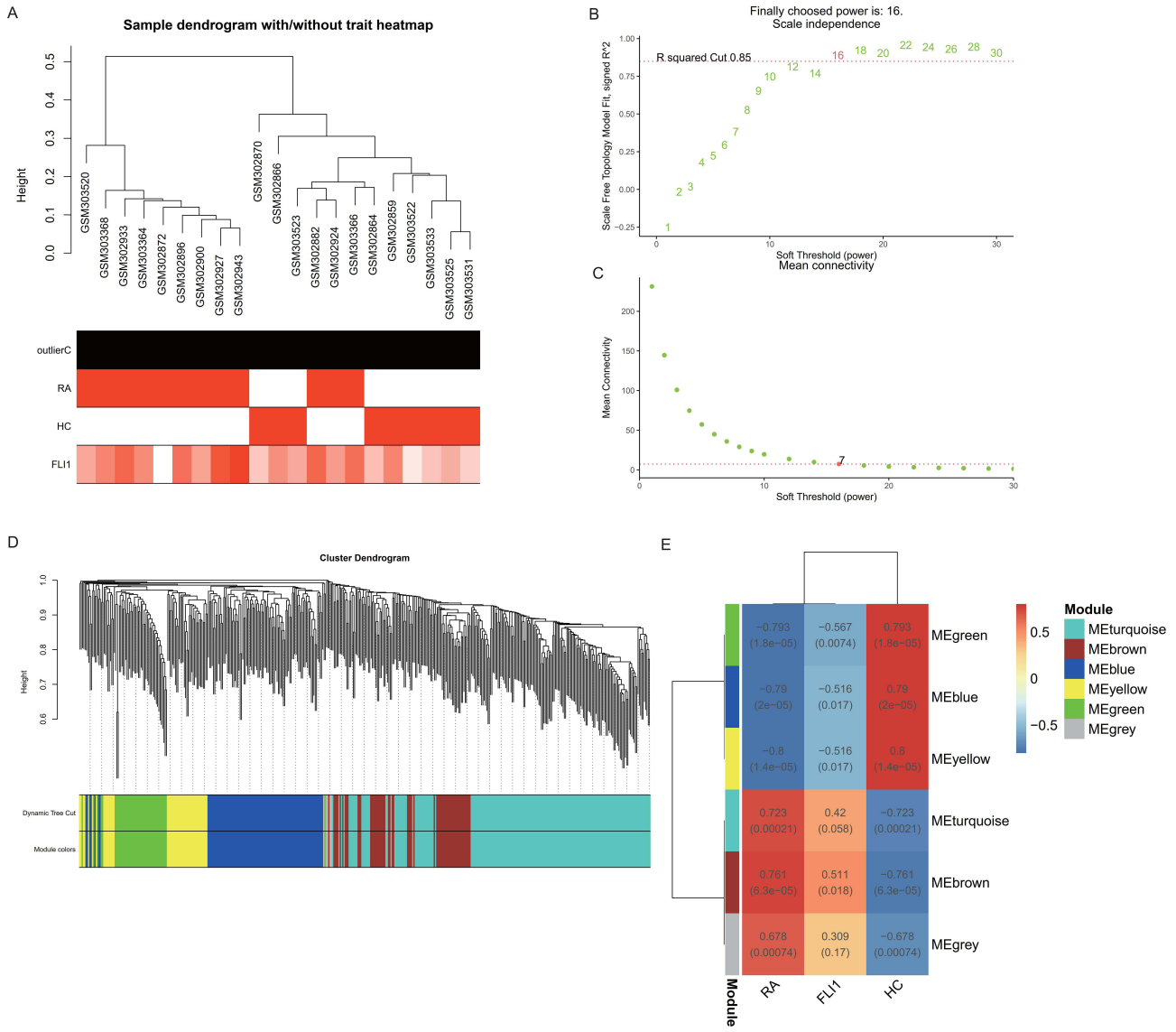

Figure 5 Co-expression module analysis. (**A**) Clustering dendrogram of 21 samples, (**B**) The connection between different soft threshold powers and the scale-free fit index, (**C**) Relationship between average connectivity and various soft threshold powers, (**D**) Clustering dendrogram of genes with various colors representing different modules, (**E**) Relationships between three traits and six modules.

negative, while it was positive with memory B-cells, plasma cells, and CD8 T-cells. The correlation was negative with eosinophils, monocytes, and quiescent CD4 memory T-cells. The memory B-cells, follicular helper T-cells, M1 macrophages, activated dendritic cells, quiescent NK cells, monocytes, and neutrophils exhibited a robust positive correlation with DCK. M1 macrophages demonstrated a substantial positive correlation with activated dendritic cells, resting NK cells, activated mast cells, monocytes, resting CD4 memory T-cells, and neutrophils were significantly negatively correlated.

Effects of Interfering With FLI1 on AGA, DCK, LRRC15, MAN2A1, and TES in RA Synoviocytes

To determine the function of FLI1 in patients with RA, si-FLI1 was constructed and verified in three dimensions: silencing efficiency, concentration, and time to screen the best si-FLI1. Finally, the si1 group was identified as the study group (Figure 9A–C). Considering that the treatment may be affected by time and concentration, different concentrations and periods were set. The best efficacy was achieved at 100 pmol and 48 h. The expressions of AGA, DCK, LRRC15,

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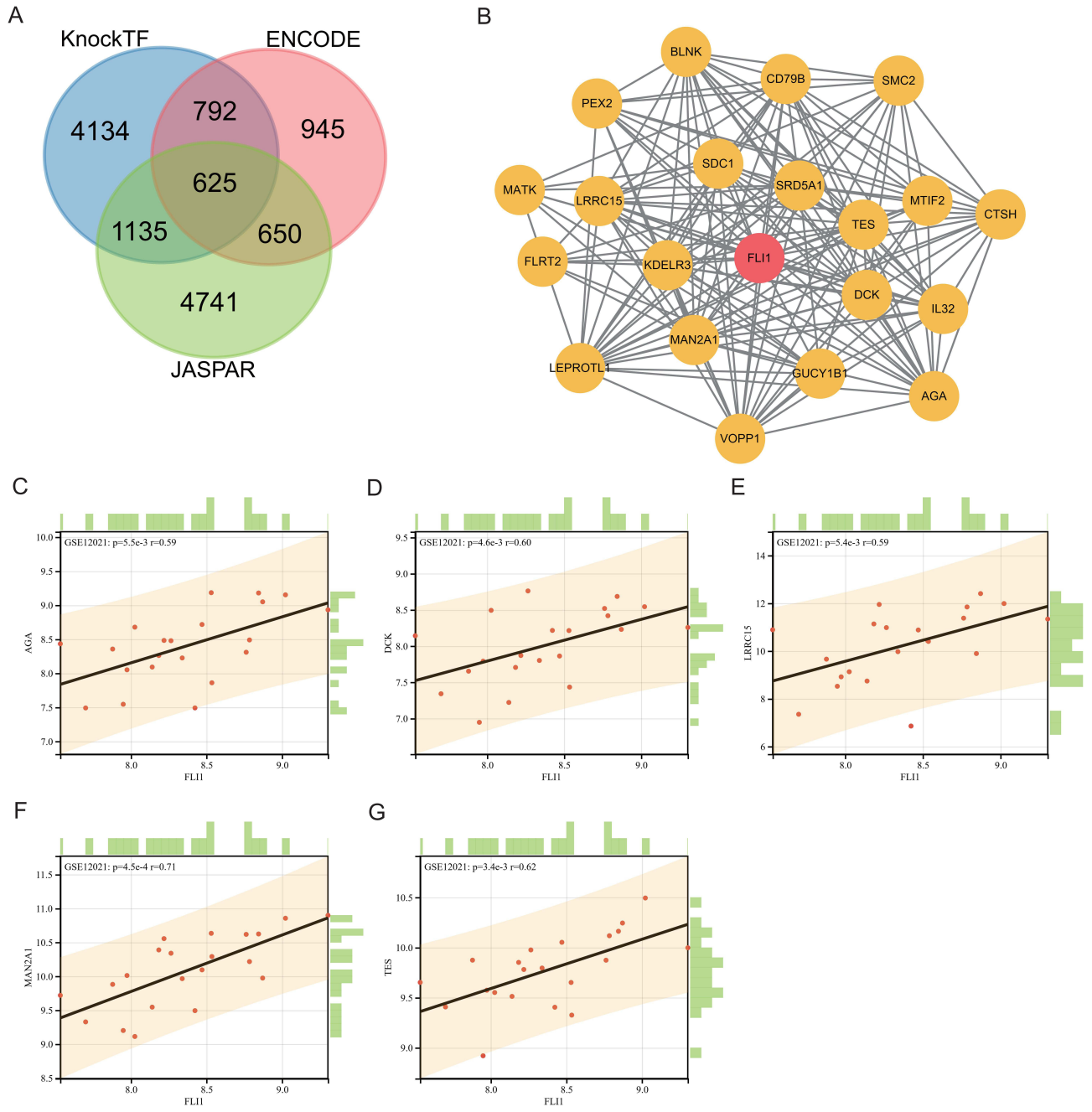

Figure 6 PPI network construction and identification of the target genes. (**A**) Target genes of FLI1, (**B**) Co-expression network in FLI1 and key module target genes, (**C-G**). Correlational analysis of AGA, DCK, LRRC15, MAN2A1, TES, and FLI1.

MAN2A1, and TES were measured after silencing FLI1, and the results suggested that, in the RA-FLS group, the expressions of AGA, DCK, LRRC15, and MAN2A1 were substantially higher than those in the si-NC group ($P < 0.01$). In addition, the RA-FLS group exhibited a substantially higher level of TES expression than the si-NC group ($P < 0.01$). The si-FLS group exhibited the same expression as the si-NC group ($P < 0.01$). The expressions of AGA, DCK, LRRC15, MAN2A1, and TES were noticeably reduced in the si-FLI1 group ($P < 0.01$) compared with those in the si-NC group (Figure 9D–H). These results indicated that the inhibition of FLI1 expression reduced the expressions of AGA, DCK, LRRC15, MAN2A1, and TES.

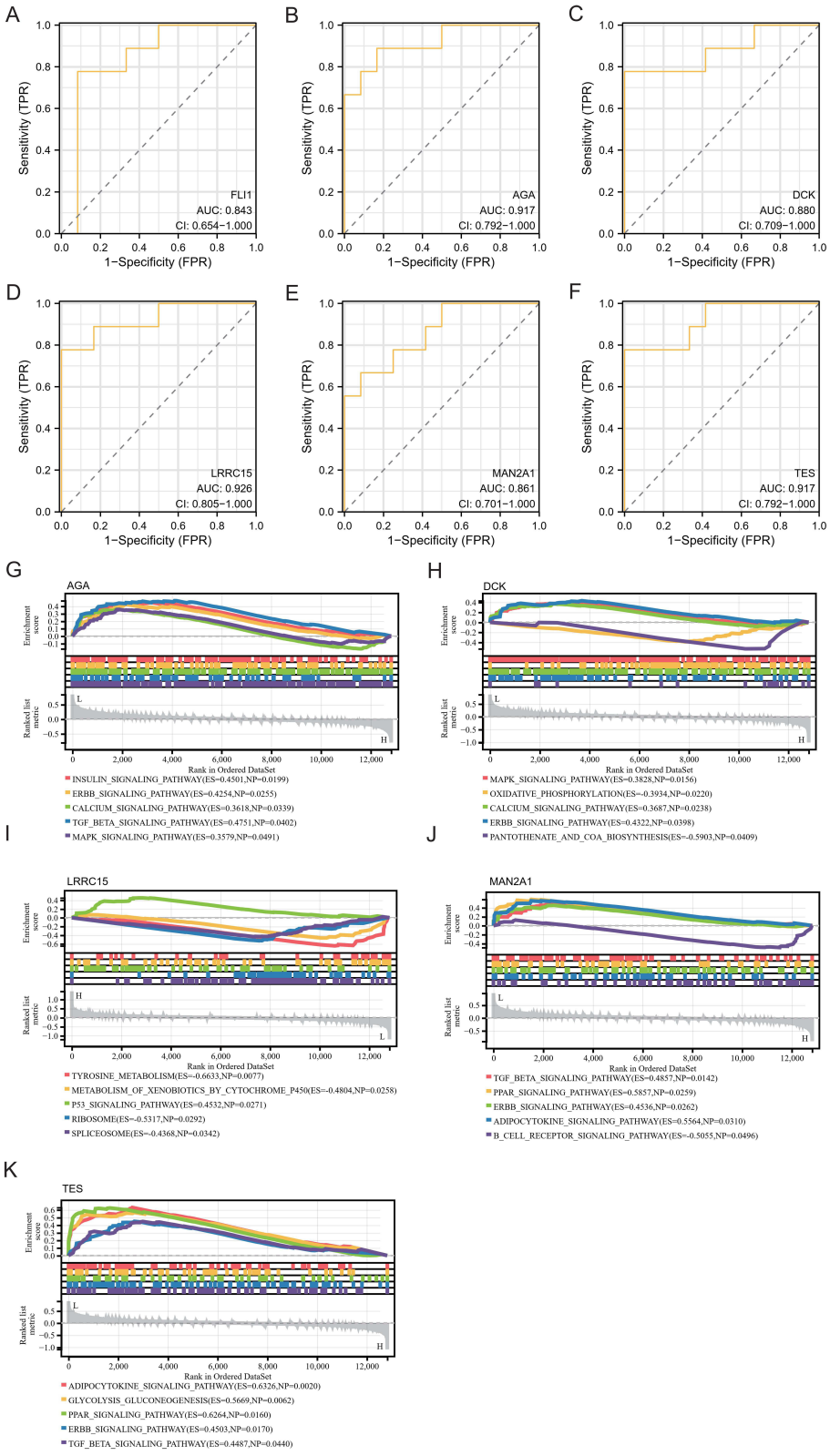

Figure 7 Expressions and GSEA-KEGG analysis of the key modules. (**A-F**). ROC analysis of FLI1 as well as 5 target genes in GSE12021, (**G-K**). GSEA-KEGG analysis of 5 target genes of FLI1 in GSE112021.

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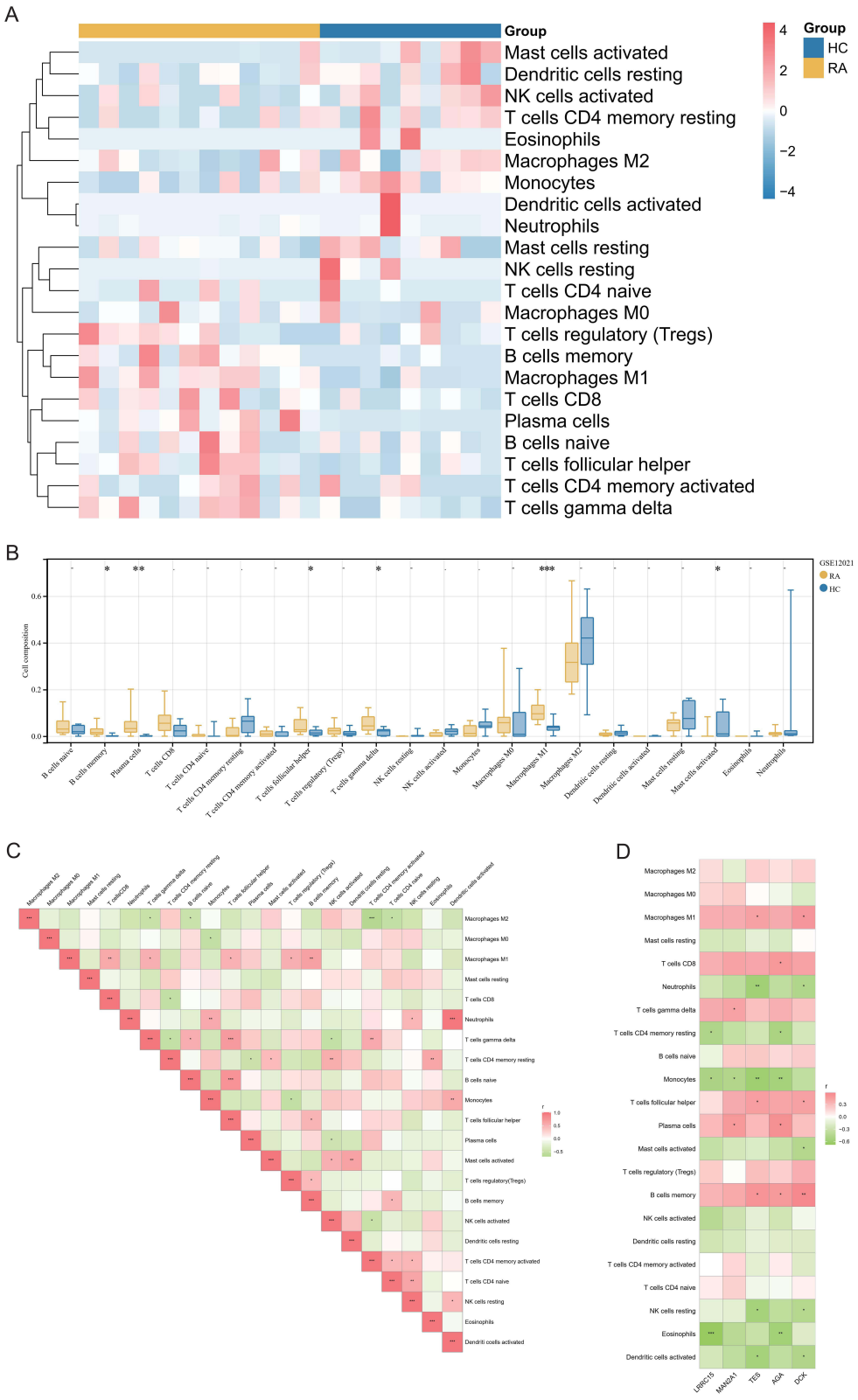

Figure 8 CIBERSORT analysis to estimate immune infiltration in RA. (**A**) Twenty-two subpopulations' expressions in RA among tumor immune cells, (**B**) Comparison of CIBERSORT immune cell fractions in RA and HC, (**C**) A correlation matrix that illustrates the activity of 22 immune cells and their proportions in RA, (**D**) A correlation matrix that illustrates the correlation between the 22 immune cell proportions in RA and the primary target genes.

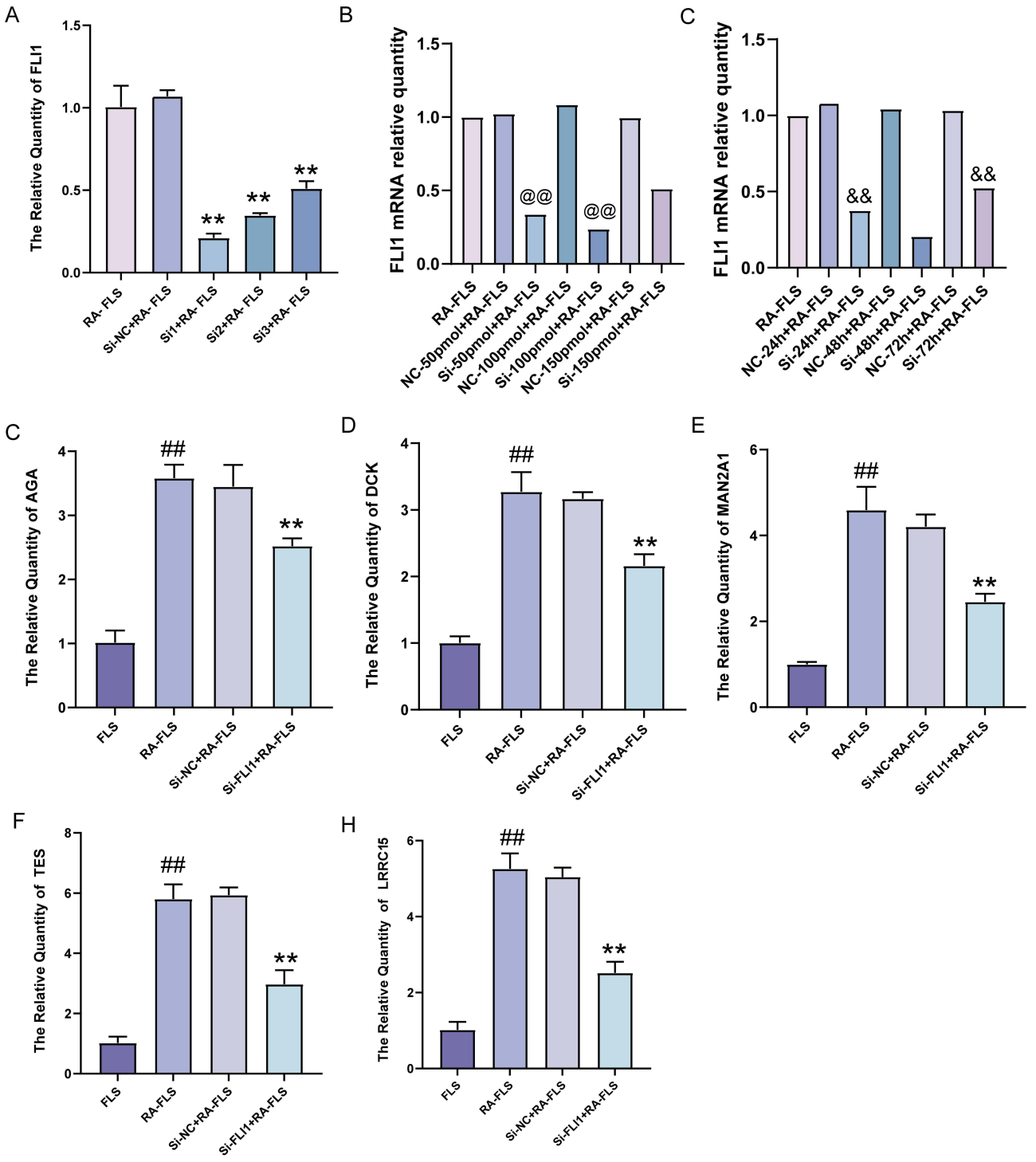

Figure 9 Expression of target genes after silencing FLI1. (**A**) FLI1 silencing screen, (**B**) FLI1 concentration screen, (**C**) FLI1 time screen, (**D-H**). Silencing of the FLI1 expression of AGA, DCK, LRRC15, MAN2A1, and TES in each group. $^{\#\#}P < 0.01$, compared to the FLS group (one-way ANOVA); $^{**}P < 0.01$, compared to the RA-FLS group (one-way ANOVA). $^{@@}P < 0.01$, compared to the si-150pmol+RA-FLS group (one-way ANOVA); $^{\&\&}P < 0.01$, compared to the si-48h+RA-FLS group (one-way ANOVA).

Effect of FLI1 Interference on RA Synovial Cell Function

To confirm the impact of FLI1 interference on RA synoviocyte functions, si-FLI1 was utilized to silence FLI1, and the proliferation and apoptosis of the synoviocytes were monitored by dividing them into groups. The viability of the cells in each group was initially assessed using a CCK8 cell proliferation assay. The si-FLI1+RA-FLS group exhibited the lowest

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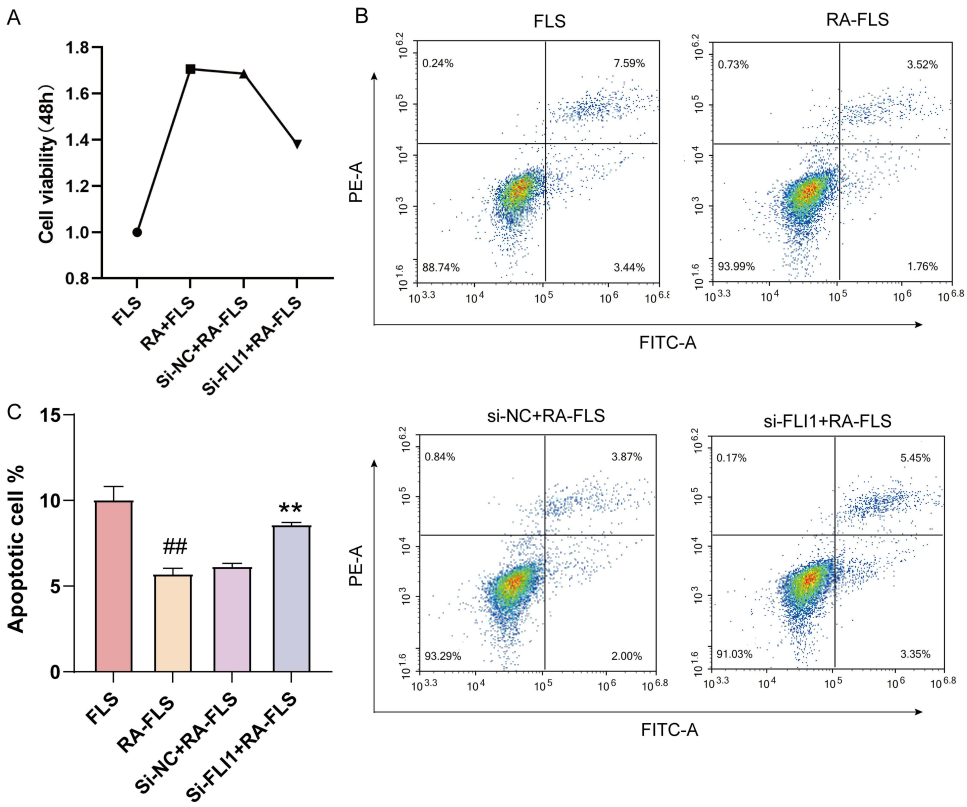

Figure 10 Proliferation and apoptosis of RA synoviocytes after silencing FLI1. (**A**) Proliferation of cells in the groups silencing FLI1, (**B** and **C**) Apoptosis of cells in groups silencing FLI1.$^{\#\#}P < 0.01$, compared to the FLS group (one-way ANOVA); $^{**}P < 0.01$, compared to the RA-FLS group (one-way ANOVA).

cell viability (Figure 10A). Subsequently, flow cytometry was implemented to identify apoptosis in each cohort (Figure 10B). Apoptosis evaluation included early and late apoptotic cells, the proportion of apoptosis in the si-FLI1 +RA-FLS group was higher than that in the RA-FLS group ($P < 0.01$) (Figure 10C).

Discussion

RA is a systemic autoimmune disease that is distinguished by inflammatory arthritis and extra-articular involvement.[24–26] In various patients or stages, RA exhibits a diverse array of clinical manifestations and complications. These variations pose difficulties in diagnosis in the clinic and lead to misdiagnosis or incorrect diagnosis.[27–29] Under normal conditions, the body cannot trigger an immune response against its tissues.[30] However, antibodies or immune effectors against self-substances are inadvertently produced by the immune system when this self-tolerance mechanism is compromised or damaged, which triggers an abnormal immune response. The immune system erroneously attacks and destroys the body's normal tissues, organs, or systems, thereby ultimately triggering inflammation, dysfunction, and even organ failure.[31–33] As a complex immune disease, the treatment for RA involves providing symptomatic relief, controlling disease progression, and preventing complications. The treatment approaches include drug therapy (eg, immunosuppressants and hormonal drugs), physical therapy, cell therapy, and psychological support.[34–36] The long course of RA makes it difficult for patients to adhere to the treatment, but failure to control the disease promptly aggravates the development of the disease.[37] In addition, the degradation of joint bone and patient outcomes are inextricably linked to the progression of inflammation and the level of disease activity in RA.[38–40] Nevertheless, the immune and inflammatory diagnostic biomarkers of RA must be comprehensively defined. Consequently, the early detection and treatment of RA necessitate the identification of specific immune and inflammatory diagnostic markers.

In RA, autoantibodies, such as RF and CCP, and laboratory indicators, such as ESR, CRP, IL-6, IL-8, and TNF-α, which are essential in the diagnosis and pathological process of the disease, are significantly abnormal.[39,41–43] Thus, the

immunoinflammatory response is essential for the development of RA, which may be related to hematopoiesis and the regulation of immune cells.[6,44–46] DEGs for RA were obtained from the GSE1919 and GSE12021 datasets. Subsequently, Venn diagram analysis was performed with the 27 ETS genes; finally, the DEG FLI1, which is upregulated in RA, was obtained.

FLI1 has been clinically validated. Clinical samples were obtained from NC and RA patients for the determination of the FLI1 content and RA-related immunoinflammatory indicators. Correlational analysis was performed to reveal that FLI1 was positively correlated with RF, CCP, ESR, and CRP. Some studies found that the expression of FLI1 in the synovial tissues of patients with RA was significantly increased[47,48] and correlated with the disease activity,[15,49] which is consistent with our current findings. To further evaluate the diagnostic efficacy of FLI1, the RA group was categorized into high and low disease activities. The subject characteristic curve was employed to assess the diagnostic efficacy of FLI1, which indicated that the efficacy of FLI1 in combination with RF, CCP, ESR, and CRP in the diagnosis of RA was significantly increased when compared with that of the single index. Hence, FLI1 may be an important indicator for assessing RA disease activity.

Past studies have reported that FLI1-regulated target genes are associated with the production of multiple inflammatory mediators, which can exacerbate the inflammatory response in immune system diseases and joint damage in arthritis.[17,50–52] Evidence highlights the potential impact of racial genetic diversity on FLI1 functions. The FLI1 enhancer rs10181488 variant correlates with elevated pro-inflammatory cytokine production, particularly in the African American cohorts.[9] This finding is consistent with our observation of FLI1-driven upregulation of IL-6/TNF-α, suggesting that racial differences in FLI1 regulatory elements may modulate RA severity or treatment response. Our cohort consisted primarily of Asians, which limited the generalizability to other populations. Therefore, cross-validation in our subsequent studies in different cohorts is deemed necessary. These studies established that FLI1 plays a significant role in the diagnosis of RA. To identify the downstream target genes, genes exhibiting high co-expression levels were selected via WGCNA analysis. The target genes of FLI1 were then predicted using KnockTF, JASPAR, and ENCODE databases. The target genes of AGA, DCK, LRRC15, MAN2A1, and TES were identified. These five target genes were analyzed using ROC curves, and the AUC values indicated a strong discriminatory ability for RA samples. Finally, GESA–KEGG analysis on AGA, DCK, LRRC15, MAN2A1, and TES was performed to predict their biological functions, identifying a connection between the MAPK-signaling pathway, the p53-signaling pathway, oxidative phosphorylation, other immunoinflammatory pathways, and the FLI1 target genes.[53–59] Moreover, the target genes were subjected to an immune-infiltration analysis. The association of FLI1 and AGA, DCK, LRRC15, MAN2A1, and TES with immune inflammation was verified from both clinical and bioinformatics perspectives.

RA is a chronic inflammatory condition that is characterized by the presence of TNF-α, IL-1, IL-6, and IL-10.[60–62] The activation and proliferation of inflammatory cells and the joints are facilitated by the MAPK-signaling pathway. This pathway stimulates inflammatory cell activation and proliferation as well as joint tissue destruction and the expression of matrix metalloproteinases (MMPs).[63–65] In RA, the expression and activity of MMPs, which can break down the main components of articular cartilage, such as type II collagen and proteoglycans, are significantly increased,[66–68] thereby destroying the cartilage structure.[69,70] Moreover, MMPs interact with TNF-α and IL-1β to form a positive feedback loop that exacerbates inflammation and tissue damage.[71,72] Furthermore, oxidative phosphorylation can occur in the mitochondria, causing mitochondrial dysfunction and oxidative stress, which aggravates cellular and tissue damage and worsens inflammatory responses.[73] The calcium-signaling pathway is linked to bone metabolism,[74–76] and the progression of RA can be influenced by any of these signaling pathways. These results substantiate the critical role of FLI1-regulated target genes in the inflammatory response to RA. Moreover, cellular experiments were implemented to ascertain the influence of FLI1 silencing on the functionality of RA synoviocytes, which signified that the inhibition of FLI1 expression increased apoptosis in RA cells, thereby affecting disease development. This finding suggests that FLI1 may promote the inflammatory response in RA and inhibit apoptosis of RA synovial cells by regulating its target genes, including AGA, DCK, LRRC15, MAN2A1, and TES.

Conclusion

In this study, bioinformatics analyses, clinical trials, and cellular experiments were integrated, which helped understand the role of FLI1 in the pathogenesis of RA. The FLI1 content and RA-associated immunoinflammatory indexes were measured in conjunction with clinical samples of RA, followed by correlational analyses, which enhanced the clinical relevance of the study results. The target genes of FLI1 were predicted using WGCNA analysis and KnockTF, JASPAR, and ENCODE databases. In addition, the roles of these target genes in the disease process of RA were experimentally verified to reveal the biological mechanisms of FLI1 regulation.

This study has some limitations, such as the lack of depth and breadth. Despite the preliminary evidence provided by this study regarding FLI1's involvement in RA, additional research is required to clarify the precise action mechanism and regulatory network. Therefore, in the future, we intend to focus on investigations to uncover the action mechanism. Furthermore, the findings' statistical power and generalizability may have been restricted by the study's small sample size. Therefore, we plan to increase the sample size in future studies and include patients with RA belonging to different races and disease stages. Through multicenter collaboration, we collected diverse samples and conducted extensive studies to verify the generalizability of FLI1 as a diagnostic and therapeutic target for RA.

In conclusion, this study examined the pertinent FLI1 targets for RA treatment using various databases and novel bioinformatics techniques. The findings suggested that FLI1 may serve as a potential immune-inflammatory biomarker for RA, with implications for disease stratification and therapeutic targeting. Through cellular experiments, we found that FLI1 could increase RA synovial cell viability and inhibit synovial cell apoptosis by promoting inflammation. We hope that the preliminary exploration of the FLI1-signaling pathway may facilitate the identification of a novel pathway for balancing the immune system and inhibiting excessive inflammation, thereby providing a more precise and effective therapeutic regimen for patients with RA.

Data Sharing Statement

All data analyzed in this study are included in this publication article file, available from the corresponding author upon reasonable request.

Consent for Publication

This paper is our original work. We certify that this manuscript has not been published in part or whole elsewhere in any language, and it has not been submitted to any other journal for review. We certify that all authors named deserve authorship and that all authors have agreed to be so listed and have read and approved the manuscript.

Institutional Review Board Statement

The study was conducted in accordance with the Declaration of Helsinki and approved by the First Affiliated Hospital of Anhui University of Traditional Chinese Medicinal Review Board (approval number 2023AH-52; approval date July 27, 2023).

Informed Consent Statement

Informed consent was obtained from all subjects involved in the study.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

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

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