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TRANSLATIONAL SCIENCE

EPO promotes the progression of rheumatoid arthritis by inducing desialylation via increasing the expression of neuraminidase 3

Gan Wu,[1,2] Ben Cao,[3] Haige Zhai,[3] Bin Liu,[3] Yuan Huang,[3] Xiaowei Chen,[4] Hanzhi Ling,[4] Sunwang Ling,[3] Shengwei Jin,[1,2] Xinyu Yang,[5] Jianguang Wang [1,2,3]

Correspondence to
Professor Jianguang Wang, Institute of Autoimmune Diseases, School of Basic Medical Sciences, Wenzhou Medical University, Wenzhou, Zhejiang, China; wjg@wmu.edu.cn, Professor Xinyu Yang, Department of Medicinal Chemistry, School of Pharmaceutical Sciences, Wenzhou Medical University, Wenzhou, Zhejiang, China; yangxy@wmu.edu.cn and Professor Shengwei Jin, Department of Anesthesia and Critical Care, The Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University, Wenzhou, Zhejiang, China; jinshengwei69@163.com

GW and BC contributed equally.

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ABSTRACT

Objective Erythropoietin (EPO) known as an erythrocyte-stimulating factor is increased in patients with rheumatoid arthritis (RA). Nevertheless, the function of EPO in the process of RA and relative mechanism needs to be further clarified.

Methods The level of EPO in serum and synovial fluid from patients with RA and healthy controls was determined by . Collagen-induced arthritis (CIA) mice were constructed to confirm the role of EPO on RA pathogenesis. Differentially expressed genes (DEGs) of EPO-treated fibroblast-like synoviocyte (FLS) were screened by transcriptome sequencing. The transcription factor of neuraminidase 3 (NEU3) of DEGs was verified by double luciferase reporting experiment, DNA pulldown, electrophoretic mobility shift assay and chromatin immunoprecipitation-quantitative PCR (qPCR) assay.

Results The overexpression of EPO was confirmed in patients with RA, which was positively associated with Disease Activity Score 28-joint count. Additionally, EPO intervention could significantly aggravate the joint destruction in CIA models. The upregulation of NEU3 was screened and verified by transcriptome sequencing and qPCR in EPO-treated FLS, and signal transducer and activator of transcription 5 was screened and verified to be the specific transcription factor of NEU3. EPO upregulates NEU3 expression via activating the Janus kinase 2 (JAK2)-STAT5 signalling pathway through its receptor EPOR, thereby to promote the desialylation through enhancing the migration and invasion ability of FLS, which is verified by JAK2 inhibitor and NEU3 inhibitor.

Conclusion EPO, as a proinflammatory factor, accelerates the process of RA through transcriptional upregulation of the expression of NEU3 by JAK2/STAT5 pathway.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Anaemia occurs in 30%–70% of patients with rheumatoid arthritis (RA), which leads to the compensatory increase of erythropoietin (EPO).
⇒ It is still controversial whether EPO plays a proinflammatory or anti-inflammatory role in the process of RA.
⇒ Clinical trials have proved that Janus kinase (JAK) inhibitors are effective in the treatment of RA, but the role of JAK2 in RA needs to be further clarified.

WHAT THIS STUDY ADDS

⇒ The levels of EPO were increased in serum and synovial fluid of patients with RA, which were positively related to Disease Activity Score 28-joint count.
⇒ EPO is a proinflammatory factor accelerating the RA process through transcriptionally upregulating the expression of neuraminidase 3 (NEU3) by JAK2/STAT5 pathway.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Implying a vicious cycle between RA development and the increased level of EPO.
⇒ For patients with RA with high EPO levels, not only JAK1 but also JAK2 inhibition is required.
⇒ Revealing the molecular mechanism of NEU3-mediated desialylation in RA development, providing a new target for RA drug development.

variety of downstream proinflammatory cytokines.[4] Nevertheless, the effectiveness of different target of JAKs (JAK1, JAK2, JAK3 and TYK2) needs further clarification.

Erythropoietin (EPO), an erythrocyte-stimulating factor, mainly activates JAK2/STAT5 pathway to regulate the proliferation, differentiation and maturation of erythroid progenitor cells.[5] In addition, the extrahaematopoietic properties of EPO in non-haematopoietic tissues have been increasingly reported recently.[6–9] Regarding the role of EPO in RA, it has been reported that 64% of patients with RA have anaemia, with a compensatory increase of serum EPO level.[10–13] A previous clinical study reported that the concentration of EPO in serum from patients with RA was positively correlated

INTRODUCTION

Rheumatoid arthritis (RA) is a common chronic autoimmune disease characterised by synovitis and the excessive proliferation of fibroblast-like synoviocyte (FLS) with continuously secreting proinflammatory cytokines, such as interleukin 6 (IL-6), tumor necrosis factor alpha (TNF-α) and matrix metalloproteinases (MMPs), leading to aggravated joint destruction and disability.[1–3] In recent years, the inhibitors of Janus kinase (JAK), such as tofacitinib and baricitinib, have been accepted for the satisfactory effect on RA treatment by inhibiting a

with the erythrocyte sedimentation rate (ESR).[13] On the one hand, EPO was considered an anti-inflammatory factor in RA. For instance, Cuzzocrea's research found that EPO significantly reduced the disease severity, oxidative damage and the production of proinflammatory cytokines in the model of collagen-induced arthritis (CIA).[14] EPO also showed efficacy in the treatment of autoimmune diseases such as autoimmune colitis and autoimmune encephalomyelitis, by inhibiting the nuclear factor kappa B pathway and the production of adaptive immune cells.[15 16] On the other hand, in contrast to the above anti-inflammatory effects, emerging studies have revealed that EPO may accelerate the progression of these diseases. Studies have shown that treatment with EPO can induce proinflammatory factor production in RA FLS.[17] Moreover, a study has reported that serum EPO concentration in patients with RA has a positive correlation with C reactive protein,[12] and our study found that the level of EPO is elevated in serum and synovial fluid, which is positively related to disease activity in RA. Overall, the function of EPO on the process of RA is still controversial, which needs to be further explored.

Sialic acids (SAs) often exist as the terminal sugars of glycans of either glycoproteins or glycolipids on the cell surface and thus are directly involved in biological processes, such as cell-cell, cell-ligand and cell-pathogen interactions.[18] Abnormal sialylation modification has been reported in patients with RA, especially decreased anti-citrullinated protein antibodies (ACPA) sialylation,[19–21] which switches ACPA from anti-inflammatory activity to proinflammatory effects in RA.[22] In addition, our previous studies also confirmed that the decreased β-galactoside α-2,6-sialyltransferase 1 (ST6GAL1) was an important cause of ACPA desialylated in patients with RA.[23] In vivo studies showed that sialylated ACPA reduced the arthritis score of CIA mice,[20] and the decreased sialylation of FLS enhanced the inflammatory condition of microenvironment in synovial tissue.[24 25]

In this study, we clarified the effect of EPO and its downstream pathway of JAK2/STAT5 on the process of RA and prospect to provide a theoretical basis for the clinical treatment of JAK inhibitor.

RESULTS

Level of EPO increased in patients with RA and was positively associated with RA activity

To clarify the role of EPO in RA progression, the level of EPO was detected by ELISA, and results showed that the level of EPO was increased in serum and synovial fluid of patients with RA compared with that in healthy controls (figure 1A,B), which was positively associated with Disease Activity Score 28-joint count (DAS28), an index to assess the disease activity of RA (figure 1C,D). Considering that erythropoietin receptor (EPOR) is the specific receptor of EPO, the expression of EPOR at messenger RNA and protein levels was detected in synovial tissue of patients with RA and healthy controls, and results showed that it increased in patients with RA (figure 1E,F); the same tendency is verified by immunohistochemistry (IHC) (figure 1G). Moreover, EPOR was mainly expressed in CD90+FLS of synovial tissues (figure 1H, online supplemental figure S1A). Next, the overexpression of EPOR was also identified in RA FLS by qPCR, western blot and immunofluorescence (figure 1I–K). The above results demonstrated that the level of EPO and its receptor EPOR were increased in patients with RA, which was positively associated with RA activity.

EPO aggravated the joint destruction in arthritis model

To evaluate the function of EPO on the process of RA, CIA model was established (figure 2A). Since the serum EPO did not increase in CIA mice compared with the control group, we then intervened with EPO (3000 U/kg) in CIA mice (figure 2B). Morphological observation of the CIA mice showed that EPO-treated mice exhibited more severity of paw swelling and were ranked at higher clinical scores (figure 2C,D). Consistently, H&E staining and safranin O-fast green staining showed that EPO promoted joint damage and bone erosion (figure 2D,E). Furthermore, EPO elevated the concentrations of the pro-inflammatory cytokines of TNF-α, IL-6 and IL-1β and decreased the anti-inflammatory cytokine of IL-10 in the serum of the CIA mice (figure 2F–I). Meanwhile, we constructed the collagen antibody induced arthritis (CAIA) model to observe the effect of inhibition of EPO elevation on arthritis by injection of an EPO-neutralising monoclonal antibody (EPO mAb) (online supplemental figure S2A,B). The results showed that EPO mAb therapy decreased the level of proinflammatory cytokines and the clinical score of joints and alleviated the joint destruction in the CAIA model (online supplemental figure S2C–I). Overall, the results indicated that EPO aggravates joint injury and inflammatory response in the arthritis model.

RNA-Seq revealed that EPO increased the expression of NEU3

In order to explore the mechanism of EPO influencing the process of RA, we treated RA FLS with 10 U/mL recombinant human EPO (rhEPO) for 24 hours and performed transcriptome sequencing. Because there have been no functional studies of EPO in FLS, we selected the time and the concentration at the highest expression of EPOR, and the results showed that 10 U/mL and 24 hours were the optimal concentration and time of EPO intervention. Maybe the highest EPOR expression could not fully represent the complete functions of the EPO-EPOR axis act in FLS cells. This may be due to the limitation that transcriptome sequencing can only reflect the gene expression level under certain circumstances (figure 3A). 71 differentially expressed genes (DEGs) were screened ($|\log_2$ fold change$| > 1$ and $p < 0.05$) (figure 3B, online supplemental table S1). Gene Ontology (GO) enrichment analysis of DEGs revealed that cell adhesion molecule production, ganglioside catabolic process, oligosaccharide catabolic process and glycoprotein biosynthetic process were significantly enriched (figure 3C). The Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway enrichment analysis showed that glycan degradation was mainly affected (figure 3D). According to the GO enrichment and KEGG pathway enrichment analysis, glycan remoulding may be an important influencing factor in the transformation of FLS into a proinflammatory state. Therefore, *NEU3*, an upregulated gene involved in the pathway of glycan degradation, was selected for further verification. Results showed that the expression of NEU3 was increased in RA FLS treated with EPO (figure 3E–G), but EPO did not affect the expression of ST6GAL1 (figure 4A,B), suggesting that EPO probably modified the glycosylation of FLS by regulating the expression of NEU3.

NEU3 was essential for EPO promoting the migration and invasion of FLS

Studies reported the decreased sialylation in RA FLS, which promotes its invasion ability.[24 25] NEU3, an enzyme of desialylation,[26] might be involved in the desialylation of RA FLS. Our results showed that knockdown or inhibiting of NEU3 by small interfering RNA or 2,3-Dehydro-2-deoxy-N-acetylneuraminic
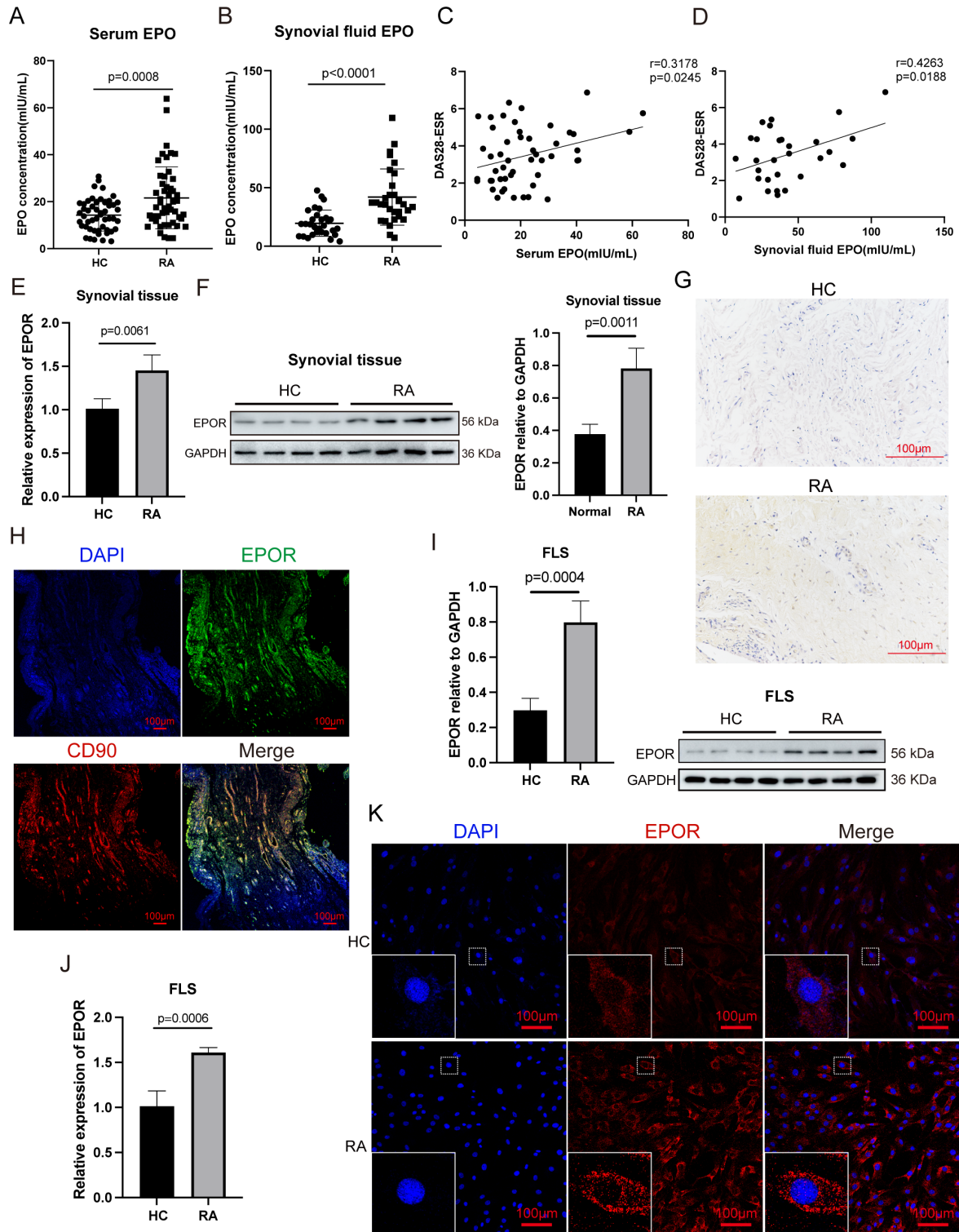
Figure 1 The overexpression of erythropoietin (EPO) and its receptor EPOR in patients with RA. (A) The concentration of EPO was detected by ELISA in serum from patients with RA (n=50) and healthy controls (n=50). (B) The concentration of EPO was detected by ELISA in synovial fluid from patients with RA (n=30) and healthy controls (n=30). (C, D) Correlation between the level of EPO in serum or synovial fluid and Disease Activity Score 28-joint count (r=0.3178, p=0.0245 and r=0.4263, p=0.0188) were evaluated by Pearson correlation coefficient. (E, F) The expression of EPOR at messenger RNA (mRNA) and protein level were detected by quantitative PCR and western blot (WB) in synovial tissue. (G) Immunohistochemistry staining images of EPOR in synovial tissue from patients with RA and healthy controls were captured at a magnification of ×200. (H) Immunofluorescence images of EPOR and CD90 in synovial tissue were captured at a magnification of ×100. (I, J) The expression of EPOR at mRNA and protein level were detected by qPCR and WB in fibroblast-like synoviocyte (FLS). (K) Immunofluorescence images of EPOR in FLS from patients with RA and healthy controls were captured at a magnification of ×200. All of the experiments were performed in triplicate. Data were presented with means ± SD. DAPI, 4,6-diamino-2-phenyl indole; DAS28-ESR, Disease Activity Score 28-joint count-erythrocyte sedimentation rate; EPO, erythropoietin; EPOR, erythropoietin receptor; FLS, fibroblast-like synoviocyte; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HC, healthy controls; RA, rheumatoid arthritis.
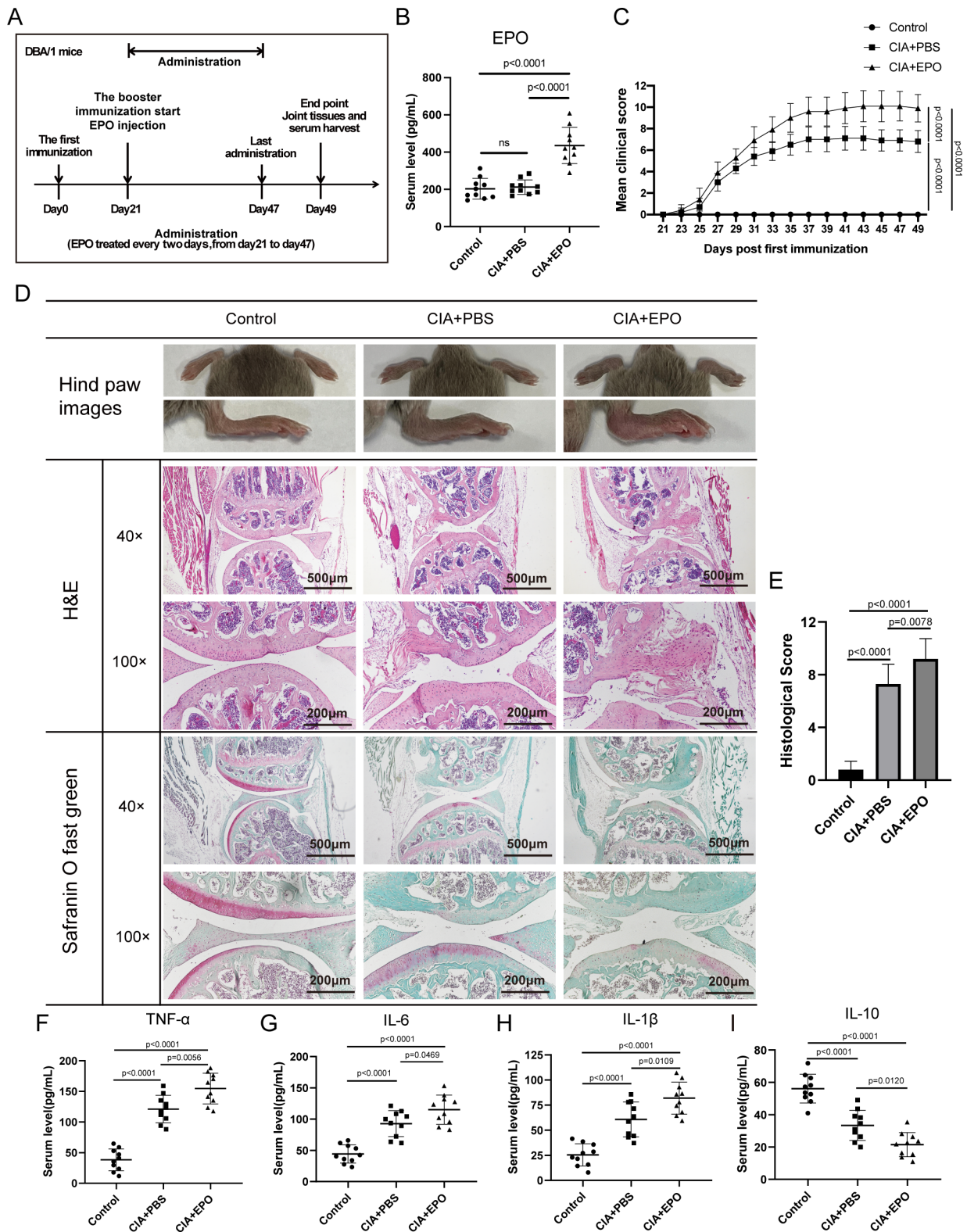
Figure 2 Erythropoietin (EPO) aggravated the joint destruction in collagen-induced arthritis (CIA) mice. (A) The timeline of the EPO-treated CIA mice experiment (n=10 per group). (B) The levels of EPO in serum were detected by ELISA. (C) Clinical scores of mice. The scoring system was defined as 0, no evidence of erythema and swelling; 1, erythema and mild swelling confined to the tarsals or ankle joint; 2, erythema and mild swelling extending from the ankle to the tarsals; 3, erythema and moderate swelling extending from the ankle to the metatarsal joints; and 4, erythema and severe swelling encompassing the ankle, foot and digits or ankylosis of the limb. Significance was tested using the analysis of variance of repeated measurement. (D) Macroscopic images and histopathological analysis of CIA mice. Macroscopic images of mice were observed on day 49 before being sacrificed (upper panel). H&E staining of knee joints (40×, 100× and middle panel). Safranin and fast green staining of knee joints (40×, 100× and lower panel). (E) Semiquantitative scores for inflammatory cell infiltration, synovial hyperplasia and bone destruction were assessed using H&E staining graded on a scale of 0 (normal) to 3 (severe) for 4 paws in 12. (F–I) The levels of TNF-α, IL-1β, IL-6 and IL-10 in serum were detected by ELISA. CIA+EPO collagen-induced arthritis+erythropoietin; CIA+PBS collagen-induced arthritis+ phosphate buffered saline; EPO, erythropoietin.
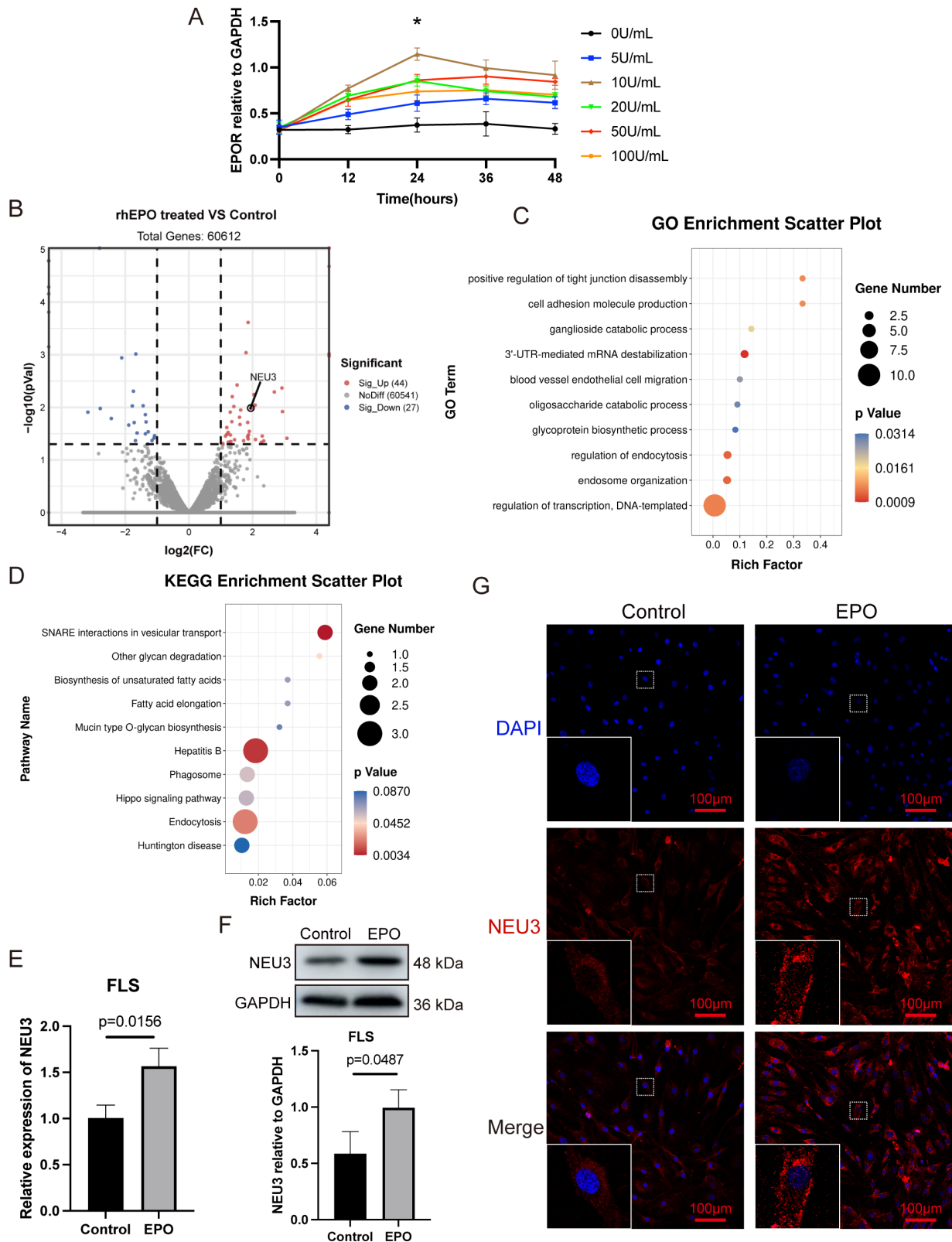
Figure 3 Erythropoietin (EPO) may increase the expression of neuraminidase 3 (NUE3). (A) The expression of receptor EPO in rheumatoid arthritis (RA) fibroblast-like synoviocyte (FLS) treated with a series of concentration gradients of recombinant human EPO (rhEPO) at a series of time gradients was detected by western blotting (WB). (B) Volcano plots of differentially expressed genes (DEGs) in RA FLS treated with EPO (n=3 per group). (C, D) The top 10 enriched go terms and Kyoto Encyclopaedia of Genes and Genomes pathway of DEGs. (E, F) The expression of NEU3 at messenger RNA and protein level were detected by quantitative PCR and WB in RA FLS treated with rhEPO. (G) Immunofluorescence images of NEU3 in RA FLS treated with rhEPO were captured at a magnification of ×200. All of the experiments were performed in Triplicate. Data were presented with means±SD. EPO, erythropoietin; EPOR, recombinant EPO; FLS, fibroblast-like synoviocyte; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GO, Gene Ontology; KEGG, Kyoto Encyclopaedia of Genes and Genomes; mRNA, messenger RNA; NEU3, neuraminidase 3; rhEPO, recombinant human EPO; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptors.
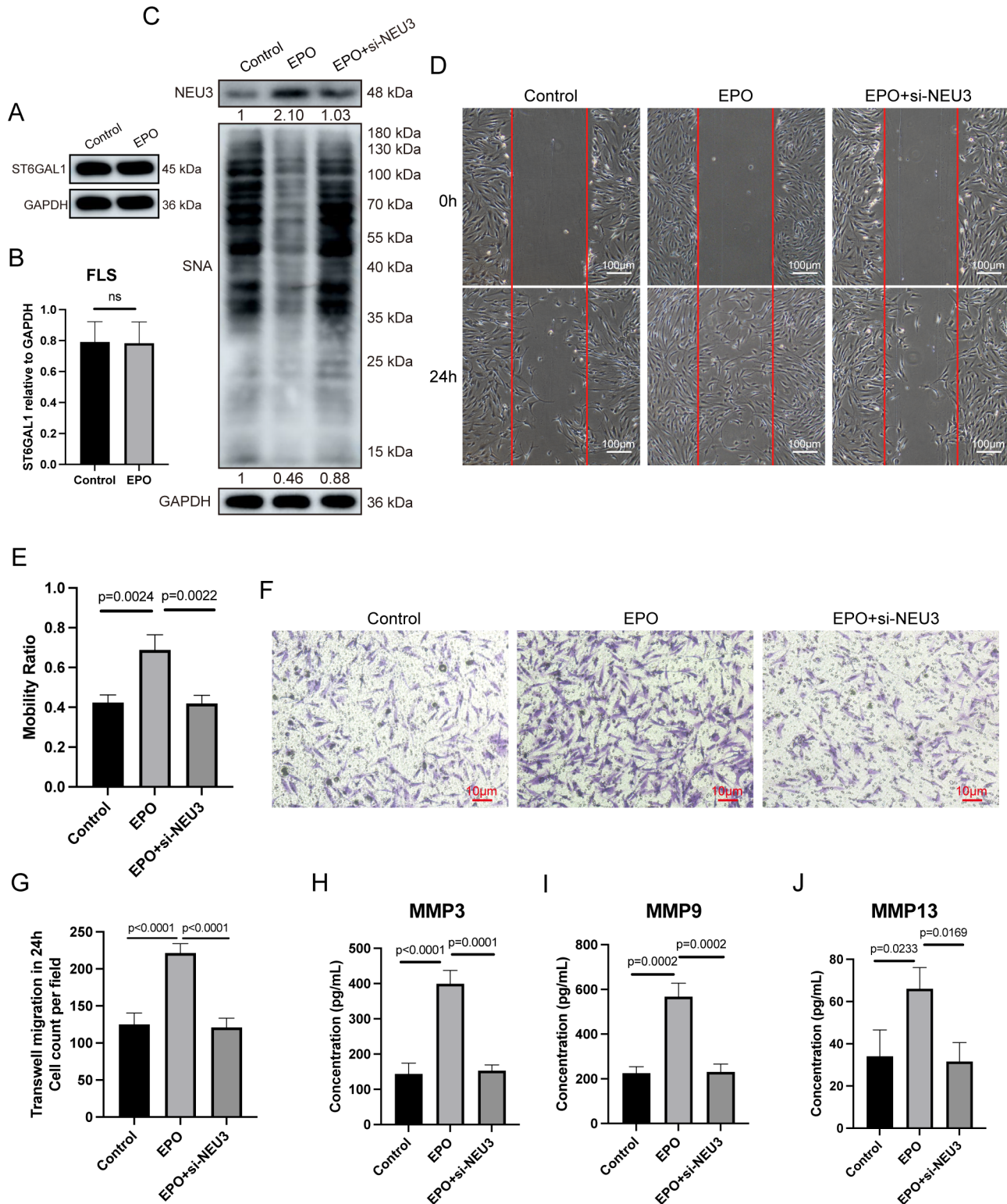
Figure 4 Erythropoietin (EPO) promoted fibroblast-like synoviocyte (FLS) migration, invasion and secretion via neuraminidase 3 (NEU3). FLSs were treated with recombinant human EPO (rhEPO) (10 U/mL) or knocked down by small interfering RNA. (A, B) The expression of β-galactoside α-2,6-sialyltransferase 1 was detected by western blotting (WB) in rheumatoid arthritis (RA) FLS treated with rhEPO. (C) The sambucus nigra lectin binding of FLS was measured by WB. (D, E) Scratch migration assay of FLS. Images of FLS were captured by a camera under an inverted microscope (original magnification ×40). The mobility ratio of each group was calculated using the following equation: migrated cellular area/scratched area×100%. (F, G) Transwell assay of FLS. Images of RA FLS on the undersurface of a filter were captured under the microscope (original magnification ×100). The number of FLS per field in different groups was quantified by software Image-Pro. (H, I) The level of matrix metalloproteinase (MMP) 3, MMP9 and MMP13 in medium supernatant was determined by ELISA. All of the experiments were performed in triplicate. Data were presented with means±SD. EPO, erythropoietin; EPO+si-NEU3, erythropoietin+small interfering RNA of neuraminidase 3; FLS, fibroblast-like synoviocyte; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MMP, matrix metalloproteinase; NEU3, neuraminidase 3; SNA, sambucus nigra lectin; ST6GAL1, β-galactoside α-2,6-sialyltransferase.

acid (DANA) could restore the reduction of sialylation in RA FLS, inhibiting the ability of migration, invasion and secretion of MMP3, MMP9 and MMP13 of RA FLS, which were induced by EPO (figure 4C–J, online supplemental figure S3A–H). To determine the direct influence of NEU3 on FLS, NEU3 was overexpressed in RA FLS, and the results showed that it could reduce the sialylation and promote the migration, invasion and secretion of MMP3, MMP9 and MMP13 of RA FLS (figure 5A–H). In addition, we constructed a severe combined immunodeficiency (SCID) mouse coimplantation model to assess the effect of NEU3 on the invasive ability of RA FLS. The results show that the invasion score elevated in the cartilage of SCID mouse transplanted with RA FLS overexpressed NEU3 (figure 5I,J). The results above indicated that EPO promotes the migration, invasion and secretion of MMPs in RA FLS by increasing the expression of NEU3.

EPO enhances the expression of NEU3 via JAK2-STAT5 pathway

To reveal the transcription regulation mechanism of EPO enhancing the expression of NEU3, first, we constructed four fragments of the NEU3 promoter (figure 6A). The P2 fragment (−1217 bp to +74 bp) of NEU3 promoter (pGL3-NEU3-P2) was selected for the screening of the transcription factor (figure 6B). STAT5 was screened as a transcription factor of NEU3, according to the DNA pulldown and liquid chromatograph-mass spectrometer identification (figure 6C), combined with JASPAR, which predicted the binding motifs of STAT5 on NEU3 promoter (figure 6D). As shown in luciferase assay, overexpression of STAT5 significantly induced NEU3 transcriptional activation and was abolished by mutation of NEU3 promoter binding to STAT5 (figure 6E). Consistently, electrophoretic mobility shift assay (EMSA) showed the same results (figure 6F). The above results suggested that STAT5 is the transcription factor binding to NEU3 promoter. Moreover, the results of chromatin immunoprecipitation-quantitative PCR showed that EPO could enhance the enrichment of STAT5 in the promoter region of NEU3 in RA FLS (figure 6G).

Next, we explore the mechanism of EPO regulating the expression of NEU3. It is known that EPO can significantly activate the JAK2-STAT5 signalling by binding to EPOR and then play a role in transcriptional regulation of downstream genes.[5] Results showed that the treatment of EPO could increase the expression of NEU3 in RA FLS by promoting the phosphorylation of JAK2 and STAT5, which was inhibited by the EPOR antagonist (EMP9), JAK2 inhibitor (AG490), STAT5 inhibitor (CAS285986) and inhibitor of JAK1 and JAK2 (baricitinib) (figure 6H,I). Additionally, EPO promotes the migration and invasion of FLS through JAK2-STAT5 signalling pathway pathway, which are verified by JAK2 inhibitor (AG490) and inhibitor of JAK1 and JAK2 (baricitinib) (figure 6J–P). Overall, these results supported that EPO regulates the transcription of NEU3 through JAK2-STAT5 signalling pathway and that JAK2 inhibitor and baricitinib can inhibit EPO's adverse effects.

METHODS AND MATERIALS

Patients and samples

Clinical samples were collected from the First Affiliated Hospital of Wenzhou Medical University from September 2020 to December 2022. All patients fulfilled the 2010 American College of Rheumatology criteria. This study was approved by the Clinical Research Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University, and participants submitted consent forms. Clinical data for all participants are available in online supplemental table S2. Serum samples of healthy control were obtained from healthy volunteers. Serum and synovial fluid were obtained from patients with RA who were newly diagnosed in the outpatient department before drug treatment and who have not been given treatment such as biological agents. Synovial tissues of patients with RA and synovial fluid, cartilage of normal subjects, were obtained from the patients undergoing joint replacement surgeries or knee surgery for traumatic injuries, who have not been given biological agents too. None of the patients had been treated with EPO. FLSs were isolated from synovial tissues according to the method described previously.[27] All FLSs of passages 3–5 were used for the experiment.

Protein preparation and western blot

The total protein was extracted from synovium or FLS, and protein concentration was measured with the bicinchoninic acid (BCA) method. 50 µg protein was separated on 12% sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) gels, transferred to polyvinylidene fluoride (PVDF) membranes and blocked for 1 hour. Afterwards, the corresponding primary antibodies were added and then incubated with matching horse-radish peroxidase-conjugated secondary antibodies. Antibodies used in this study are available in online supplemental table S3. The signals were assessed with the enhanced chemiluminescence (ECL) kit on Amersham Imager 600 (GE, USA). Protein grey value detection was analysed by Image J software.

Transcriptome sequencing and bioinformatics analysis

RA FLSs were cultured and incubated with rhEPO (10 U/mL) for 24 hours, and three independent samples of each group were collected by Trizol reagent. The quantity and purity of total RNA were analysed by Bioanalyzer 2100 and RNA 6000 Nano Kit (Agilent, USA), and high-quality RNA samples with RIN number >7.0 were used to construct a sequencing library. The sequencing library was used for transcriptome sequencing which was performed by Illumina NovaSeq 6000 (LC-Bio Technologies (Hangzhou) Co, Ltd, China). DEG analysis was performed by DESeq2 software (http://www.bioconductor.org/packages/release/bioc/html/DESeq2.html). The genes with the parameter of p<0.05 and absolute fold change ≥2 were considered DEGs, which were subjected to enrichment analysis of GO (http://www.geneontology.org/), functions and KEGG (http://www.kegg.jp) pathways.

Scratch migration assay

RA FLSs were seeded in six-well plates (2.5×10⁵ cells/well) and pretreated with rhEPO (10 U/mL), rhEPO (10 U/mL) + DANA (1 mM), rhEPO (10 U/mL) + AG490 (10 µM) or rhEPO (10 U/mL) + baricitinib (1 µM) for 24 hours. A scratch was made along the diameter of the well using a 10 µL pipette tip (Axygen Corning, USA). Images were captured at 0 hour and 24 hours by a camera under the microscope (×40 magnification). The mobility ratio was analysed by Image J and was calculated using the following equation: migrated cellular area/scratched area×100%.

Transwell assay for invasion

Transwell invasion assays were performed using transwell chamber with 8 µm pores (Corning, USA). RA FLSs were pretreated with rhEPO (10 U/mL), rhEPO (10 U/mL) + DANA (1 mM), rhEPO (10 U/mL) + AG490 (10 µM) or rhEPO (10 U/mL) + baricitinib (1 µM) for 24 hours. Then, cells were trypsinised and resuspended with serum-free Dulbecco's modified
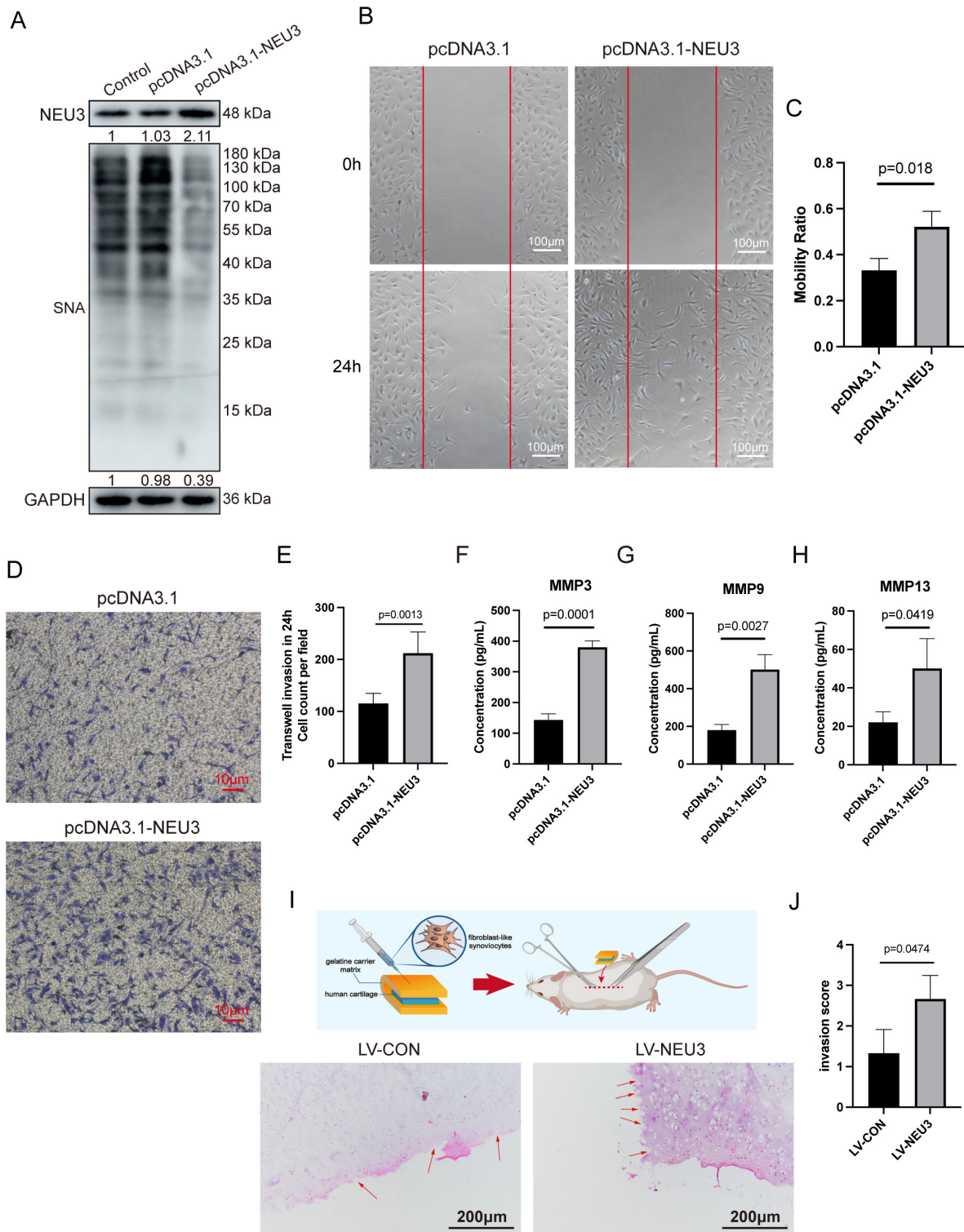
Figure 5 Neuraminidase 3 (NEU3) directly promotes the migration, invasion and secretion of matrix metalloproteinases (MMPs) of fibroblast-like synoviocyte (FLS). FLSs were transfected with pcDNA3.1 or pcDNA3.1-NEU3. (A) The SNA binding of FLS was measured by western blotting. (B, C) Scratch migration assay of FLS. Images were captured by a camera under an inverted microscope (original magnification ×40). The mobility ratio of each group was calculated using the following equation: migrated cellular area/scratched area×100%. (D, E) Transwell assay of FLS. Images of FLS on the undersurface of a filter were captured under the microscope (original magnification ×100). The number of FLS per field in different groups was quantified by software Image-Pro. (F–H) The level of MMP3, MMP9 and MMP13 in medium supernatant was determined by ELISA. (I) FLS infected with LV-CON or LV-NEU3 and cartilage were transplanted into severe combined immunodeficiency (SCID) mice (n=3 mice per group). H&E staining of the cartilage removed from SCID mice with a magnification of ×100. The red arrows indicate rheumatoid arthritis FLS invaded the cartilage. (J) The score of invasiveness of RA FLS into the cartilage in SCID mice. The level of invasiveness was scored as follows: 0, no or minimal invasion; 1, visible invasion (two-cell depth); 2, invasion (five-cell depth); and 3, deep invasion (more than ten-cell depth). All of the experiments were performed in triplicate. Data were presented with means±SD. NEU3, neuraminidase 3; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LV-CON, lentivirus-control; LV-NEU3, lentivirus-neuraminidase 3; MMP, matrix metalloproteinase; NEU3, neuraminidase 3; SNA, sambucus nigra lectin.
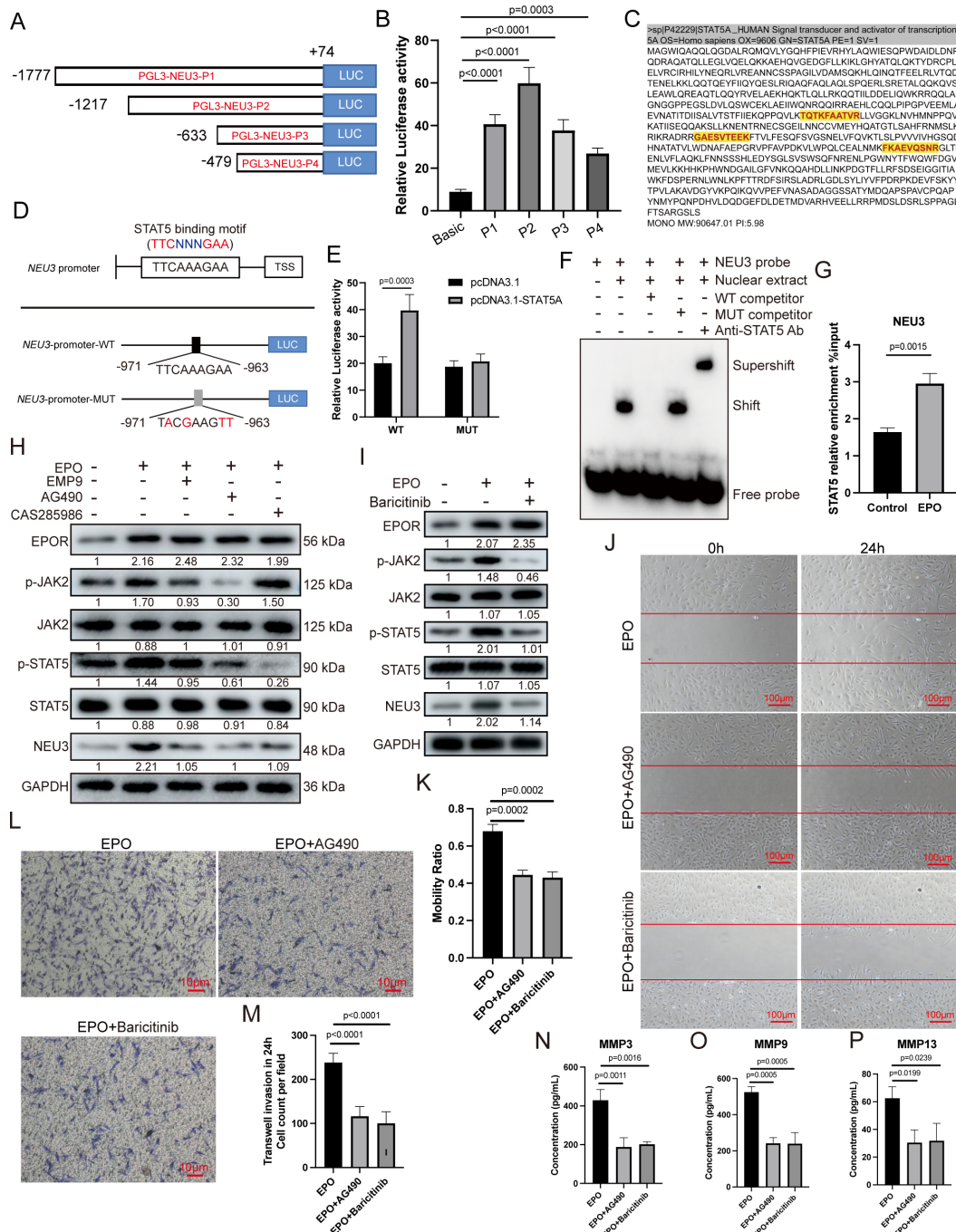
Figure 6 Erythropoietin (EPO) increased the expression of neuraminidase 3 (NEU3) via Janus kinase 2 (JAK2)-STAT5 pathway. (A) Schematic representation of truncated fragments of NEU3 promoter. (B) Double luciferase reporter gene assay of NEU3 promoter fragments. (C) The amino acid coverage of identified STAT5A sequence by LC-MS (yellow). (D) The sequence of STAT5 binding motif in the promoter of NEU3 was predicted by JASPAR (upper panel) and the sequence of NEU3 promoter wild type (WT) and NEU3 promoter mutation (MUT) (lower panel). (E) Double luciferase reporter gene assay of WT and MUT NEU3 promoter. (F) Electrophoretic mobility shift assay was used to determine the binding of NEU3 probes to nuclear extract protein of fibroblast-like synoviocyte (FLS). (G) Chromatin immunoprecipitation-quantitative PCR of STAT5 binding to the promoter of NEU3 in rheumatoid arthritis (RA) FLS. (H, I) The expression of EPOR, total-JAK2, p-JAK2, total-STAT5, p-STAT5 and NEU3 in RA FLS treated with the inhibitors (EMP9, AG490, CAS285986 and baricitinib) was detected by western blotting. (J, K) Scratch migration assay of FLS treated with recombinant human EPO (rhEPO) (10 U/mL), rhEPO (10 U/mL) + AG490 (10 μM) or rhEPO (10 U/mL) + baricitinib (1 μM). Images were captured by a camera under an inverted microscope (original magnification ×40). The mobility ratio of each group was calculated using the following equation: migrated cellular area/scratched area×100%. (L, M) Transwell assay of FLS treated with rhEPO (10 U/mL), rhEPO (10 U/mL) + AG490 (10 μM) or rhEPO (10 U/mL) + baricitinib (1 μM). Images of FLS on the undersurface of a filter were captured under the microscope (original magnification ×100). The number of FLS per field in different groups was quantified by software Image-Pro. (N–P) The level of MMP3, MMP9 and MMP13 in medium supernatant of FLS treated with rhEPO (10 U/mL), rhEPO (10 U/mL) + AG490 (10 μM) or rhEPO (10 U/mL) + baricitinib (1 μM) was determined by ELISA. All of the experiments were performed in triplicate. Data were presented with means±SD. EPO, erythropoietin; EPOR, EPO receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; JAK2, Janus kinase 2; LUC, luciferase; MMP, matrix metalloproteinase; MUT, mutation; NEU3, neuraminidase 3; STAT5, signal transducer and activator of transcription 5; WT, wild type.

Eagle's medium at a final concentration of 1×10^5/mL. 200 µL cell suspension was put into the upper chamber of the transwell insert. 500 µL DMEM with 10% fetal bovine serum as chemo-attractant was placed in the lower wells. The plate was incubated at 37°C under 5% CO_2 for 24 hours. Cells that migrated to the lower phase of the upper chamber were fixed in methanol for 30 min and stained with crystal violet for 3 min at room temperature. Pictures were taken under the microscope, and the cell number was quantified by the software Image-Pro. The assay was performed in triplicate.

FLS-cartilage-SCID model construction

To determine the ability of FLS to invade cartilage in vivo, the FLS-cartilage-SCID model was constructed. The normal human cartilage was cut into 5–8 mm³ pieces. The sterile sponge soaked with FLS (5×10^5) and a piece of cartilage were inserted under the skin at the left flank of SCID mouse under sterile conditions. After 60 days, mice were sacrificed, the implants were removed and embedded in Tissue-Tek OCT compound (Miles, Elkhart, Indiana, USA) for frozen section, and the histological changes of cartilage invasion by FLS cells were evaluated by H&E staining. Thus, the cartilage in H&E results is the normal cartilage implanted subcutaneously into the SCID mice; if the cartilage was invaded by FLS cells, the cartilage will present a bumpy state at the edge, and conversely, it is a smooth surface.

DNA pulldown assay

A biotin-labelled double-stranded oligonucleotide probe containing DNA binding sequence for the -1217 bp to $+74$ bp region of NEU3 promoter sequence was synthesised by Tsingke Biotech (Beijing, China). Briefly, 1 mg of nuclear protein extract was mixed and incubated with 10 µg of probe and 100 µL of streptavidin-magnetic beads. The binding complex was then magnetically separated and resuspended in 30 µL of loading buffer and boiled at 100°C for 5 min. The collected samples containing the bound proteins were separated by SDS-PAGE for further analysis and identification.

Electrophoretic mobility shift assay

The biotin-labelled oligonucleotide probe (5'-CCTATGTT CTTTCAAAGAACCCT-3') was synthesised for EMSA corresponding to the STAT5 binding sites in the NEU3 promoter. Nuclear extract was mixed with the labelled probe in a total volume of 10 µL of binding buffer. After incubation for 20 min at room temperature, the reaction mixtures were then separated by electrophoresis with 6% non-denaturing polyacrylamide gel in $0.5 \times$ tris-borate-EDTA buffer (pH 8.3) at 100 V for 1 hour. The bound and free probes in the gel were transferred to a nylon membrane (Amersham, USA) at 380 mA for 1 hour. The probes on the membrane were detected using chemiluminescent EMSA kit (Beyotime, China) as instructed. For competition experiments, a 100-fold molar excess of the unlabelled double-stranded probe was added 10 min before the addition of the unlabelled probe and mutated probe.

Statistical analysis

Statistical analysis was conducted in GraphPad Prism 8 (GraphPad Software, La Jolla, California, USA), and the data were presented as the mean±SD. The Shapiro-Wilk method was used to determine whether the data were normally distributed, and the homogeneity of variance was tested by the Levene method. If the measurements between two groups were normally distributed, the unpaired Student's t-test was used; otherwise the Mann-Whitney U test was used. One-way analysis of variance (ANOVA) test with post hoc contrasts by Tukey test was applied to compare the means of multigroups. To test the difference between the data of the same group of subjects at different time periods (three times or more), ANOVA of repeated measurements was used.

DISCUSSION

It has been reported that 64% of patients with RA are accompanied by anaemia, resulting in compensatory elevation of EPO levels.[10–13] So, how does elevated EPO affect the progression of RA disease? Up to now, it is still controversial whether EPO plays a proinflammatory or anti-inflammatory role on the process of RA. A previous clinical study reported that the concentration of EPO in serum from patients with RA was positively correlated with ESR.[13] In this study, we found that the levels of EPO were increased in serum and synovial fluid of patients with RA and positively correlated with DAS28, reminding that EPO might play a proinflammatory role in RA. In vivo experiments, we found that EPO aggravated joint swelling, joint destruction and bone erosion in CIA mice, which was contrary to the conclusion that EPO relieved arthritis in CIA mice in Cuzzocrea's study.[14] Our results show that EPO plays a proinflammatory role in RA.

Synovial tissue is the main lesion site of RA, consisting of macrophages and FLS. It is important consensus that excessively proliferated FLS plays an important role in RA progress by forming invasive pannus, leading to cartilage damage.[1] In this study, GO and KEGG enrichment analysis of transcriptome sequencing of EPO-treated FLS. The results showed that glycosylation degradation pathway was highly enriched, and the corresponding DEGs of NEU3, a kind of sialidase that hydrolyses terminal SA residues of glycoproteins and glycolipids, were selected for further study. Sialylation modification could mediate cell recognition and adhesion processes.[28] Studies have shown that the sialylation level was decreased in RA, and the desialylation of ACPA exacerbated the development of RA.[20 21] In addition, the decreased sialylation level of FLS promotes its migration and invasion, leading to the transition to a pathogenic state.[24 25] Sialylation is regulated by sialyltransferase and neuraminidase (NEU), mediating the reaction of sialylation and desialylation, respectively. Here, we innovatively revealed that the treatment of EPO could reduce the sialylation of FLS by upregulating the expression of NEU3, enhancing the migration and invasion ability of FLS, accompanying the release of MMPs and ultimately promoting the damage of cartilage, which was inhibited by NEU3 inhibitor or NEU3 knockdown. These results suggested that NEU3 would be a good therapeutic target in RA. Moreover, we found that STAT5 is a specific transcription factor binding to NEU3 promoter and confirmed that EPO promoted NEU3 expression by activating JAK2/STAT5 pathway.

JAKs, as a non-receptor tyrosine protein kinase, have four isoforms, including JAK1, JAK2, JAK3 and TYK2, which have been proved to play an important role in RA progress. Targeted JAK inhibitors are used for the treatment of RA. In the recent European Alliance of Associations for Rheumatology recommendations for the management of RA, JAK inhibitors were recommended for RA treatment failing in traditional disease-modifying anti-rheumatic drugs treatment. Previous studies have shown that inhibition of JAK1 might be the main reason for the therapeutic effect of JAK

inhibitors in RA.[29] Biochemically, all JAK inhibitors show the greatest potency at inhibiting JAK1, with varying levels of other JAK isoforms.[30] Although these inhibitors have similar efficacy in RA, the reported incidences of adverse events vary, including severe infections,[31] venous thromboembolism,[32] natural killer cell reduction[33] and anaemia.[34] JAK2 inhibition is considered one cause of anaemia.[35] Our study found that EPO played a proinflammatory role through JAK2/STAT5 pathway, which was prevented by the inhibition of JAK2, indicating that the inhibition of JAK2 is necessary for RA treatment, especially in patients with high EPO level. The above conclusions show that the inhibition of JAK2 may be a double-edged sword in the treatment of RA, suggesting that JAK2 inhibition treatment is beneficial in patients with RA with elevated EPO, while it may increase the risk of anaemia in patients with normal or lower EPO. Therefore, further clinical trials are urgently needed to evaluate the dose-response relationship between EPO levels and JAK2 inhibitor.

In summary, we first prove that EPO is a proinflammatory factor, which accelerates RA process through transcriptionally upregulating the expression of NEU3 by JAK2/STAT5 pathway. This study suggests that not only JAK1 but also JAK2 inhibition is required for patients with RA with high EPO levels.

Author affiliations
[1]Department of Anesthesia and Critical Care, The Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University, Wenzhou, Zhejiang, China
[2]Key Laboratory of Pediatric Anesthesiology, Ministry of Education, Wenzhou Medical University, Wenzhou, Zhejiang, China
[3]Institute of Autoimmune Diseases, School of Basic Medical Sciences, Wenzhou Medical University, Wenzhou, Zhejiang, China
[4]Department of Immunology and Rheumatology, The First Affiliated Hospital of Wenzhou Medical University, Wenzhou, Zhejiang, China
[5]Department of Medicinal Chemistry, School of Pharmaceutical Sciences, Wenzhou Medical University, Wenzhou, Zhejiang, China

terminology, drug names and drug dosages), and is not responsible for any error and/or omissions arising from translation and adaptation or otherwise.

ORCID iD
Jianguang Wang http://orcid.org/0000-0003-0597-021X

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