Dear Editor,

Rheumatoid arthritis (RA), which is the most common inflammatory arthritis disease, as well as a kind of most prevalent autoimmune disease, affects 1–3% population all over the world. Currently available treatments can reduce the disability rate of patients with RA to some extent, but they cannot completely block the inflammatory joint destruction and relieve the pain that comes with it. Our previous study found that Neuron Navigator 2 (NAV2) increased significantly in RA and was regulated by transcription factor E2F1. However, it is still unclear why NAV2 is elevated at the genetic level in RA fibroblast-like synoviocytes (FLS). Additionally, RA's exact pathological mechanisms and therapeutic targets are also not fully understood. Hence, an in-depth study of the pathogenesis of RA is urgently needed.

In this study, super-enhancer (SE) identification in RA FLS was performed through ChiP-seq against the H3K27ac modification. SE refers to extra-long cis-acting elements of 8–20 kb in length that have transcriptional enhancement activity, which can assemble key transcription factors and cofactors in high density and activate the expression of identity determining genes in stem cells. Thus, SE plays an important role in the modulation of cells fate and can be recognized as a valuable biomarker and therapeutic target to identify and intervene with disease-related genes. The results displayed that significantly increased ChIP-seq signal of H3K27ac in human RA FLS (CHIPMD) when compared to human osteoarthritis (OA) FLS (CHIPCK) (Supplementary Fig. S1a, b). The enrichment level of H3K27ac in the coding gene region showed that the overall level of H3K27ac increased significantly in RA FLS (Supplementary Fig. S1c). Moreover, we also found that in human RA FLS, the signal strength of SE was significantly stronger than typical enhancers (Supplementary Fig. S1d). Compared with 642 SE-associated genes identified in OA FLS, 1368 SE-associated genes were identified in RA FLS which indicated that more genes were driven to express in RA to promote disease progression (Supplementary Fig. S1e, f). GO analysis and KEGG pathway analysis were used to investigate the functional enrichment of the identified SE-related targets and pathways (Supplementary Fig. S2a, b). The SEs were associated with genes enriched in axon guidance, cell adhesion, and cell motility. Additionally, regulation of the actin cytoskeleton pathway could be stimulated in RA. These results indicated that the SE-associated genes and signaling pathways in RA FLS were hyperactive and may promote the progression of RA. Interestingly, the SE signal of NAV2 showed a significant increase in primary human RA FLS (Supplementary Fig. S3). Then the results of the expression of Vimentin showed that the cells derived from synovium tissues were primary FLS and the expression of NAV2 was markedly upregulated by using an immunofluorescence double staining experiment (Fig. 1a). Also, the obvious increase of NAV2 was confirmed in the primary synovial cells from RA synovium samples through Western blot analysis (Fig. 1b). Next, we found that the expression of NAV2 was significantly elevated in AIA rat synovial tissues. The clinical symptoms were evaluated every 5 days from Day 10 to Day 30. Images of rats' hind paws displayed remarkable inflammatory exacerbation and soft tissue swelling as time went on. Micro-CT deeply showed the typical symptoms of bone destruction, swollen joints, synovial membrane hyperplasia with severe infiltration of inflammatory cells, as well as pannus formation in joint tissues (Supplementary Fig. S4a). Arthritis scores and hind paw volumes also showed more severity and higher incidence of arthritis in the model group when compared to the control group (Supplementary Fig. S4b, c). These results indicated that the AIA rats model was successfully established in this study. Interestingly, the NAV2 expression was also remarkably increased in the inflamed joints on the protein level (Fig. 1c). In previously oncology-related studies, NAV2 expression has been found to be increased in cancers such as colon cancer and uterine sarcoma. Moreover, the overexpression of NAV2 is also associated with the poor prognosis of colorectal cancer (CRC) and promotes CRC invasion through the slingshot-1L (SSH1L)/Cofilin-1 signaling pathway. So we speculated that NAV2 could regulate this pathway in RA. We first showed that the SSH1L/Cofilin-1 signaling pathway was activated obviously in inflamed joints of AIA rats (Fig. 1c).

Human RA FLS were treated with TNF-α (20 ng/ml) for 0, 1, 3, 6, 12, 24, and 48 h. As illustrated in Supplementary Fig. S5a–e, protein expression levels of IL-6, iNOS, MMP-3, and MMP-9 were significantly increased time-dependently. Among the results, the protein levels of IL-6, iNOS, and MMP-9 showed a persistent significant upregulation from 0 to 12 h, whereas MMP-3 protein expression reached a peak at 24 h during TNF-α-induced cellular inflammation. Similarly, the results also showed the elevated mRNA levels of iNOS and IL-8 (Supplementary Fig. S5f, g). Collectively, the results indicated that TNF-α-stimulated FLS exhibited an activated inflammatory response state, similar to the increased pro-inflammatory mediators to active the RA external environment. Interestingly, the NAV2 mRNA level reached a peak at 1 h and then declined. Consistent with this, the expression of NAV2 protein showed a significant increase during TNF-α-induced cellular inflammation from 0 to 24 h but slightly decreased at 48 h (Fig. 1d), the peak of NAV2 might indicate NAV2 played a crucial role in the initial phase of RA progression.

NAV2 is a protein-coding gene that plays an important role in cellular growth, migration, and invasion. As illustrated in Supplementary Fig. S6a–e, after knockdown of NAV2 expression by transfection with siRNA, we could find a decrease in NAV2, accompanied by the expression of COX-2, IL-6, and MMP-9 decreased obviously. Besides, silenced NAV2 significantly decreased the mRNA level of iNOS (Supplementary Fig. S6f). More importantly, the results of immunofluorescence double staining also showed that TNF-α induced the co-expression of iNOS and NAV2 was eliminated after NAV2 was knocked down (Supplementary Fig. S7). Taken together, these findings strongly

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suggested that silencing NAV2 was sufficient to partly antagonize the inflammatory response in RA.

In vitro experiment, the results showed that the abundance of phosphorylated SSH1L (p-SSH1L) was significantly increased upon TNF-α treatment and phosphorylated Coflin-1 (p-Coflin-1) decreased dramatically (Supplementary Fig. S8a), indicating that TNF-α probably activates SSH1L/Coflin-1 signaling pathway through phosphorylation activation. Additionally, we also found that knockdown of the NAV2 gene could dramatically decrease p-SSH1L but increase p-Coflin-1 in contrast to control cells (Fig. 1e). Previous results showed that the migration and invasion of FLS were related to NAV2 expression in RA.2 Here, our results showed a decrease in F-actin polymerization and stress fiber disassembly in cells with silenced NAV2 gene (Fig. 1f). In addition, after downregulated NAV2 expression, the NAV2 knockdown (NAV2-KD) cells showed decreased TNF-α-induced EdU incorporation and BrdU absorbance (Fig. 1g–i). Moreover, the Scratch wound migration assay showed that FLS had an augmented ability to migrate when compared to the control cells. Transwell assay also showed that silencing NAV2 expression could obviously impede the invasion and motility of the cells (Fig. 1g, j–l). These experiments revealed that NAV2 plays a crucial role in promoting inflammation and metastasis of RA FLS by modulating F-actin polymerization through the SSH1L/Coflin-1 signaling pathway.

The underlying mechanisms by which NAV2 accelerates inflammatory response and associated phenotypes in RA were investigated in the following study. First, we showed that TNF-α time-dependently increased phosphorylated STAT3 (p-STAT3) significantly in human RA FLS (Supplementary Fig. S8b). Then it was interesting to find that overexpressed STAT3 in FLS resulted in upregulating NAV2 expression, whereas knockdown of STAT3 had the opposite effect (Supplementary Fig. S9a, b). To further clarify that STAT3 regulates the expression of NAV2 and then promotes the inflammation reaction in RA we performed Luciferase reporter assay and ChIP assay. Of note, elevated STAT3 expression could dramatically enhance the transcription of NAV2 (Fig. 1m), and ChIP
results showed that STAT3 could enrich the promoter region of NAV2 from −2000 to +500 (Fig. 1n, o). More importantly, according to the binding mode, it can be clearly seen that NAV2 and STAT3 proteins match well (the binding energy is −65.05 kcal/mol, Table S1). These hydrogen bonds can effectively bind the two proteins tightly to form a stable complex (Supplementary Fig. S10). RMSD measures the average distance between atoms to reveal structural changes during the simulation over time. Lower deviations of the RMSD value mean better stability. The RMSD value of the NAV2 from proteins tightly to form a stable complex (Supplementary Fig. S10). The online version contains supplementary material available at https://doi.org/10.1038/s41392-022-01050-7.

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ADDITIONAL INFORMATION

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