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ARTICLE

LncRNA NEAT1_1 suppresses tumor-like biologic behaviors of fibroblast-like synoviocytes by targeting the miR-221-3p/uPAR axis in rheumatoid arthritis

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Abstract

Fibroblast-like synoviocytes (FLSs) are the predominant effector cells in the pathological progression of rheumatoid arthritis (RA). Therefore, elucidating the underlying molecular mechanism of the biologic behaviors in RA-FLSs will be helpful in developing the potent targets for the treatment of RA. We have previously documented that the tumor-like biologic behaviors of RA-FLSs are exacerbated by urokinase-type plasminogen activator receptor (uPAR), a specifically up-regulated receptor in RA-FLSs. Here, we investigate the further mechanism of uPAR and clarify its function in RA-FLSs. We demonstrate that miR-221-3p positively correlates to uPAR and regulates uPAR level in RA-FLSs. Simultaneously, one long noncoding RNA, nuclear paraspeckle assembly transcript 1_1 (NEAT1_1) is identified, which can predictively target miR-221-3p at three sites, indicating a strong possibility of being a competing endogenous RNA in RA-FLSs. Interestingly, NEAT1_1 and miR-221-3p can colocate in the nucleus and cytoplasm in RA-FLSs. Importantly, NEAT1_1 can act as a rheostat for the miR-221-3p/uPAR axis and the downstream JAK signaling. In line with the biologic function, NEAT1_1 negatively regulates the tumor-like characters, and cytokine secretions of RA-FLSs. Collectively, our data provide new insight into the mechanisms of NEAT1_1 in modulating RA-FLSs tumor-like behaviors. The targeting of NEAT1_1 and miR-221-3p/uPAR axis may have a promising therapeutic role in patients with RA.

KEYWORDS
fibroblast-like synoviocytes, miR-221-3p, NEAT1_1, rheumatoid arthritis

1 | INTRODUCTION

Rheumatoid arthritis (RA) is a common systemic autoimmune disease characterized by chronic polyarticular synovitis, progressive destruction of joint cartilage, and bone loss.[1,2] In RA, activated fibroblast-like synoviocytes (FLSs) have a central role in promoting pannus formation and bone erosion by interacting with immune cells and secreting a variety of proinflammatory cytokines.[3] Importantly, accumulating evidence indicated that inflammatory cytokines activated RA-FLSs, which possess many biologic characteristics, such as hyperproliferation, migration, and tissue invasion, which are similar to tumor cells.[4,5] These features of RA-FLSs result in an invasive hyperplastic lining tissue formation known as pannus.[6] Therefore, targeting on RA-FLSs or combined with immune suppression can exert a therapeutic effect on

Abbreviations: ceRNAs, Competing endogenous RNAs; FLSs, Fibroblast-like synoviocytes; GO, Gene ontology; GSEA, Gene Set Enrichment Analysis; lncRNA, Long noncoding RNA; NEAT1_1, Nuclear paraspeckle assembly transcript 1_1; RA, Rheumatoid arthritis; uPAR, Urokinase-type plasminogen activator receptor

RA.[6] Conclusively, understanding the underlying molecular signaling of RA-FLSs tumor-like characteristics will not only help to illuminate the mechanism but also help to develop the potential therapeutic approach for the treatment of RA.

We have previously reported that urokinase-type plasminogen activator receptor (uPAR) is up-regulated in RA-FLSs, which can promote RA-FLSs proliferation and tumor-like behaviors.[4] But the further mechanism is still unclear. Emerging evidence has demonstrated that small noncoding RNAs (such as miRNAs) are vital in regulating mRNA expression by mediating mRNA cleavage and destabilization.[7,8] Therefore, miRNAs may have indispensable roles in modulating cell biologic processes.

The miR-221-3p is a recently identified oncogenic miRNA that regulates the development of tumor. MiR-221-3p has been reported to be up-regulated in several types of cancers, such as pancreatic, liver, non-small cell lung, colorectal, and breast cancers.[9,10] It can also promote cancer metastasis and serves as a potential biomarker for the diagnosis of cancer patients. Importantly, it has been reported that miR-221-3p can positively regulate uPAR translation by enhancing uPAR mRNA stability in breast cancer.[11] This positive regulation seems to occur through interaction with human antigen R (HuR, also known as embryonic lethal abnormal vision-like 1), which is an AU-/U-rich element-(ARE)-binding protein. Furthermore, miR-221-3p can promote tumor-like behaviors of RA-FLSs.[12] However, whether the miRNA can regulate the expression of uPAR in RA-FLSs and the underlying mechanism remains unclear.

The expression of miRNA is regulated by multiple mechanisms, one of which is long noncoding RNAs (lncRNAs). LncRNAs are the prominent class of transcripts with lengths exceeding 200 nucleotides, which have been verified to act as competing endogenous RNAs (ceRNAs) by combining with miRNAs to eliminate the effect of the miRNAs on their targeted transcripts. In this way, lncRNAs play essential roles in regulating a series of cellular biologic processes. It has been documented that the expression changes of lncRNAs correlated to numerous human diseases, such as cancer, neurologic disorders, cardiovascular disorders, RA, and other autoimmune diseases.[13] Importantly, many studies have shown that lncRNAs play as ceRNAs in modulating the pathogenesis of RA.[14,15] However, whether there exists any lncRNA that can function as a ceRNA of miR-221-3p in RA-FLSs is unclear.

In this study, one lncRNA-nuclear paraspeckle assembly transcript 1_1 (NEAT1_1) was identified by analyzing our previous microarray data (GSE128813) and bioinformatic analysis data (ENCORI, http://starbase.sysu.edu.cn/), which can predictively target on miR-221-3p with three sites. Considering that there was no significant difference in expression of NEAT1_2 between RA and controls in our microarray data, we focused on NEAT1_1 in subsequent experiments. The expression of NEAT1_1 was significantly lower in RA-FLSs compared to healthy control (HC)-FLSs. Importantly, NEAT1_1 was negatively correlated to miR-221-3p, which consequently resulted in the down-regulated uPAR expression and the alleviated tumor-like behaviors in RA-FLSs. Therefore, NEAT1_1 modulates the tumor-like characteristics of RA-FLSs through the miR-221-3p/uPAR axis. Conclusively, our data provide new insight into the mechanisms of NEAT1_1 in modulating RA-FLSs tumor-like behaviors. The targeting of NEAT1_1 and miR-221-3p/uPAR axis may have a promising therapeutic role in patients with RA.

2 | MATERIAL AND METHODS

2.1 | Patients

Synovial tissues were collected from active RA patients undergoing synovectomy of the knee joint or total knee replacement surgery at the Third Affiliated Hospital of Sun Yat-sen University (Guangzhou, China). RA patients were diagnosed according to the 2010 American College of Rheumatology/ European League against Rheumatism classification criteria. The HC group synovial tissues were taken from five patients who underwent arthroscopic surgery for severe joint trauma and had no other joint abnormalities or systemic disease. The basic clinical parameters for RA patients are provided in the Supporting Information Table S1. This research was approved by the ethics committee of the Third Affiliated Hospital at Sun Yat-sen University, and all subjects provided written informed consent in accordance with the Declaration of Helsinki. For experiments, FLSs from each donor were employed in one experiment. At least three donors'-derived FLSs were used in all experiments.

2.2 | Isolation and identification of FLSs

FLSs were isolated from synovial biopsy specimens of patients with RA or traumatic arthritis and cultured as previously described.[4] FLSs from passages 3 to 5 were used for the following experiments. The morphologic characteristics of FLSs have fibroblast-like appearance and were spindle-shaped when observed under a light microscope. Expression levels of surface markers on FLSs were detected for characterization using flow cytometry. FLSs from passage 3 were trypsinized, centrifuged, and stained with commercial monoclonal antibodies against CD68 APC and CD90 FITC (Biolegend, San Diego, CA, USA) for 20 min.

2.3 | Transfection

Two types of adenoviral vectors were used in our study: Ad-LncRNA NEAT1_1, a replication-defective adenovirus expressing human LncRNA NEAT1_1, and Ad-NC, an empty vector adenovirus only expressing GFP without additional exogenous genes. Both vectors were purchased from Genechem (Shanghai, China). RA-FLSs were seeded into 6-well plates (1.5×10^5 cells/well) 18 h before transfection, and when RA-FLSs reached 60% confluence, adenovirus particles were transfected at a multiplicity of infection of 500. The culture supernatants were replaced with DMEM containing 10% FBS after 6 h incubation. Subsequent experiments were performed 2 d after infection with Ad-NEAT1_1 or Ad-NC.

RA-FLSs were transfected with miR-221-3p mimics or inhibitor, uPAR small interfering RNAs (siRNAs), NEAT1 siRNAs, and their negative controls (synthesized by GenePharma, Shanghai, China) using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) as previously described.[4,16] Their sequences are shown in Supporting Information Table S2.

2.4 | Quantitative real-time reverse transcription PCR

Total RNA was extracted from RA-FLSs using TRIzol RNA Reagent (Invitrogen) in accordance with the manufacturer' s instructions. NanoDrop ND-2000 (Thermo Fisher Scientific, Waltham, MA, USA) was used to measure the quantity and quality of RNA samples. To determine the relative expression and transfection efficiency of lncRNA NEAT1_1, cDNA was synthesized by equal amounts of RNA from different samples using the PrimeScriptTM RT reagent Kit with gDNA Eraser (Takara Bio-technology, Tokyo, Japan) and detected using TB GreenTM Premix Ex TaqTMII PCR (Takara Bio-technology). The relative expression of miRNAs was measured by a Hairpin-itTM miRNA RT-PCR Quantitation Kit (95°C, 15 s; 62°C, 30 s; 72°C, 30 s, 40 cycles) (GenePharma). All qPCR reactions were performed on ABI 7500 real-time PCR amplification equipment (Applied Biosystems, Foster City, CA, USA). The PCR primers are listed in Supporting Information Table S3. The relative expression of target genes was normalized to the internal reference genes GAPDH and U6 and was calculated using the $2^{-\triangle\triangle Ct}$ method.

2.5 | Western blot

Total proteins were extracted from cells with RIPA lysis buffer mixed with PMSF and phosphatase inhibitor cocktail I (MedChemExpress, Monmouth Junction, NJ, USA). An equal amount of protein was separated on 10% or 12% SDS-PAGE gels based upon the molecular weight of the target protein and transferred to polyvinylidene difluoride membranes (Millipore, Burlington, MA, USA). Membranes were blocked with 5% BSA at room temperature for 1 h and then incubated overnight at 4°C with primary antibodies (anti-uPAR antibody, Cell Signaling Technology, Danvers, MA, USA, #12863S; anti-p-PI3K (Ser249) antibody, Cell Signaling Technology, #13857S; anti-PI3K antibody, Cell Signaling Technology, #4263S; anti-p-Akt (Ser473) antibody, Cell Signaling Technology, #4060S; anti-Akt antibody, Cell Signaling Technology, #9272S; anti-p-JAK1 antibody, Cell Signaling Technology, #74129S; anti-JAK1 antibody, Cell Signaling Technology, #3344S; and anti-GAPDH, Abcam, Cambridge, United Kingdom, #ab8245). Subsequently, proteins were detected by an ECL system reagent (KeyGEN BioTECH, Nanjing, China) after being incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. Protein expression was calculated with Image J software and normalized to GAPDH expression.

2.6 | Gene set enrichment analysis (GSEA) and gene ontology (GO) analysis

GSEA and GO analysis were used to identify characteristic biologic processes in which NEAT1_1-related mRNAs may participate. GSEA is a computational method that can determine whether a predefined set of genes show a statistically significant and consistent difference between two biologic states. GO analysis covers three domains: biological process, cellular component, and molecular function.

2.7 | RNA FISH

The intracellular colocalization analysis of LncRNA NEAT1_1 and miR-221-3p was performed by RNA fluorescence in situ hybridization (RNA FISH) experiment. According to the RNA FISH kit of GenePharma, RA-FLSs were seeded on 15 mm glass bottom cell culture dish at a density of 2×10^4 cells/dish. Cells were fixed with 4% paraformaldehyde for 15 min at room temperature. After being permeabilized in Triton X-100 and washed with 10× saline sodium citrate (SSC), the cells are hybridized with probes that specifically target NEAT1 and miR-221-3p overnight in 37°C incubator. The nucleus was stained with DAPI on the next day of hybridization and imaged using a confocal laser microscope (LSM 710, Carl Zeiss, Jena, Germany) to observe the intracellular distribution of NEAT1 and its colocalization relationship with miR-221-3p. Colocalization statistical analysis was performed using Imaris software.

2.8 | Cell migration assay

Cell migration experiments were carried out in a 24-well transwell chamber (Corning, Cambridge, MA, USA) with an aperture of 8 μm. At 48 posttransfections, a total of 1×10^4 cells (1.5×10^4 cells in the rescue experiment) were suspended in 20% FBS and inoculated in the upper chamber. Then, 600 μl of medium containing 40% FBS was added as a chemical inducer in the lower chamber. After incubation at 37°C in 5% CO_2 for 24 h, nonmigratory cells were removed from the upper surface of the filter with cotton swabs. Cells that had migrated through the membrane were fixed in 4% paraformaldehyde (Boster, Wuhan, China) for 20 min, stained with crystal violet for another 20 min, and counted under a microscope. The number of migrated cells was calculated as the average number of cells passing through the membrane in five randomly selected regions.

2.9 | Wound healing assay

The RA-FLSs were seeded in the 6-well plate and scratched with 200 μl pipette tips. The plate was washed twice with PBS to remove detached cells, and the remaining cells were grown in DMEM containing 10% FBS. After 24 h of incubation, the migration rate was quantified by counting cells removed from the reference line.

2.10 | Cell invasion assay

For cell invasion experiments, the upper chamber wells were coated with 50 μl of diluted BD Matrigel basement membrane matrix (BD Biosciences Influx, Franklin Lakes, NJ, USA). The matrigel was diluted 1:10 with DMEM. After air-drying and consolidation of matrigel, subsequent experiments were similarly performed as described in the migration analysis.

2.11 | ELISA

After transfection for 48 h, the cell supernatants of RA-FLSs were collected. The secretions of proinflammatory cytokines (IL-6, IL-8, IL-1β, TNF-α) were measured with (ELISA kits, Jiangsu Meimian industrial Co., Ltd, Yancheng, China) as recommended by the manufacturer.

2.12 | Cell cycle analysis

Cell cycle phase analysis was assessed using flow cytometry with the Cell Cycle Detection Kit (KeyGEN BioTECH, China) according to the manufacturer's protocol. The transfected RA-FLSs were digested with 0.25% trypsin, washed twice with cold PBS, and fixed in 70% ethanol at 4°C overnight. Fixed cells were then rewashed with PBS and treated with 500 μl of propidium iodide (PI)/ RNase A staining buffer at 37°C in the dark for 30 min. Samples were analyzed with a flow cytometer (FACSCalibur, BD Bioscience Influx). Fluorescence emitted from the PI–DNA complex was estimated at 488 nm, and 10,000 cells in each sample were analyzed using Modfit software.

2.13 | Flow cytometry analysis of apoptosis

The Annexin-V-PE/7-AAD apoptosis detection kit (Elabscience Biotechnology, Wuhan, China) was used to measure apoptosis. In brief, 48 h after transfection, RA-FLSs were digested with EDTA-free trypsin (Invitrogen) and washed twice with cold PBS. Approximately 1×10^5 cells were collected and resuspended in a mixture comprising 500 μl of binding buffer and 5 μl of Annexin-V-PE and 5 μl of 7-AAD, according to the manufacturer's protocol, and then incubated in the dark at room temperature for 15 min. Finally, samples were analyzed by flow cytometry (FACSCalibur, BD Bioscience Influx).

2.14 | Statistics

The data presented were derived from at least three independent experiments. Statistical analysis was performed by SPSS version 25.0 software (SPSS, Inc., Chicago, IL, USA). Experimental data are presented as the mean ± SD. Student's *t*-test was used for data comparison between two groups, and differences were considered statistically significant when *P*-values were less than 0.05.

3 | RESULTS

3.1 | miR-221-3p positively regulates the expression of uPAR in RA-FLSs

MiR-221-3p has been verified up-regulated in various types of cancers and related to tumor metastasis.[9,10] We have reported that RA-FLSs possess tumor-like behavior such as hyperproliferation, migration, and invasion, in which uPAR played a key role.[4] Therefore, we ought to determine whether miR-221-3p expressed in RA-FLSs and the correlation between miR-221-3p and uPAR in RA-FLSs. RA-FLSs were isolated from synovial tissues of RA patients and identified using their morphologic characteristics and surface markers, CD68- and CD90 (Thy-1) + (Supporting Information Fig. S1).

The expressions of miR-221-3p and uPAR in RA-FLSs were determined using qPCR, individually. The results indicated that, compared to HC-FLSs, both the expressions of miR-221-3p and uPAR were significantly higher in RA-FLSs (Fig. 1A, left and middle panel). Importantly, the expression of uPAR was positively correlated to miR-221-3p (Fig. 1A, right panel). To further determine the result that uPAR was positively correlated to miR-221-3p, we developed miR-221-3p knockdown and overexpression systems using miR-221-3p inhibitor and mimic, respectively. The expressions of uPAR in the two systems were determined using Western blotting. As expected, the expressions of uPAR were decreased in miR-221-3p knockdown system and elevated in the overexpression system individually in protein level (Fig. 1B, C), although it was not significant in mRNA level in miR-221-3p knockdown system (Fig. 1B, left panel). In our previous study, we documented that uPAR modulates the activation of PI3K/Akt signaling.[4] Here, however, we found that phosphorylation levels of PI3K and Akt in the miR-221-3p knockdown system were not statistically significant compared to the negative control group (Supporting Information Fig. S2). Together, these results demonstrate that miR-221-3p is positively related to uPAR and regulates the expression of uPAR in RA-FLSs.

3.2 | lncRNA NEAT1_1 is down-regulated and negatively correlated to miR-221-3p in RA-FLSs

The expression of miRNA is regulated by multiple mechanisms, one of which is long noncoding RNAs (lncRNAs). It has been accepted that lncRNAs modulate miRNA in two ways: competitive binding to miRNAs as miRNA sponges and acting as precursor molecules of miRNAs.[17] Moreover, lncRNA MIR222HG is the precursor of miR-221-3p.[18] To determine by which approaches that lncRNA regulates miR-221-3p expression in RA-FLSs, we firstly analyzed the expression of lncRNA MIR222HG by qRT-PCR. However, the results showed that the expression of lncRNA MIR222HG in RA-FLSs was not significant compared to HC-FLSs (Supporting Information Fig. S3). Thus, lncRNA MIR222HG may not modulate the expression of miR-221-3p by acting as a precursor molecule of miR-221-3p in RA-FLSs.

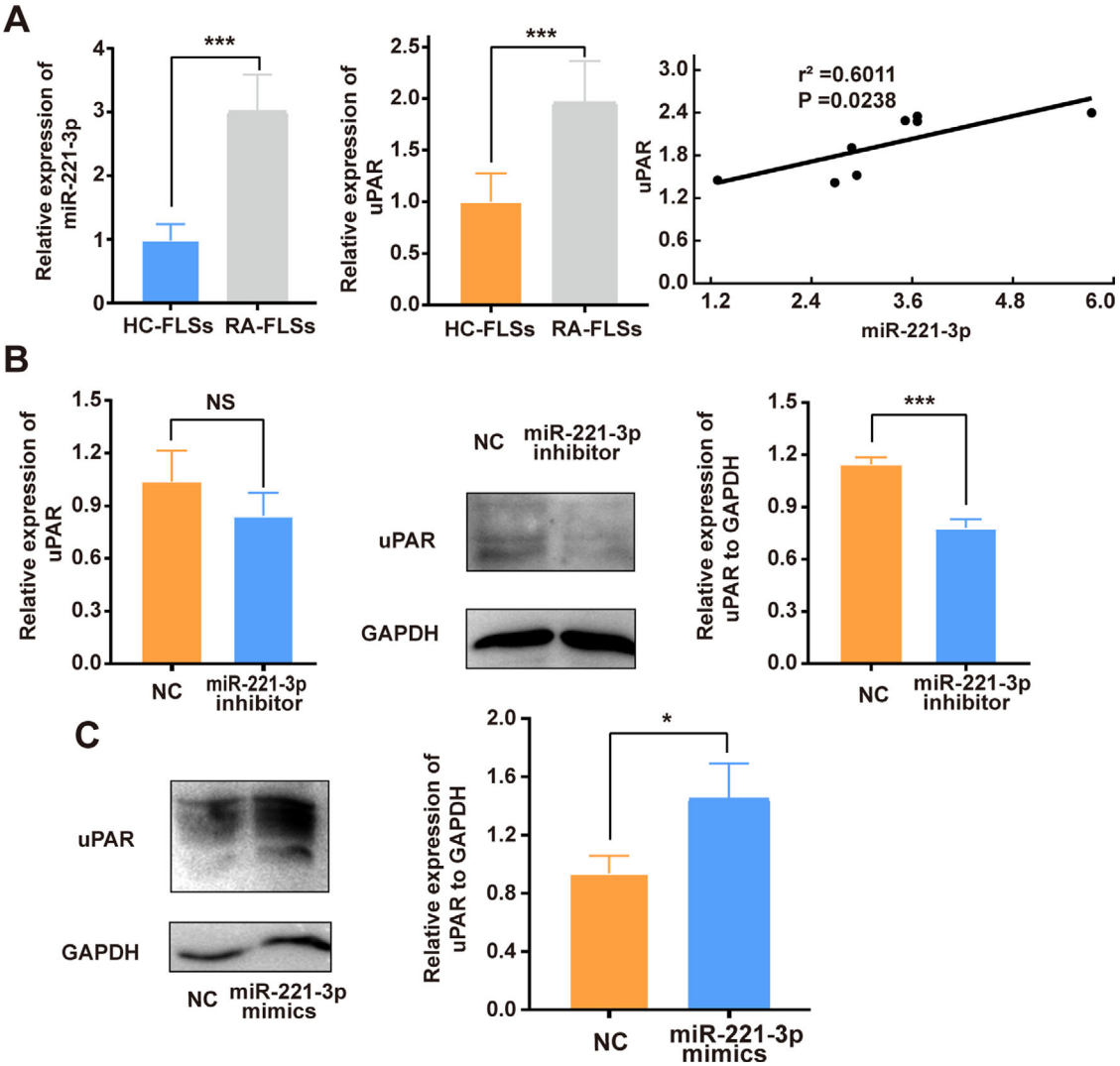

FIGURE 1 **miR-221-3p positively regulates the expression of uPAR in RA-FLSs.** HC-FLSs or RA-FLSs were obtained from joint trauma patients or RA patients, respectively. Cells were maintained in DMEM and passages 3–6 were used for the follow-up experiments. (**A**) The expressions of miR-221-3p (*panel left*) and uPAR (*panel middle*) in HC-FLSs ($n = 5$) and RA-FLSs ($n = 8$) were detected by qPCR. The correlation of miR-221-3p and uPAR in RA-FLSs was analyzed with the Spearman correlation assay (*panel right*). (**B**) The expressions of uPAR in miR-221-3p knockdown system were determined using qPCR (*panel left*) and Western blotting (*panel middle*). The Western blotting results were summarized by normalizing the mean gray value of interest protein to GAPDH (*panel right*). (**C**) The expressions of uPAR in miR-221-3p overexpression system were determined using Western blotting (*panel left*). The Western blotting results were summarized by normalizing the mean gray value of interest protein to GAPDH (*panel right*). The data indicate the mean ± SD of three independent experiments ($n = 3$, $^*P < 0.05$, $^{**}P < 0.01$. NS, not significant). HC, healthy control; RA, rheumatoid arthritis; fibroblast-like synoviocytes, FLAs; uPAR, urokinase-type plasminogen activator receptor

Consequently, we turned to assume that lncRNAs regulate miR-221-3p by competitively binding to it and function as ceRNAs of miR-221-3p in RA-FLSs. Given the results that miR-221-3p was up-regulated in RA-FLSs, we hypothesized that there exist any number of down-regulated lncRNAs that can regulate miR-221-3p level. To screen which lncRNAs that related to rheumatic diseases may function through interacting with the miR-221-3p, we analyzed the possibility lncRNA that can interact with miR-221-3p, using our previous microarray data (GSE128813) and bioinformatic analysis website (ENCORI, http://starbase.sysu.edu.cn/). Of note, we showed that one lncRNA NEAT1_1 was down-regulated in RA-FLSs that can predictably bind to miR-221-3p (Fig. 2A). To confirm the result, we further analyzed the expression of NEAT1_1 by RNA sequencing results. Consistent with the bioinformatic result, the expression of NEAT1_1 in RA-FLSs was dramatically declined compared to HC-FLSs (Fig. 2B). Next, we analyzed the possible biologic functions of NEAT1_1 using GSEA and GO analysis. The results showed that NEAT1_1 may correlate with cytokine signaling in the immune system, cell migration, and other nine pathways that related to extracellular matrix degradation, bone metabolism balance, and miRNA metabolism ($P < 0.05$) (Fig. 2C). Using qRT-PCR analysis, we further confirmed the result that the expression of NEAT1_1 was significantly decreased in RA-FLSs compared to HC controls (Fig. 2D). Collectively, these data suggest that NEAT1_1 is down-regulated and negatively correlated to miR-221-3p in RA-FLSs

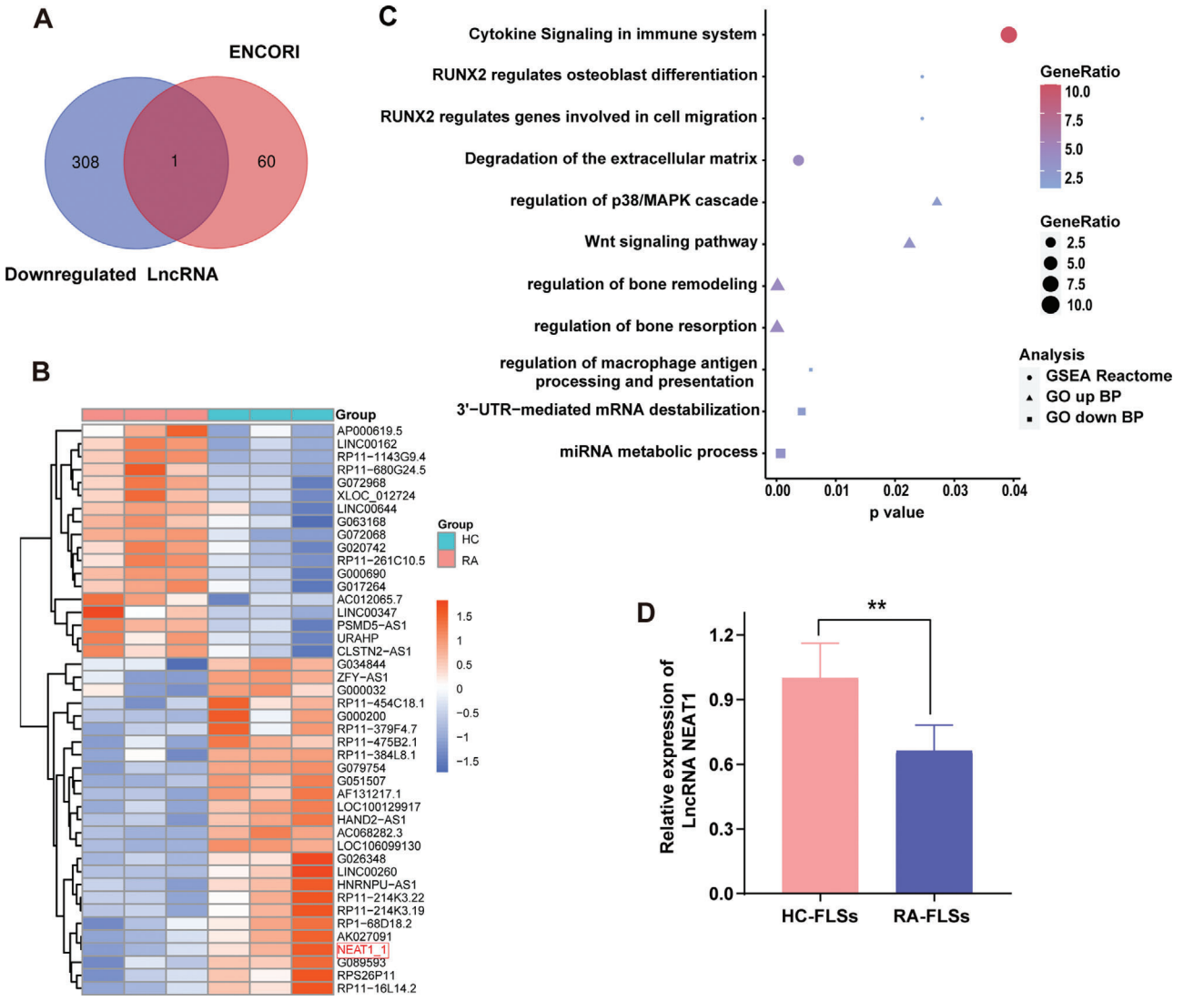

FIGURE 2 **Long noncoding RNA (lncRNA) nuclear paraspeckle assembly transcript 1_1 (NEAT1_1) is down-regulated in rheumatoid arthritis-fibroblast-like synoviocytes (RA-FLSs).** lncRNAs that may interact with miR-221-3p were blasted with microarray data (GSE128813) and bioinformatic analysis website. RNA-seq data of RA-FLSs vs. HC-FLSs were performed to verify the bioinformatic results. qPCR analysis of RA-FLSs vs. HC-FLSs were used to confirm the RNA-seq results. (**A**) lncRNAs that may interact with miR-221-3p were blasted with microarray data (GSE128813) and bioinformatic analysis website and showed with the Venn diagram. (**B**) The heat map was used to show the RNA-seq data of distinguishable lncRNA expression profiles in RA-FLSs vs. HC-FLSs (fold changes > 1.5 and $P < 0.05$, $n = 3$). (**C**) The possible biologic functions of the blasted lncRNA NEAT1_1 were analyzed by gene set enrichment analysis (GSEA) and gene ontology (GO) analysis. The bubble chart was used to show the partial enrichment results. The color and size of each dot represents the enriched gene number in the GSEA reactome and GO biological process. (**D**) The expression of NEAT1 was verified by qPCR in HC-FLSs ($n = 5$) and RA-FLSs ($n = 8$). The data indicate the mean ± SD of three independent experiments ($n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, NS, not significant)

and may act as a ceRNA of miR-221-3p to regulate the biologic function of RA-FLSs.

3.3 | NEAT1_1 negatively modulates miR-221-3p/uPAR axis and the downstream signaling

Although NEAT1_1 is negatively correlated to miR-221-3p in RA-FLSs, it still unclear that how NEAT1_1 regulates miR-221-3p and uPAR. To determine this, we firstly investigated the colocalization of NEAT1_1 and miR-221-3p in RA-FLSs using confocal imaging. The results indicated that NEAT1_1 and miR-221-3p were both colocated in the nucleus and cytoplasm and the Pearson's correlation and overlap coefficient indicated meaningful change, which suggested that NEAT1_1 may function as a ceRNA of miR-221-3p (Fig. 3A). Furthermore, we established a NEAT1_1 overexpression system using an adenoviral vector carrying GFP to investigate whether NEAT1_1 can down-regulate the miR-221-3p/uPAR axis (Fig. 3B). As expected, the expression of miR-221-3p in NEAT1_1 overexpression system was dramatically decreased but uPAR mRNA level didn't change. However, the

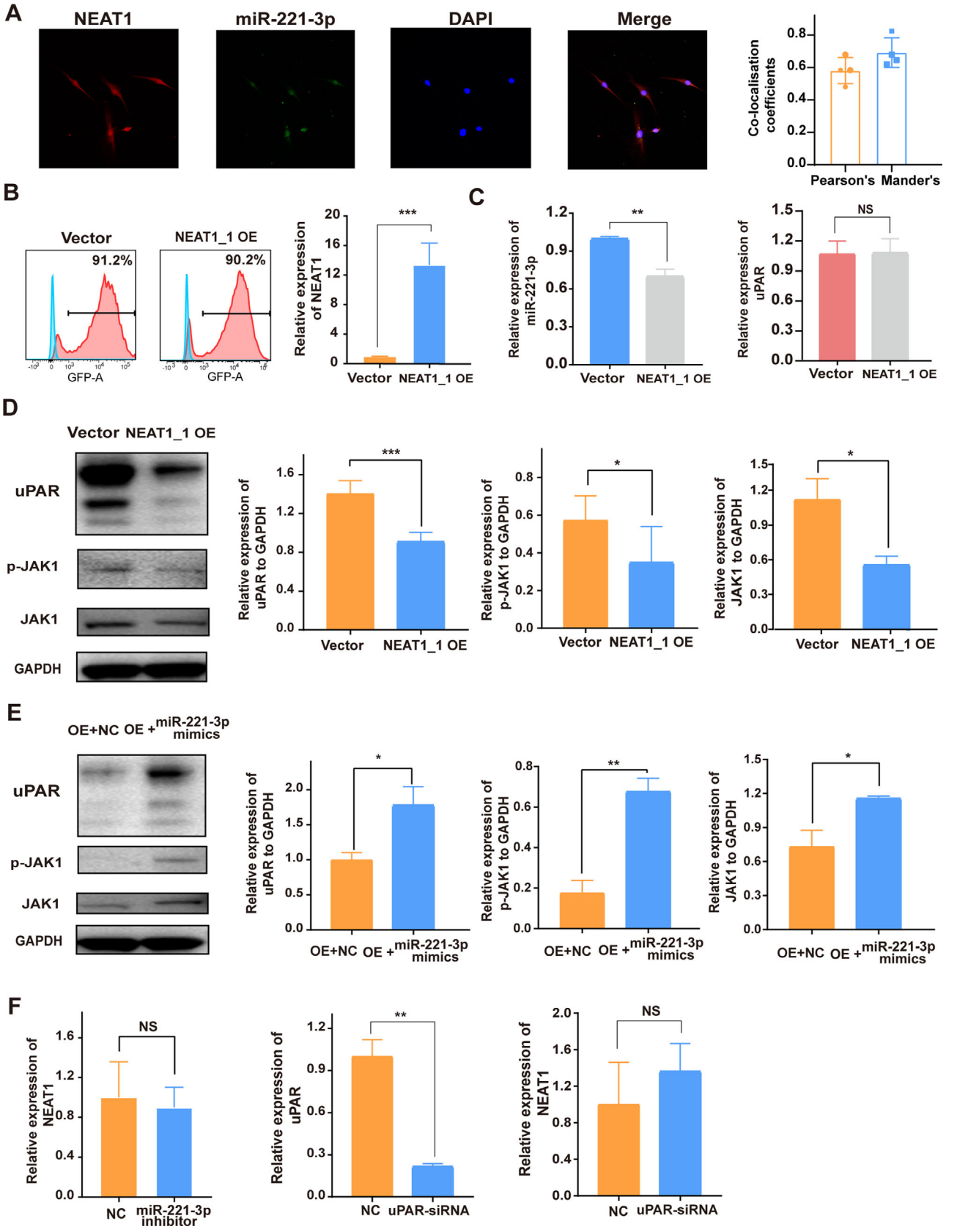

FIGURE 3 **Nuclear paraspeckle assembly transcript 1_1 (NEAT1_1) negatively modulates miR-221-3p/uPAR (urokinase-type plasminogen activator receptor) axis and the downstream signaling** (**A**) The colocalization of NEAT1_1 and miR-221-3p in rheumatoid arthritis-fibroblast-like synoviocytes (RA-FLSs) was investigated by RNA FISH assay. Representative images of NEAT1_1 (red), miR-221-3p (green) and nuclei (blue) (*panel*

(Continues)

FIGURE 3 (Continued)
left) were shown. The Pearson's correlation and overlap coefficient were shown from four independent experiments (*panel right*). (**B**) Overexpression of NEAT1_1 in RA-FLSs was conducted using the adenovirus-mediated transfection system. The efficiency of NEAT1_1 overexpression in RA-FLSs was evaluated by GFP using flow cytometry (*panel left and middle*). The relative expression of NEAT1_1 was verified by qPCR (*panel right*). (**C**) The expressions of miR-221-3p (*panel left*) and uPAR (*panel right*) in NEAT1_1 overexpression system were determined using qPCR. (**D**) The expressions of uPAR, p-JAK1, and JAK1 in NEAT1_1 overexpression system were determined using Western blotting (*panel left*). The Western blotting results were summarized by normalizing the mean gray value of interest protein to GAPDH (*panel right*). (**E**) The expressions of uPAR, p-JAK1, and JAK1 in both miR-221-3p and NEAT1_1 overexpression system were determined using Western blotting (*panel left*). The Western blotting results were summarized by normalizing the mean gray value of interest protein to GAPDH (*panel right*). (**F**) The relative expressions of NEAT1 in miR-221-3p knockdown system (*panel left*) and uPAR knockdown system (*panel right*) were determined using qPCR. The data indicate the mean ± SD of three independent experiments ($n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, NS, not significant). OE, overexpression

uPAR expression was significantly reduced on protein level detected by western blotting. Importantly, combined with the developed miR-221-3p overexpression system, we showed that enhanced miR-221-3p can significantly rescue the down-expressed uPAR (Fig. 3E, left panel). Inversely, both the expressions of NEAT1_1 in miR-221-3p knockdown system and uPAR knockdown system were not changed (Fig. 3F), indicating that NEAT1_1 can regulate miR-221-3p and uPAR, but miR-221-3p and uPAR cannot modulate NEAT1_1 in turn. Conclusively, NEAT1_1 can directly interact with miR-221-3p and suppress the expression of uPAR on protein level.

We further conducted the Western blotting to investigate whether NEAT1_1 can regulate the downstream signaling of miR-221-3p/uPAR axis. As shown in Figure 3D, both the phosphorylated JAK1 and total JAK1 were significantly reduced in NEAT1_1 overexpressed RA-FLSs. Interestingly, the phosphorylated JAK1 can be rescued in both miR-221-3p and NEAT1_1 overexpression systems (Fig. 3E), indicating that miR-221-3p can reverse the modulating effect of NEAT1_1 on uPAR signaling. However, the phosphorylation levels of PI3K and Akt were not significant in the NEAT1_1 overexpression system (Supporting Information Fig. S4). Thus, these results indicate that NEAT1_1 can act as a rheostat for miR-221-3p/uPAR axis and the downstream JAK signaling.

3.4 | NEAT1_1 modulates the tumor-like biologic behaviors of RA-FLSs

The chronic inflammatory environment activated RA-FLSs show lots of tumor-like behaviors, such as hyper-proliferation, apoptosis resistance, migration, and tissue invasion.[4,5] Here, we explored the proliferation, apoptosis, migration, and invasion capacities of RA-FLSs to confirm their tumor-like characters compared to HC-FLSs. The results revealed that the population of G2/M phase cell cycle in RA-FLSs was much higher than HC-FLSs (Supporting Information Fig. S5A). Also, RA-FLSs showed lower apoptosis population than HC-FLSs (Supporting Information Fig. S5B), indicating an anti-apoptosis character. Moreover, we used a transwell chamber system and matrigel system to investigate the migration and invasion capacities of RA-FLSs. Indeed, the data showed that both the migration and invasion capacity of RA-FLSs were significantly higher than HC-FLSs (Supporting Information Fig. S5C, D). Thus, consistent with our previous data,[4,5] we confirmed that RA-FLSs possess tumor-like biologic characters.

Next, we investigated whether NEAT1_1 can regulate the tumor-like biologic behaviors of RA-FLSs by modulating miR-221-3p/uPAR axis. Using the established NEAT1_1 overexpression system, we examined the function of NEAT1_1 on RA-FLSs migration capacity. In order to obtain an accurate result of RA-FLSs migration, wound-scratch assay and transwell chamber system were employed individually. As expected, the migration capacities of RA-FLSs in the two systems were consistently decreased in NEAT1_1 overexpressed condition (Fig. 4A, B). To further explore the modulatory effect of NEAT1_1 on RA-FLSs migration, we designed two siRNAs to down-regulate NEAT1_1 level, and one of which was called NEAT1-siRNA#1 was verified effective using qPCR analysis (Supporting Information Fig. S6). The migration capacity of RA-FLSs in the NEAT1_1 knockdown system was detected using the transwell chamber system. The result revealed that RA-FLSs migration capacity in the NEAT1_1 knockdown system was reversely enhanced (Fig. 4C). Thus, we showed that NEAT1_1 can regulate RA-FLSs migration capacity. Furthermore, the invasion capacities of RA-FLSs in NEAT1_1 overexpression system and knockdown system were determined using the matrigel system. As expected, the overexpressed NEAT1_1 suppressed the invasion capacity of RA-FLSs, whereas down-expressed NEAT1 promoted this capacity (Fig. 4D, E). Collectively, these results suggest that NEAT1_1 can act as a rheostat for regulating the tumor-like behavior of RA-FLSs and may exert a protective role in patients with RA.

3.5 | NEAT1_1 inhibits expression levels of cytokines in RA-FLSs

Due to the long-term exposure to inflammatory milieu, RA-FLSs have been impelled to secrete an abnormal profile of inflammatory cytokines, which contribute to the aggravated severity of RA disease. Indeed, the GSEA and GO analysis results showed that IL-1β, IL-6, and IL-8 were significantly elevated in RA-FLSs compared to HC-FLSs. To determine the modulatory effect of NEAT1_1 on RA-FLSs inflammatory cytokine secretion, we detected mRNA levels of IL-6, IL-8, IL-1β, and TNF-α in NEAT1_1 overexpressed RA-FLSs. The results suggested that the overexpression of NEAT1_1 significantly decreased IL-6 and IL-8 (Fig. 4F), wheres IL-1β and TNF-α were not significant (Supporting Information Fig. S7A). We further conducted the ELISA to detect the secretion levels of IL-6, IL-8, IL-1β, and TNF-α in the supernatants of NEAT1_1 overexpressed RA-FLSs. The results showed that only

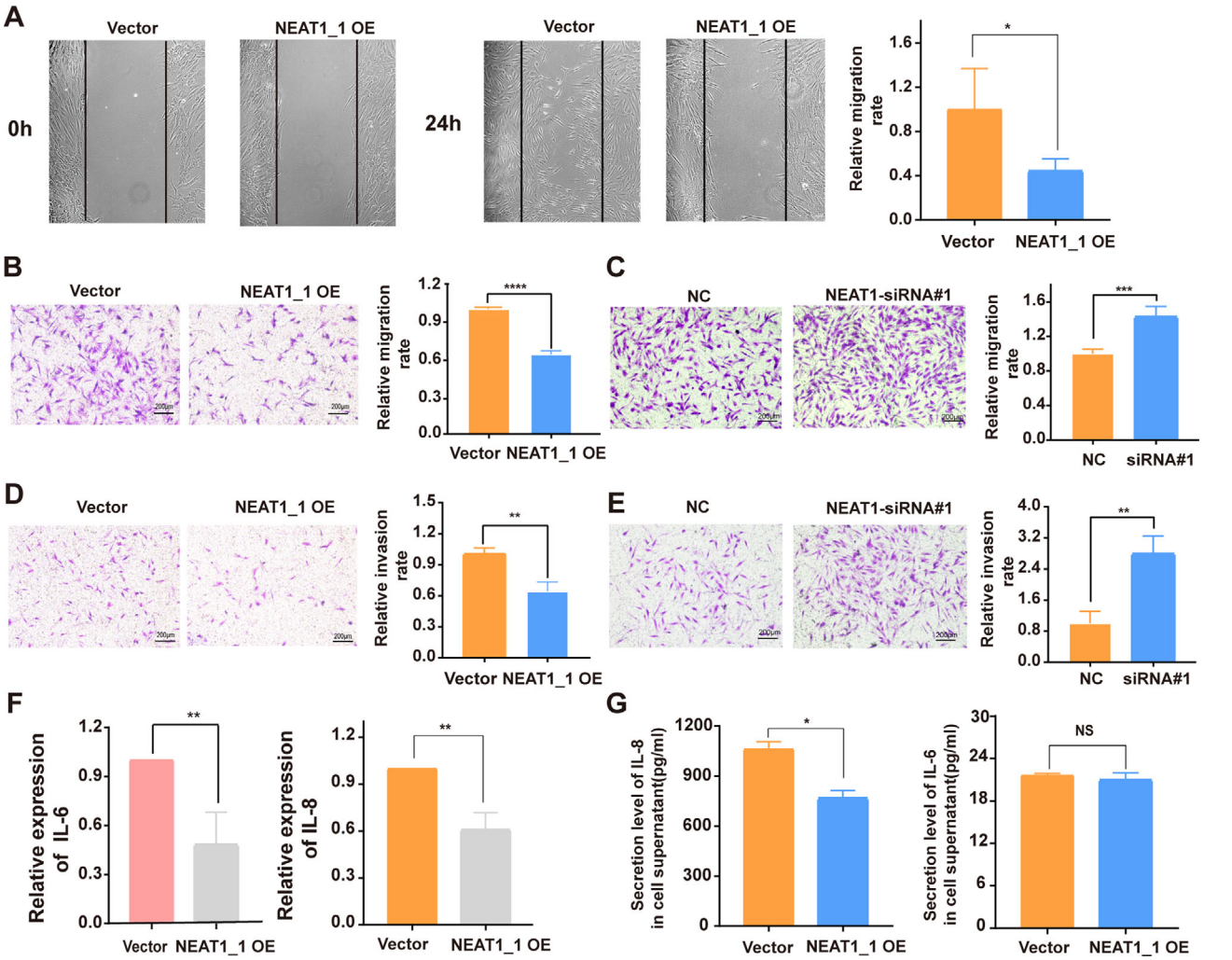

FIGURE 4 **Nuclear paraspeckle assembly transcript 1_1 (NEAT1_1) modulates the tumor-like biologic behaviors of rheumatoid arthritis-fibroblast-like synoviocytes (RA-FLSs).** RA-FLSs in NEAT1_1 overexpression system or knockdown system were prepared for migration, invasion, and cytokines detections. (**A**) The migration capacity of NEAT1_1 overexpressed RA-FLSs was determined by wound-scratch assay. Photographs of the cells at 0 and 24 h were obtained using microscope imaging (*panel left* and *middle*). The results were summarized by calculating the related migration rate (*panel right*). (**B**–**E**) The migration capacities of RA-FLSs in NEAT1_1 overexpression system (**B**) and knockdown system (**C**) were detected using the transwell chamber system. The invasion capacities of RA-FLSs in NEAT1_1 overexpression system (**D**) and knockdown system (**E**) were determined using the matrigel system. Representative images (original magnification, ×40) are shown. The results were summarized by calculating relative migration or invasion rates. (**F**) The relative expression of IL-6 (*panel left*) and IL-8 (*panel right*) in the NEAT1_1 overexpression system was verified by qPCR. (**G**) The secretion levels of IL-8 (*panel left*) and IL-6 (*panel right*) in the supernatants of NEAT1_1 overexpressed RA-FLSs were detected by ELISA. The data indicate the mean ± SD of three independent experiments ($n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, NS, not significant). OE, overexpression

IL-8 was significantly decreased in NEAT1_1 overexpressed RA-FLSs (Fig. 4G, left panel), whereas IL-6, IL-1β, or TNF-α were not significantly affected (Fig. 4G, right panel, Supporting Information Fig. S7B). Thus, NEAT1_1 mainly regulates IL-8 secretion in RA-FLSs.

3.6 | NEAT1_1 has no effect on apoptosis and cell cycle of RA-FLSs

As we have showed earlier, RA-FLSs possess hyperproliferation and apoptosis resistance properties (Supporting Information Fig. S5A, B). The imbalance between proliferation and apoptosis in RA-FLSs contributes to synovial hyperplasia. It is important to explore whether NEAT1_1 can modulate RA-FLSs apoptosis and cell cycle. Therefore, we investigated the apoptosis and cell cycle of RA-FLSs in NEAT1_1 overexpression system and knockdown system, respectively. Strongly, the results indicated that both apoptosis and cell cycle of RA-FLSs in NEAT1_1 overexpression system and knockdown system were not significantly changed (Supporting Information Fig. S8A–D). Also, we detected the apoptosis changes of RA-FLSs in NEAT1_1 overexpression system combined with miR-221-3p overexpression system. The results still did not show significant changes in RA-FLSs (Supporting

Information Fig. S8E). Thus, NEAT1_1 does not affect apoptosis and cell cycle of RA-FLSs via the miR-221-3p/uPAR axis.

3.7 | NEAT1_1 regulates the migration and invasion of RA-FLSs by interacting with miR-221-3p

Using the NEAT1_1 overexpression system and knockdown system, we have verified that NEAT1_1 can modulate migration, invasion, and IL-8 secretion of RA-FLSs. Also, NEAT1_1 regulates miR-221-3p with a single-track model. However, whether NEAT1_1 affects migration, invasion, and IL-8 secretion of RA-FLSs by interacting with miR-221-3p is still unclear. Herein, we explored the tumor-like characters of NEAT1_1 overexpressed RA-FLSs in the miR-221-3p overexpression system. As expected, the overexpression of miR-221-3p can significantly rescue migration capacities of NEAT1_1 overexpressed RA-FLSs in wound-scratch system and transwell chamber system (Fig. 5A, B). Accordingly, the invasion capacity of NEAT1_1 overexpressed RA-FLSs was dramatically rescued in the miR-221-3p overexpression system (Fig. 5C). We further conducted the ELISA assay to detect the secretion of IL-8 on the same condition. Consistently, IL-8 production of NEAT1_1 overexpressed RA-FLSs was rescued in the miR-221-3p overexpression system (Fig. 5D). Together, these data suggest that NEAT1_1 regulates the tumor-like biologic characters of RA-FLSs by interacting with the miR-221-3p/uPAR axis.

4 | DISCUSSION

RA is a systemic autoimmune disease that directly affects the synovial joints and often causes cartilage destruction, bone erosion, and even severe disability. Recently, the etiology and pathogenesis of RA have been studied in more depth. It has been substantiated that the susceptibility genes, environmental factors, and immune system disorders can definitely contribute to the pathologic progression of RA.[1,2,19] Although the studies of biologic development for RA pathology have provided new possible approaches for RA treatment, the current therapies for RA are still unsatisfactory.[20] Due to the long-term exposure to inflammatory milieu, RA-FLSs have been impelled to secrete an abnormal profile of inflammatory cytokines, which may initially and perpetually result in cartilage invasion and bone destruction in RA.[21] A number of researches have revealed the functions of RA-FLSs metabolomics, inflammatory stimulation, immune activation, and somatic mutation epigenetics in remodeling the pathologic characteristics of RA.[6] Epigenetic factors, including DNA methylation, histone modification, and regulation of noncoding RNA, are promising areas for uncovering the progression of RA and seeking the potent targets for RA therapy.[22] Therefore, it is essential to explore the underlying mechanisms and pathways of epigenetics contributing to the abnormal behaviors of RA-FLSs.

The tumor-like biologic characters of RA-FLSs make them more aggressive in promoting the progression of RA. We have documented that uPAR is up-regulated in RA-FLSs, which can promote RA-FLSs tumor-like behaviors. MiR-221-3p is a new identified miRNA that regulates the development of tumor in several types of cancers. Importantly, miR-221-3p can promote tumor-like behaviors of RA-FLSs. Here, we confirmed that both miR-221-3p and uPAR were highly expressed in RA-FLSs. More importantly, uPAR was positively correlated to miR-221-3p in RA-FLSs, indicating miR-221-3p can promote the tumor-like behaviors of RA-FLSs by synergistically interacting with uPAR. To further verity whether miR-221-3p can modulate uPAR signaling, we detected the expressions of uPAR in miR-221-3p knockdown system or overexpression system. As we expected, uPAR expressions were dynamically changed with altered expression of miR-221-3p in RA-FLSs. Increasingly, evidence has revealed that lncRNAs exert their regulatory effects on mRNA through interacting with miRNAs. In terms of mechanism, for the regulation of miR-221-3p, one lncRNA NEAT1_1 has been screened based on our microarray data (GSE128813) and the bioinformatics program ENCORI. Interestingly, lncRNA NEAT1 is an oncogene that is up-regulated in various cancers, resulting in poor overall survival.[23] However, contradictory findings show that NEAT1 is down-regulated in some cancers such as leukemia[24] and is a direct target of the tumor suppressor p53, suggesting that NEAT1 may be a tumor suppressor instead.[25] Therefore, it is of vital to clarify the function and mechanism of NEAT1_1 in RA-FLSs.

NEAT1 produces two transcripts, NEAT1_1 and NEAT1_2, which differ in their 3′ UTR processing. NEAT1_1 completely overlaps with the long NEAT1_2 isoform at the 5′ end. But the function of each isoform on cell phenotypes has remained unclear. In this study, we designed the primers of the overlapping sequence to identify the expression of NEAT1_1 in RA-FLSs. Indeed, the results revealed that NEAT1_1 was down-regulated in RA-FLSs compared to HC-FLSs. In addition, consistent with our results, another study that analyzed datasets containing 53 synovial tissues from 33 RA patients and 20 healthy donors also indicated that lncRNA NEAT1 (log2FC = −1.63; P = 7.64e-06) can be a significant regulator in RA with lower expression compared to the HC group.[26] In contrast, it is also indicated that NEAT1 is up-regulated in PBMCs[27] and PBMC-derived exosomes[28] in patients with RA. Thus, these results suggest that altered NEAT1 involves in RA pathogenesis, and its circulating levels may represent a biomarker for RA diagnosis.

NEAT1_1 and miR-221-3p are colocated in the nucleus and cytoplasm in RA-FLSs. Furthermore, the expressions of miR-221-3p and uPAR of RA-FLSs were accordingly reduced in the NEAT1_1 overexpression system. Inversely, the expressions of NEAT1_1 in miR-221-3p or uPAR knockdown systems were not elevated, indicating that NEAT1_1 regulates miR-221-3p and uPAR with a single-track model. Interestingly, others have provided proofs that NEAT1_2 protects doxorubicin-induced cardiac senescence by sponging miR-221-3p.[29] This indicates that both the isoforms of NEAT1 exert their functions by sponging miR-221-3p. Thus, NEAT1_1 may act as an upstream signaling molecule of the miR-221-3p/uPAR axis. To verify this, we further explored the function of NEAT1_1 in modulating the tumor-like behaviors of RA-FLSs in the NEAT1_1 overexpression system. As expected, overexpression of NEAT1_1 reduced the migration, invasion, and cytokine secretion of RA-FLSs, whereas it had no effect on apoptosis and cell cycle. Consistent with others,

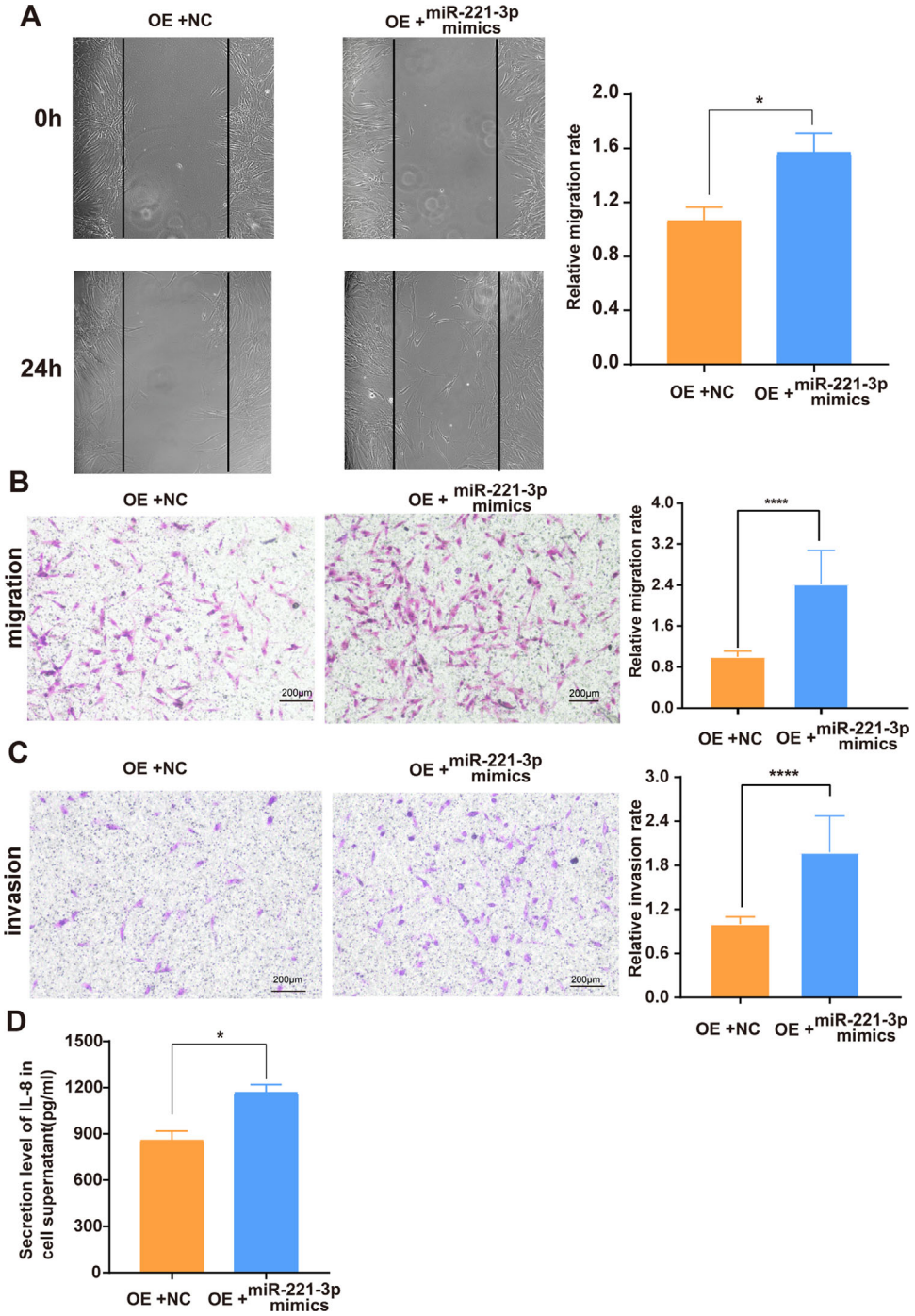

FIGURE 5 Nuclear paraspeckle assembly transcript 1_1 (NEAT1_1) regulates the migration and invasion of rheumatoid arthritis-fibroblast-like synoviocytes (RA-FLSs) by interacting with miR-221-3p. NEAT1_1 overexpressed RA-FLSs were prepared in miR-221-3p overexpression system for migration, invasion, and cytokines detections. (A, B) The migration capacities of RA-FLSs in both miR-221-3p and NEAT1_1 overexpression system were determined by wound-scratch assay (A, *panel left*) and transwell chamber system (B, *panel left*), individually. The results were summarized by calculating the related migration rate (*panel right*). (C)The invasion capacities of RA-FLSs in both miR-221-3p and NEAT1_1 overexpression system were determined using matrigel system (*panel left*). The results were summarized by calculating the related invasion rate (*panel right*). (D) The secretion of IL-8 in the supernatants of RA-FLSs on the same condition were detected by ELISA. The data indicate the mean ± SD of three independent experiments ($n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, NS, not significant). OE, overexpression

NEAT1_1 is not required for the 2D growth and proliferation of cancer cells.[30]

To further investigate whether NEAT1_1 works through the miR-221-3p/uPAR axis, we performed rescue experiments. The results suggested that miR-221-3p overexpression can partially restore the effect of NEAT1_1 overexpression on migration, invasion, and cytokine secretion in RA-FLSs. These data imply that NEAT1_1 negatively regulates tumor-like characters of RA-FLSs by sponging miR-221-3p. Additionally, the decreased NEAT1_1 in RA-FLSs may contribute to the increased rheumatoid synovial aggression, cartilage erosion, and bone destruction. Recently, others showed that NEAT1 was up-regulated in human FLSs-RA cell lines, and can promote proliferation, migration, invasion, and inflammatory cytokine secretion and suppressed apoptosis in RA-FLSs.[31] Considering that lncRNAs exhibit tissue, developmental stage, space-time, and cell subtype specificity,[32] NEAT1 may have diverse functions across different cell types. As for the downstream of miR-221-3p/uPAR axis, NEAT1_1 mainly regulates the activation of JAK1, but PI3K/Akt signaling just slightly altered without statistical significance. It has been documented that miR-221-3p and NEAT1 can directly regulate the PI3K/Akt signaling.[33,34] However, miR-221-3p and NEAT1 not only modulates PI3K/Akt signaling via uPAR, but also some other pathways, which forms a sophisticated network. Taken together, our data indicate that NEAT1_1 may play a protective role in RA-FLSs.

In summary, our studies clarified that miR-221-3p is an upstream molecule of uPAR that can regulate the downstream signaling of uPAR and the tumor-like characters in RA-FLSs. As a direct target of miR-221-3p, NEAT1_1 may play a protective role in RA pathology through its sponging interacting with miR-221-3p. Our data provide new insights into the mechanisms of NEAT1_1 in modulating RA-FLSs tumor-like behaviors. The targeting of NEAT1_1 and miR-221-3p/uPAR axis may have a promising therapeutic role in patients with RA.

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ETHICAL APPROVAL AND CONSENT TO PARTICIPATE

The research was approved by the ethics committee of the Third Affiliated Hospital at the Sun Yat-sen University and all subjects were given the written informed consent in accordance with the Declaration of Helsinki.

CONSENT FOR PUBLICATION

Consent for publication has been obtained from all authors.

AVAILABILITY OF SUPPORTING DATA

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

DISCLOSURES

The authors declare no conflicts of interest.

AUTHORSHIP

Y. Lu, YP and MW designed the experiments. MW, YC, XB and Y. Liu performed the experiments. XL and ZH collected synovial tissues. MW, YS, WW and BM analyzed these data. MW wrote the manuscript. Y. Lu and YP edited the manuscript. Yixiong Chen and Manli Wang contributed equally to this work.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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