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Biological regulation on synovial fibroblast and the treatment of rheumatoid arthritis by nobiletin-loaded tetrahedral framework nucleic acids cargo tank

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ABSTRACT

The hyperplasia and destruction of synovial tissue have an important impact on the development of rheumatoid arthritis (RA), the abnormal proliferation and migration of synovial fibroblast in synovial tissue is similar to tumor cells. Targeting anomalous synovial fibroblast and designing a high bioavailability nano drug delivery system can reduce the dosage for the treatment of rheumatoid arthritis and it is of great significance to reduce toxic and side effects and improve curative effect. In this experiment, the nobiletin-loaded tetrahedral framework nucleic acids cargo tank was established, carrying anti-inflammatory small molecule monomer drug nobiletin with minimal bioavailability. Both *in vitro* cell experiments and *in vivo* animal studies proved the nano cargo tank enhance the role of nobiletin in reducing the invasiveness of pathological synovial fibroblast and promote their apoptosis, effectively alleviate the disease development of rheumatoid arthritis.

Synovial tissue is a kind of mesenchymal tissue around joints, which is mainly composed of fibroblasts. In rheumatoid arthritis, synovial fibroblast in inflamed synovial tissue proliferates and migrates abnormally, secretes chemokines and inflammatory factors, intensifies systemic reaction, secretes enzymes that destroy tissues and bones, and destroys articular cartilage [1]. The growth characteristics of synovial fibroblast are similar to tumor cells [2–5]. At present, synovial fibroblast is a promising therapeutic target. Currently available treatments include nonsteroidal anti-inflammatory antipyretic, anti-inflammatory, biological drugs and small molecule monomers, *etc*. Therapy for rheumatoid arthritis requires to be improved through adequate safety and effectiveness to satisfy medical needs [6].

Nobiletin is a flavonoids from citrus peel [7], it has been proved to have anti-inflammatory and anti-cancer properties, including inhibiting cell proliferation, anti-metastasis and induction of apoptosis [7]. However, it is insoluble in water and ether, leading to its low bio availability and poor plasma stability [8–10]. Tetrahedral framework nucleic acids (tFNAs) have unique drug delivery characteristics with good biocompatibility, stable three-dimensional structure and are considered to be a highly promising method of conventional drug treatment [11–15]. It can enter cells carrying small molecules [16–22] and has anti-inflammatory properties and tissue penetration. Flavonoids have shown the ability of direct interaction with DNA [23] and could preferentially bind to the DNA structure by intercalating with double stranded DNA. The classic intercalation is dominant binding mode of interactions *via* hydrogen bonding between the hydroxyl groups of the flavonoids and the guanine and cytosine residues in the DNA [24]. Studies have shown that tFNAs could permeate cells *via* caveolin-mediated endocytosis and sustains stable. The underlying advantages of tFNA-Nob were the improved water solubility and cell penetration that results from tFNA transportation. According to the preliminary research of our research [18,23], we want to realize a nano tank to carry nobiletin into synovial cells, increasing the drug effects of anti-inflammatory and promoting the apoptosis of pathological synovial fibroblast, enhancing the plasma stability of nobiletin, and reducing the invasiveness of abnormal synovial fibroblast effectively and safely, and reduce rheumatoid arthritis effectively.

As shown in Fig. 1a, four separate stranded DNA synthesized tFNAs, the mediation instrument was used to shake violently for 8 h to obtain the cargo tank of the composite drug system tFNA-Nob, flavonoid monomer nobiletin entered DNA base pairs by classical intercalation [18,23]. Characteristics of four single-stranded DNA (ssDNA) and tFNAs were detected by capillary electrophoresis

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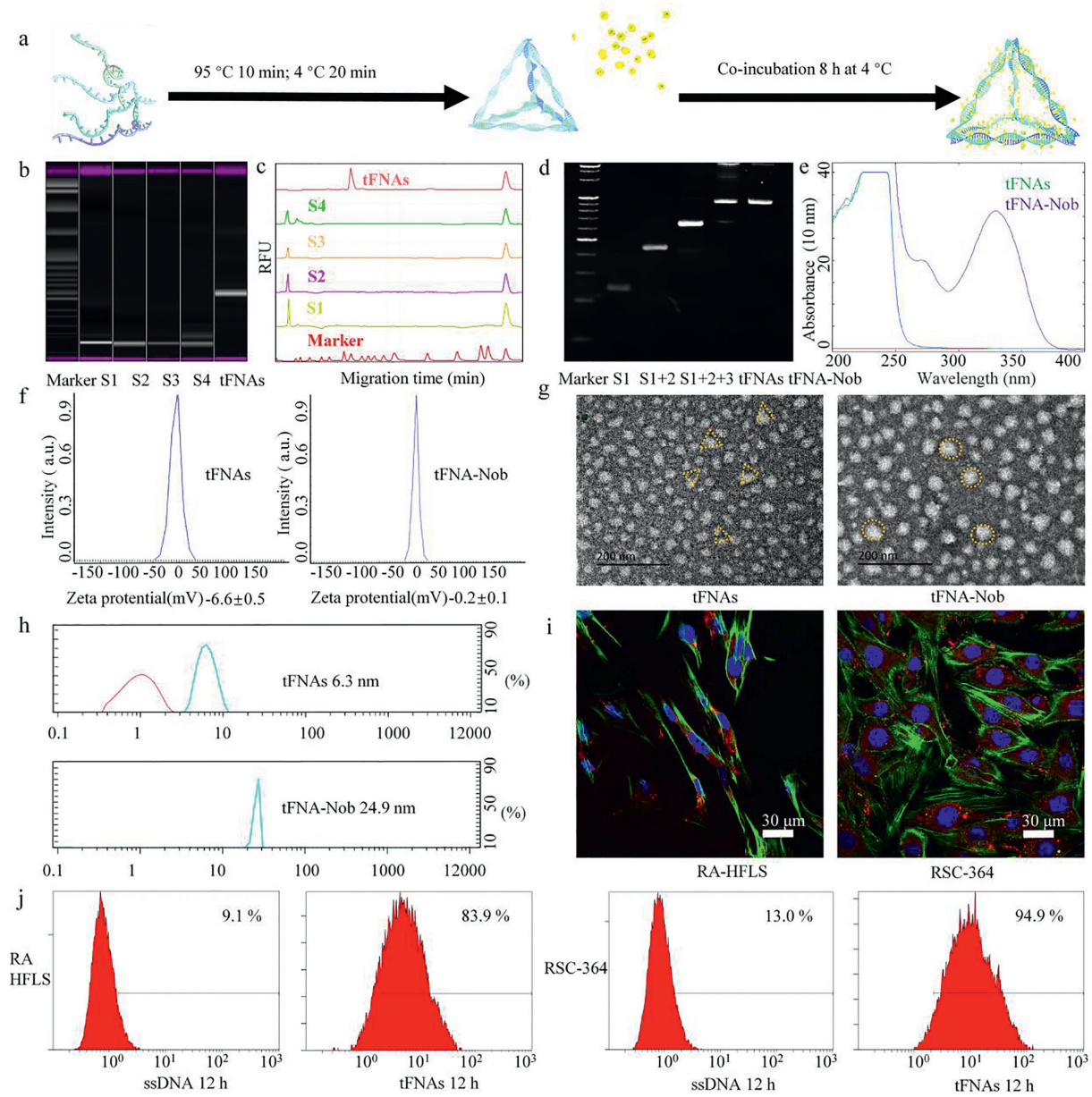

Fig. 1. Characterization and cellular uptake of tFNA-Nob. (a) The synthesis process of tFNA-Nob. (b) Capillary electrophoresis images showed the successful preparation of tFNAs. (c) The preparation of tFNAs was analyzed by capillary electrophoresis. (d) The results of polyacrylamide gel electrophoresis confirmed the preparation of tFNAs and tFNA-Nob. (e) UV wavelength scanning showed that the wavelengths of tFNAs and tFNA-Nob were 260 nm and 340 nm, respectively. (f) The zeta potential of tFNAs and tFNA-Nob was measured. (g) The DLS results showed the shape and size of tFNAs and tFNA-Nob. (h) The particle sizes of tFNAs and tFNA-Nob were 6.3 nm and 24.9 nm, respectively. (i) The tFNAs synthesized by one ssDNA was labeled with Cy5 and the presence of Cy5 in the cell was proved by immunofluorescence picture, verified that tFNAs could enter RA-HFLS and RSC-364. Red: cy5. Scale bar: 30 μm. (j) Comparing the three-dimensional structure of single DNA double stranded ssDNA and tFNAs, tFNAs can enter the cells in large quantities at 12 h in RA-HFLS and RSC-364 cells (accounting for 83.9% and 94.9% respectively).

(Figs. 1b and c). Polyacrylamide gel electrophoresis (PAGE) showed that four single-stranded DNA synthesized tFNAs and tFNA-Nob, which moved the slowest, indicating that the synthesis was successful, it proved that nobiletin entered the three-dimensional structure of tFNAs to form a nano cargo tank, the structure of tFNAs were not damaged after taking nobiletin, and the strip disappeared, which means the overall structure of the nano cargo tank composite drug system was more stable (Fig. 1d). The ultraviolet wavelength scanning demonstrated that the wavelengths of tFNAs and tFNA-Nob are 260 nm and 340 nm, respectively. It proves that the nano cargo tank is a composite, not a simple mixed system (Fig. 1e). Zeta potential showed that tFNAs have a negative charge (-6.6 ± 0.5 mV). After carrying the neutral small molecule nobiletin, the absolute value of the negative charge decreases (-0.2 ± 0.1 mV) (Fig. 1f). Transmission electron microscope showed that tFNAs have a triangular pyramid structure, after nobiletin is inserted into tFNAs, the volume of the composite drug system becomes larger and circular (Fig. 1g). Particle sizes of tFNAs and tFNA-Nob measured by the particle size measuring instrument are 6.3 nm and 24.9 nm, respectively (Fig. 1h). The precipitation of insoluble nobiletin disappeared after being carried by tFNAs (Fig. S3 in Supporting information). Above phenomena proved nobiletin-loaded tetrahedral framework nucleic acids cargo tank composed successfully. Rat synovial cell line (RSC-364) [24] and human rheumatoid arthritis synovial cells (RA-HFLS) [25] were used in our *in vitro* studies. According to our previous research [26], immunofluorescence detected that tFNA could enter two kinds of cells (Fig. 1i) [27]. Flow cytometry verified that different

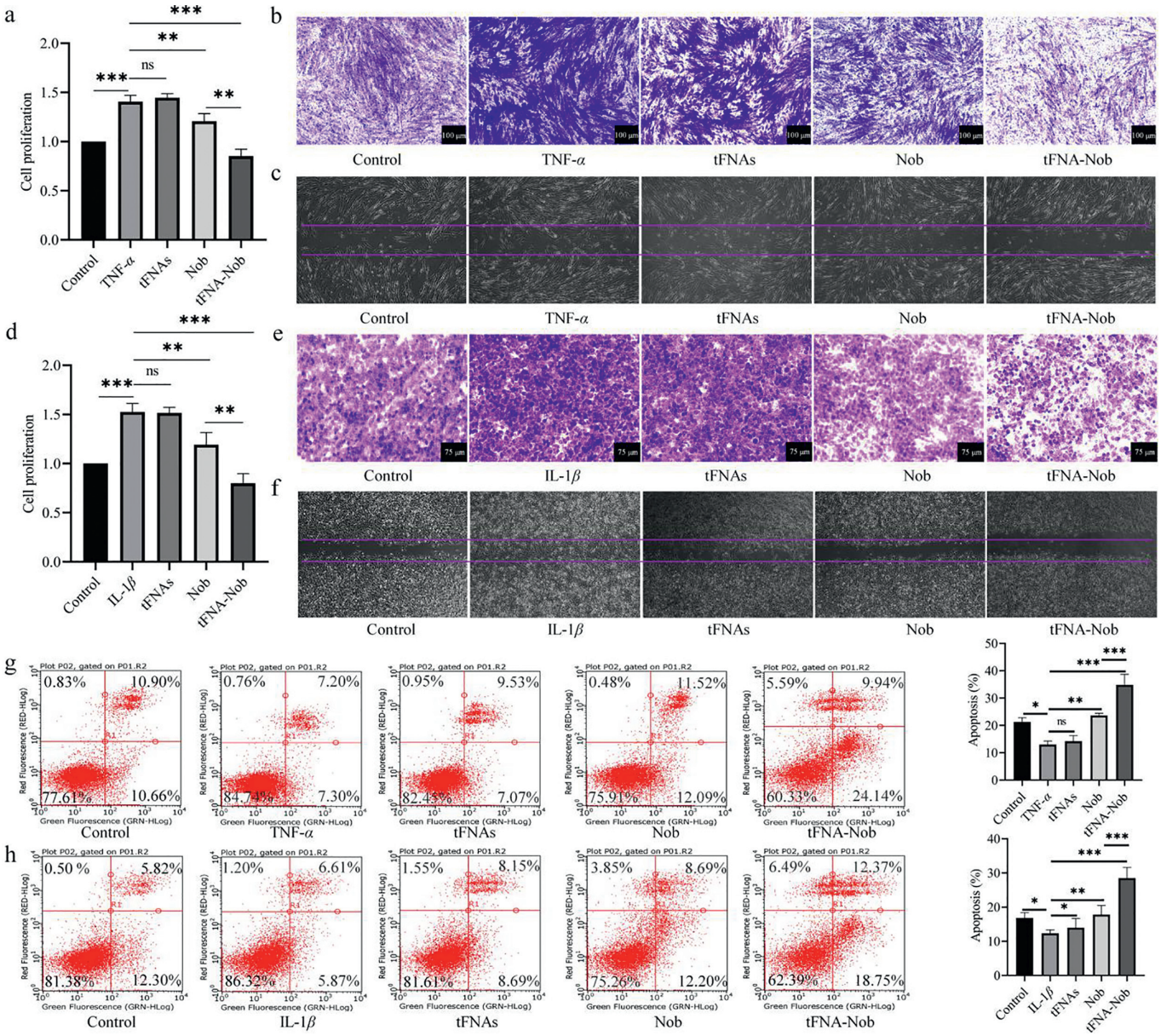

Fig. 2. tFNA-Nob regulates the biological behavior of RA-HFLS and RSC-364. (a) Effect of tFNA-Nob on the proliferation of RA-HFLS, statistical method: one-way ANOVA. The data are expressed as mean ± standard deviation ($n = 3$). (b) Effect of RA-HFLS on vertical migration of RA-HFLS (scale bar: 100 μm). (c) Effect of RA-HFLS on horizontal migration of RA-HFLS. (d) Effect of tFNA-Nob on proliferation of RSC-364. (e) Effect of tFNA-Nob on vertical migration of RSC-364 (scale bar: 75 μm). (f) Effect of tFNA-Nob on horizontal migration of RSC-364. (g) The effect of tFNA-Nob on RA-HFLS cell apoptosis was statistically analyzed by flow cytometry. Statistical method: one-way ANOVA. The data are expressed as mean ± standard deviation ($n = 3$). (h) The effect of tFNA-Nob on apoptosis of RSC-364 cells was statistically analyzed by flow cytometry. Statistical method: one-way ANOVA. The data are expressed as mean ± standard deviation ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, ns: not significant.

from single DNA, tFNAs could be taken up by cells in a large amount after 12 h culture with cells (Fig. 1j).

As shown in Supporting information, when the mixing ratio of tFNAs and nobiletin is about 1:160, the particle size of the composite system reaches saturation and stability, At the same time, the changes in drug loading rate and entrapment efficiency of tFNAs and nobiletin of different proportions were determined by absorbance. After the calculation of encapsulation efficiency and drug loading rate of tFNA-Nob drug system, the ratio of tFNA:nobiletin means 1:80 is selected as the synthesis specification of subsequent experiments (Fig. S1 in Supporting information), and the experiment of simulating drug sustained release in dialysis bag is carried out, it is found that the drug release speed is slower and more after tFNA loading. It is speculated that tFNA-Nob system can slow down and stabilize the drug (Fig. S2 in Supporting information). The above results show that the composite drug system with citrus inserted into tFNAs has been successfully synthesized and can be used in further research experiments.

200 nmol/L tFNAs were selected which has no significant effect on cell proliferation to be used as a carrier only [28], the compound drug group adopted the ratio of 1:80, then compared with the same concentration of nobiletin. Adding TNF-α (10 ng/L) to RA-HFLS cells for 24 h [29] and IL-1β (10 ng/L) to RSC-364 cells [30] for 24 h to simulate the inflammatory state, the success of the model was verified by cell counting kit-8 (CCK-8), which showed the induction of inflammatory factors promoted the proliferation of synovial fibroblast. Figs. 2a and d respectively count the cell viability of RA-HFLS and RSC-364 cells model after the treatment by tFNAs and tFNA-Nob composite drug system. tFNA-Nob can significantly inhibit abnormal cell proliferation compared with the same

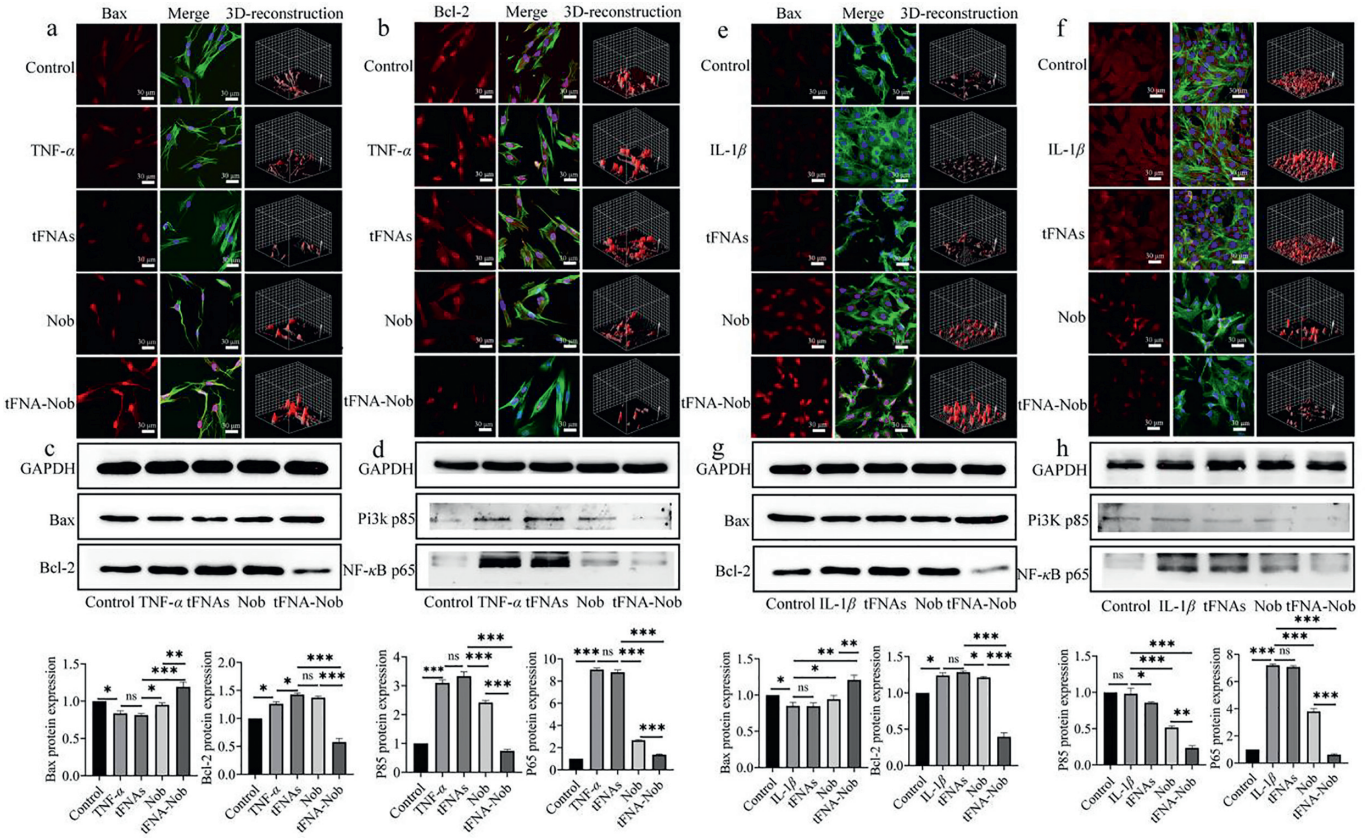

Fig. 3. Effect of tFNA-Nob on the expression of apoptotic protein in synovial cells. (a) Effect of tFNA-Nob on Bax protein expression in RA-HFLS (scale bar: 30 μm). (b) Effect of tFNA -Nob on Bcl-2 protein expression of RA-HFLS (scale bar: 30 μm). (c) Western blot analysis of Bax and Bcl-2 protein of RA-HFLS by tFNA-Nob. Statistical method: unpaired *t*-test. The data are expressed as mean ± standard deviation ($n = 3$). (d) Western blot analysis of p85 and p65 protein of RA-HFLS by tFNA-Nob. Statistical method: unpaired *t*-test. The data are expressed as mean ± standard deviation ($n = 3$). (e) Effect of tFNA-Nob on Bax protein expression of RSC-364 (scale bar: 30 μm). (f) Effect of tFNA-Nob on Bcl-2 protein expression of RSC-364 (scale bar: 30 μm). (g) Western blot analysis of Bax and Bcl-2 protein of RSC-364 by tFNA-Nob. Statistical method: unpaired *t*-test. The data are expressed as mean ± standard deviation ($n = 3$). (h) Western blot analysis of Pi3Kp85 and NF-κBp65 protein of RSC-364 by tFNA-Nob. Statistical method: unpaired *t*-test. The data are expressed as mean ± standard deviation ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, ns: not significant.

concentration of the nobiletin group. Under the same experimental operation the ability of the compound drug group to inhibit the vertical migration and horizontal migration of RA-HFLS was enhanced (Figs. 2b and c), the same trend was displayed in RSC-364 (Figs. 2e and f), statistical results were shown in supplementary material. It is understood that abnormal proliferation and migration of synovial cells are related to uncontrolled apoptosis. Therefore, we also explored the effect of the drug group on synovial cell apoptosis through flow cytometry. As shown in Figs. 2g and h, after inflammatory factor modeling, the ability of tFNA-Nob to promote synovial cell apoptosis was greatly enhanced compared with the single nobiletin group.

We further proved the expression of apoptosis related proteins Bax and Bcl-2 by taking immunofluorescence photos under confocal microscope and Western blot. Similarly, after stimulation with inducer and incubation with drug group, the results showed that the tFNA-Nob promoted the expression of Bax protein and inhibited the expression of Bcl-2 protein in two different synovial cells than the single drug group (Figs. 3a-c, e-g), 3D reconstruction using imageJ can clearly and intuitively see the protein expression content. In the study of apoptosis-related signaling pathways, it is recognized that the activation of Akt pathway can promote survival and growth stimulation, thus inhibiting apoptosis. It is speculated that the activation of Akt signaling pathway may be related to the abnormal proliferation and migration of fibroblast-like synovial cells in rheumatoid arthritis. Western blot verified that the Pi3Kp85 protein of its regulatory subunit was activated under the stimulation of inflammatory factors, and the activation was inhibited after drug treatment also through the downstream NF-κB subunit protein p65 was verified by Western blot to explore whether the drug also regulates this pathway and inhibits the abnormal biological behavior of synovial fibroblast (Figs. 3d and h).

In vivo, male wister rats aged 7–8 weeks were used to make collagen induced arthritis (CIA) disease model [31]. It is injected into the tail root of wister rats through bovine type II collagen and emulsifier of incomplete Freund adjuvant. CIA wister rat model was established after 2 weeks of immune induction, we made a general observation and evaluated the arthritis index, and then injected drugs into the diseased joints to ensure that the drug concentration was 500 nmol/L tFNAs. Similarly, the ratio of tFNAs:nobiletin was 1:80, and the control group was injected with normal saline. The drug was injected into ankles every other day for 2 weeks/6 times and joint samples were collected for histopathological evaluation. The experiment showed that the joints of CIA rats are significantly red and swollen. Injection of tFNAs cannot alleviate the lesions, but both nobiletin group and tFNA-Nob group could alleviate the arthritis lesions to a certain extent, and the effect of tFNA-Nob group was better (Fig. 4a). Hematoxylin-eosin (HE) staining found that the synovial tissue at the joints of CIA rats significantly proliferated, thickened, invaded bone tissue and cartilage, and more inflammatory cells were seen. After treatment with tFNAs, nobiletin and tFNA-Nob, tFNAs group had almost no therapeutic effect. Nobiletin and tFNA-Nob had a certain therapeutic effect, and the therapeutic effect of tFNA-Nob was the best (Fig. 4b). Toluidine blue staining (TB) showed that the bone tissue and cartilage tissue of CIA rats were damaged,

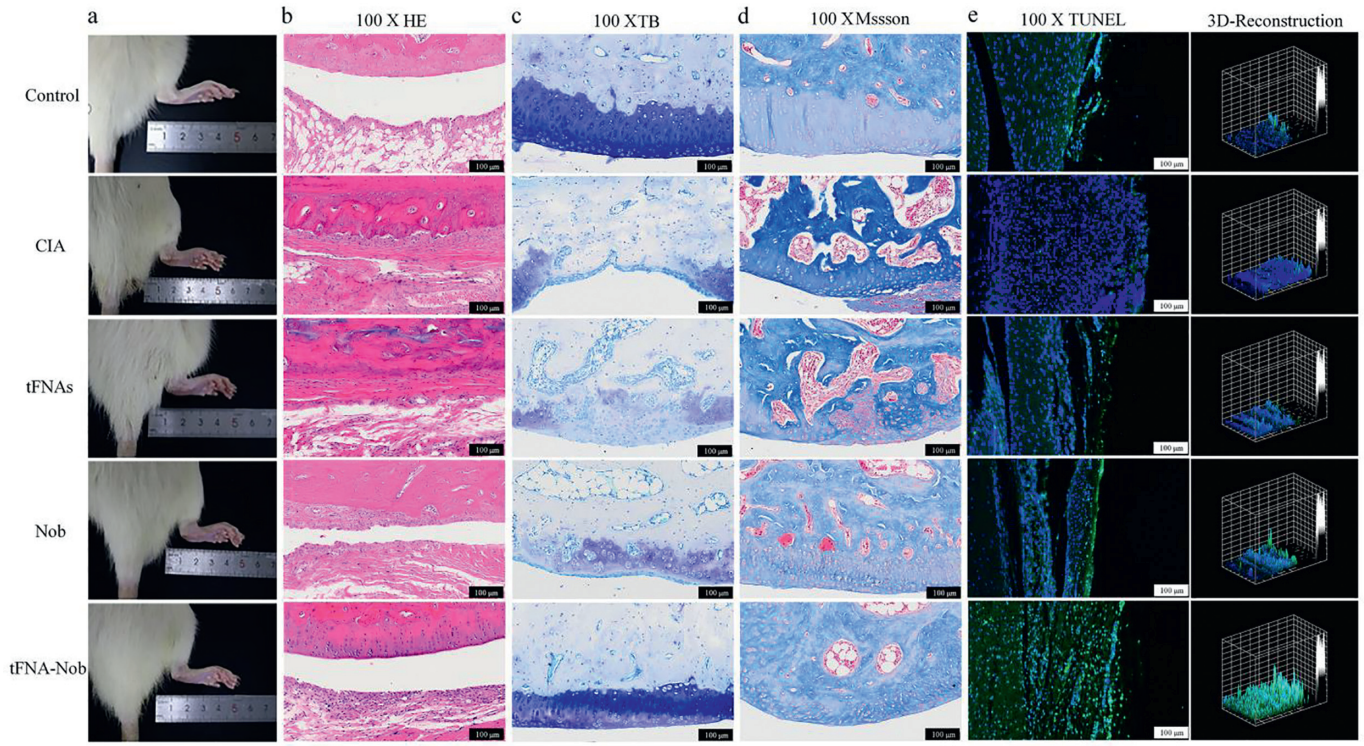

Fig. 4. Treatment of tFNA-Nob in CIA rats. (a) Joint records before and after treatment. (b) He staining of joints. The thickness of synovium and the number of inflammatory cells were observed. (c) Toluidine blue staining (TB) of joints. The destruction of bone tissue and cartilage were observed. (d) Masson staining of joints. The fibrosis of joints was observed. (e) TUNEL staining and three-dimensional reconstruction analysis (green: apoptotic cells, blue: nucleus). The number of apoptotic cells and synovial thickness were observed. Scale bar: 100 μm.

and tFNA-Nob had a good recovery effect after treatment (Fig. 4c). Masson staining demonstrated that the degree of fibrosis of CIA rats' joints was seriously increased, and the bone tissue was damaged by inflammation (Fig. 4d). Then we further carried out TdT-mediated dUTP Nick-End Labeling (TUNEL) staining on the joint synovium. Compared with normal rats, CIA rats have fewer apoptotic cells, thicker synovium and increased number of synovial fibroblast, while after the three groups of drug treatment, the thickness of synovium and number of synovial fibroblast decreased. tFNAs could not promote apoptosis, and tFNA-Nob promoted the number of apoptotic cells to increase significantly, which was consistent with the drug regulation on the apoptosis behavior of two different synovial cells in the cell experiment (Fig. 4e). Previous studies have proved that inflammatory immune response can promote osteoclast formation, inhibit osteoblast formation, destroy the balance between osteoclasts and osteoblasts, and lead to bone resorption [32]. In this experiment, tFNA-Nob inhibited inflammation by promoting synovial tissue apoptosis, alleviated the immune cascade, antagonized the secretion of inflammatory factors, regulated activated osteoclasts (Figs. S5e and f in Supporting information). As a result, the destruction of bone and joint was relieved, and the reduced bone mineral density and bone volume fraction tend to be normal (Figs. S5c and d in Supporting information). This study was approved by the Ethics Committee of The West China Hospital of Stomatology, Sichuan University. tFNA-Nob efficiently transmitted the nobiletin into the synovial tissue of the diseased joint. As a carrier with tissue permeability and cell penetrating ability, tFNAs improved the bioavailability of nobiletin, nobiletin was released slowly and stably and exerted its pharmacological effects of promoting apoptosis and anti-inflammatory, induced the apoptosis of abnormally proliferating synovial cells and reduced the secretion of inflammatory proteins, thus protecting soft tissue and bone tissue in joint, reducing arthritis score and alleviating rheumatoid arthritis (Figs. S5a and b in Supporting information, Fig. 4a). Compared with the same concentration of nobiletin, tFNA-Nob can significantly reduce the course of arthritis in CIA rats.

In general, we have developed a new composite system of nucleic acids cargo tank loaded with small molecule monomer drug nobiletin, detected the appropriate proportion of tFNAs:nobiletin, and then verified that the tFNA-Nob had better ability to inhibit the proliferation and migration of synovial fibroblast than the single nobiletin with the same concentration *in vitro* by inhibiting PI3K/Akt and NF-κB signaling pathway and promoting apoptosis. *In vivo* experiments verified the therapeutic effect of the tFNA-Nob CIA rats compared with nobiletin, apoptosis of synovial tissue was increased, joint damage was relieved, and the course of rheumatoid arthritis was inhibited. This result may be related to tFNAs which can increase the bioavailability and water solubility, cell permeability and tissue permeability of nobiletin as a nano cargo tank.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cclet.2022.05.063.

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