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Bioactive Materials

Controlled TPCA-1 delivery engineers a pro-tenogenic niche to initiate tendon regeneration by targeting IKKβ/NF-κB signaling

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ABSTRACT

Tendon repair remains challenging due to its poor intrinsic healing capacity, and stem cell therapy has emerged as a promising strategy to promote tendon regeneration. Nevertheless, the inflammatory environment following acute tendon injuries disrupts stem cell differentiation, leading to unsatisfied outcomes. Our study recognized the critical role of NF-κB signaling in activating inflammation and suppressing tenogenic differentiation of stem cells after acute tendon injury via multiomics analysis. TPCA-1, a selective inhibitor of IKKβ/NF-κB signaling, efficiently restored the impaired tenogenesis of stem cells in the inflammatory environment. By developing a microsphere-incorporated hydrogel system for stem cell delivery and controlled release of TPCA-1, we successfully engineered a pro-tenogenic niche to initiate tenogenesis for tendon regeneration. Collectively, we recognize NF-κB signaling as a critical target to tailor a pro-tenogenic niche and propose the combined delivery of stem cells and TPCA-1 as a potential strategy for acute tendon injuries.

1. Introduction

Tendon, a connective tissue that is mainly composed of aligned collagenous fiber, is responsible for force transmission from muscle to bone during movement. Tendon injuries often occur in athletes and individuals when subjected to trauma, excessive mechanical loading, and repetitive musculoskeletal activities [1]. Aging, chronic inflammation, and other pathological factors like diabetes are found to increase the risk of tendon disorders, frequently resulting in partial or complete tendon rupture [2]. Tendon disorders account for up to 30 % of musculoskeletal diseases, and ~30 million tendon-related procedures were conducted each year worldwide, resulting in a great economic and societal burden [3,4]. Unfortunately, tendons exhibit a very limited intrinsic reparative capacity, and the current conservative (like corticosteroid injections and shockwave therapy) and operative (suturing, autograft/allograft/artificial graft implantation) treatments yield unsatisfied outcomes, including scar tissue formation and tendon adhesion, with a high retear rate of up to 40 % [5]. Therefore, it is urgent to

develop new intervention strategies to improve the clinical outcomes of patients with tendon injuries.

With the emergence of regenerative medicine, stem cell therapy has attracted great attention from researchers and clinicians for tendon regeneration. Stem cells possess various compelling capacities including self-renewal, multi-lineage differentiation, paracrine functions, and immunomodulatory properties [6,7]. In 2007, Bi et al. first identified and isolated tendon stem/progenitor cells (TSPCs, ~3–4% of tendon resident cells) in human tendons [8]. Emerging evidence has revealed that TSPCs can self-renew and differentiate into various cell types to regulate tendon homeostasis, making them a critical cellular candidate for tendon healing. The transplantation of exogenous stem cells is a compelling strategy to treat various tendon disorders, while normal tendons are hardly obtained from patients in clinical practice, which limits the application of human TSPCs. In contrast, the delivery of stem cells derived from other clinically available tissues like bone marrow, adipose, and periodontal ligaments could be a more available strategy to promote tendon repair [7,9–11]. Up to now, the safety and therapeutic effects of human bone marrow-derived mesenchymal stem cells (hBMSCs) and adipose-derived MSCs (hADSCs) injections in treating tendon disorders have been demonstrated in clinical trials [10]. MSCs injection was found to relieve pain and improve arthroscopic and radiological parameters, as well as functions in patients with tendon disorders [9,10]. Despite this, the therapeutic effects of MSCs injections are still unsatisfactory and even not obvious in several clinical trials [6, 12], which could be attributed to the following reasons: 1) the heterogeneity in tendon pathologies and cell sources [10,13]; 2) the low cell retention and survival *in situ* of tendon lesions after MSCs injection [14–16]; 3) the pathological differentiation of stem cells in the pro-inflammatory tendon microenvironment [7,9,10,17–19]. Due to the high similarity of tendon and ligament in structure and function, clinically-available periodontal ligament is a promising stem cell source to replace hBMSCs and hADSCs for tendon repair. Human periodontal ligament stem cells (hPDLSCs) could be isolated from periodontal ligaments of surgically extracted teeth, and exhibited the typical characteristics of MSCs like clone formation and multi-lineage differentiation [20]. Moshaverinia et al. compared the tenogenic capacity of hPDLSCs with hBMSCs, and found that hPDLSCs exhibited a higher potential for tendon regeneration, as evidenced by the higher expressions of tendon markers like scleraxis (SCX) and tenomodulin (TNMD) both *in vitro* and *in vivo* [21]. Our recent study revealed that hPDLSCs in synergy with biomimetic silk scaffold promoted *in situ* tendon regeneration in rats [11]. Specifically, the hPDLSCs seeded in silk scaffolds were found to significantly promote tendon matrix formation with increased collagen deposition and aligned fiber arrangement, as well as slightly decreased pro-inflammatory responses in injured tendons as compared to the scaffold alone. Thus, hPDLSCs could be a promising candidate to treat tendon injuries in future stem cell therapy. In addition, various delivery systems have been developed to provide a suitable environment for stem cell transport, retention, and survival *in situ* of tendon lesions [22–24]. However, the inflammatory environment of injured tendons, particularly in acute injuries is commonly ignored, which could severely hinder the functions of the transplanted stem cells and result in unsatisfied therapeutic effects.

Generally, acute injuries to the tendon trigger an immediate release of pro-inflammatory cytokines, such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), from resident immune cells like macrophages and mast cells, which creates a local inflammatory environment that attracts neutrophils and other immune cells to lesions for eliminating debris and pathogens [25]. Besides, tenocytes, the primary cell population of tendon resident cells, could be activated into an inflammatory phenotype and secret pro-inflammatory cytokines, which probably played a critical role in tendon inflammation activation and resolution [1,25]. The produced cytokines and chemokines by immune cells and tenocytes could perpetuate the pro-inflammatory niche of injured tendons, which was demonstrated to impair the functions of endogenous or

transplanted stem cells [26,27]. For instance, Zhang et al. found that IL-1β irreversibly suppressed tenogenic differentiation and induced metabolism transition in TSPCs *in vitro* [26]. Cho et al. identified a markedly up-regulated pro-inflammatory cytokines, C1q/TNF-related protein-3 (CTRP3) after acute tendon injury, which was shown to inhibit tenogenic potentials of TSPCs but promoted chondrogenic differentiation, thereby inducing heterotopic cartilage matrix formation in tendons [27]. Therefore, the manipulation of tendon inflammatory environment has been suggested to improve the therapeutic effects of stem cells for tendon regeneration. This includes the controlled administration of drugs or growth factors, and possibly combining these interventions with biomaterials as a delivery system to regulate the inflammatory cascade to favor tendon repair. However, a critical cellular and molecular process that determines tendon inflammation activation after acute injuries urgently needs to be recognized for preventative interventions and precise treatments. Recent clinical investigations have shown that nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling is involved in inflammation activation in the early- and intermediate-stages of tendinopathy [5,28]. The study of Wang et al. revealed that the activation of NF-κB signaling contributed to age-related inflammation and induced the senescence of TSPCs, and thereby resulted in degenerative rotator cuff tendinopathy [29]. In contrast, the targeted inhibition of IkB kinase β (IKKβ)/NF-κB signaling promoted inflammation resolution and improved outcomes in acute and chronic tendinopathy models [28,29]. Therefore, NF-κB signaling could be a promising target to rescue inflammation activation after acute tendon injuries and thereby tailor a suitable stem cell niche to initiate regenerative responses. However, the intrinsic mechanisms between NF-κB signaling activation and dysregulated stem cell differentiation in acute tendon injuries are unclear, and the efficiency of targeting NF-κB signaling on inducing tendon inflammation resolution and improving the therapeutic effects of stem cells has not been comprehensively investigated.

In this study, we integrated multiomics analyses to recognize the inflammation activation pathway of injured tendon with a focus on NF-κB signaling and revealed the association between tendon inflammatory microenvironments and dysregulated stem cell differentiation, as well as its underlying mechanisms after acute tendon injuries. Subsequently, we investigated the effects of TPCA-1, a small molecule that targeted IKKβ/NF-κB signaling, on rescuing the suppressed tenogenic potentials of stem cells in the inflammatory environment. Then, we developed a silk fibroin (SF) microsphere-incorporated GelMA hydrogel system to deliver hPDLSCs and TPCA-1. In this delivery system, SF microsphere was used to load TPCA-1 and control its release; GelMA hydrogel was introduced to maintain the transplanted hPDLSCs and TPCA-1-loaded SF microspheres in tendon lesion sites and provide a suitable water-abundant microenvironment for cell survival. Finally, we verified the feasibility of modulating tendon inflammatory environment by targeting IKKβ/NF-κB signaling to improve the therapeutic effects of hPDLSCs for tendon repair.

2. Materials and methods

2.1. Cell culture

All types of cells were cultured in a humidified atmosphere containing 5 % CO_2 at 37 °C. hPDLSCs were extracted from the human periodontal ligament of surgically extracted human third molars as previously reported [11]. hBMSCs from healthy adults were purchased from Cyagen Biosciences (Suzhou, China, HUXMA-01001). rTSPCs were isolated from 6-8-week-old Sprague Dawley rats according to the previously established procedure [30]. Briefly, the harvested periodontal ligaments or Achilles tendons were cut into pieces and digested in 2.5 mg/ml Type I collagenase (Biofroxx, 1940MG100) overnight at 37 °C. After centrifugation and resuspension, cells were seeded at a low density (maximum 1000 cells) in a 10 cm tissue plate (NEST Biotechnology,

704001) and cultured with low glucose DMEM (Gibco, C1185500) with supplementation of 10 % fetal bovine serum (FBS, Wisent, 086–550) and 1 % penicillin-streptomycin (P/S, Gibco, 151400122). After 10 days of culture, colonies were formed and subsequently dissociated using trypsin for passage. Cells between passages 4 and 6 were used in this study, with the culture medium changed every 2–3 days. Low-glucose DMEM with 1 % P/S and 10 % FBS was used for cell culture of all types.

To evaluate the tenogenic differentiation of stem cells, the cells were cultured in a tenogenic medium consisting of high-glucose DMEM (Gibco, C11995500BT), 50 μg/ml L-ascorbic acid 2-phosphate (Sigma, A8960), 10 % FBS, and 1 % P/S as previously reported [31].

To evaluate the effect of inflammation on the tenogenic differentiation of hPDLSCs, rTSPCs, and hBMSCs, cells were cultured in the tenogenic medium and treated with IL-1β (0, 0.1, 1, or 10 ng/ml) (GenScript, Z02922), or TNF-α (0, 0.1, 1, or 10 ng/ml) (GenScript, Z02774) for 3 and 7 days. To evaluate the effect of TPCA-1 on the inhibited tenogenic differentiation of hPDLSCs, cells were cultured in a tenogenic medium and treated with 0.1 ng/ml IL-1β or 10 ng/ml TNF-α, either alone or in combination with TPCA-1 (0, 0.1, 1, or 10 μM) (Selleck, S2824), for 3 and 7 days.

2.2. Cell proliferation assay

To evaluate the effect of IL-1β or TNF-α on the proliferation in hPDLSCs, cells were seeded at a density of 2000 cells per well into a 96-well plate and cultured in the growth medium. hPDLSCs were then treated with different concentrations (0, 0.1, 1, and 10 ng/ml) of IL-1β or TNF-α for 1, 3, 5, and 7 days. Besides, to evaluate the effect of TPCA-1 on cell proliferation, hPDLSCs also were treated with different concentrations (0, 0.1, 1, and 10 μM) of TPCA-1 for 3 days. Cell proliferation was evaluated using a CCK8-kit (APExBIO, K1018) assay. At the designated time points, the culture medium was replaced with a 10 % CCK-8 solution (v/v). After being incubated for 1 h at 37 °C, the solution was measured for absorbance using an 800 TS microplate reader (BioTek, Winooski, VT, USA) at a wavelength of 450 nm.

2.3. RNA isolation and qPCR

Total RNA was extracted from cultured cells using the RNA prep Pure Cell/Bacteria Kit (Tiangen Biotech, DP430), and reverse transcription to cDNA was performed using the ReverTra Ace® qPCR RT Master Mix (Toyobo, FSQ-201). Subsequently, qPCR was conducted following the manufacturer's protocols (SYBR® Green Premix Pro Taq HS qPCR Kit, AG11718). The sequences of primers used are summarized in Table S1. Representative results are displayed by normalizing the expression of the target gene to glyceraldehyde 3-phosphate dehydrogenase (*Gapdh/GAPDH*).

2.4. Immunofluorescent (IF) staining

The hPDLSCs were seeded at a density of 2000 cells per well in a 96-well plate and cultured in the growth medium. Cells were treated with IL-1β or TNF-α for 30 min and then stained for NF-κB antibody (Cell Signaling, 8242). Cells were washed with PBS, fixed in 4 % paraformaldehyde, permeabilized with 1 % (v/v) Triton X-100 (Beyotime, P0096), and then blocked with QuickBlock™ Blocking Buffer (Beyotime, P0260) for 20 min. Subsequently, samples were incubated with rabbit anti-NF-κB at 4 °C overnight. After being washed with PBS, cells were incubated with 488-conjugated goat anti-rabbit IgG (Proteintech, SA00013-2) for 1 h at room temperature, while nuclei were stained with DAPI (Beyotime, C1002) for 5 min. The samples were observed and imaged using fluorescence microscopy (Carl-Zeiss, Oberkochen, Germany). The ratio of nuclear and cytoplasmic fluorescence for NF-κB was determined using ImageJ software (NIH, Bethesda, MD).

2.5. Western blot (WB) analysis

The hPDLSCs were seeded at a density of 5×10^4 cells per well in the 6-well plates and cultured in the growth medium treated with IL-1β or TNF-α for 30 min. Total protein extraction and WB experiments were performed as previously described [32]. Cells were lysed in RIPA lysis buffer (Beyotime, P0013B), and total proteins were extracted [32]. Nuclear protein was extracted using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, Waltham, MA) according to the protocol of manufacturer. Protein concentrations were assessed using a BCA protein assay kit (Beyotime, P0009). The obtained proteins were separated by gel electrophoresis and then transferred onto polyvinylidene difluoride membranes (Beyotime, P2012). After blocking with QuickBlock™ Blocking Buffer (Beyotime, P02016) for 15 min, the membranes were incubated overnight at 4 °C with rabbit anti-pNF-κB (Cell Signaling, 3033), rabbit anti-NF-κB (Cell Signaling, 8242), rabbit anti-IκBα (Cell Signaling, 9242), mouse anti-β-actin (Cell Signaling, 4967), and rabbit anti-PCNA (Proteintech, Biotin-60097). After being washed twice with Tris Buffered Saline with Tween (TBST), the membranes were incubated with HRP (Horseradish peroxidase) labeled Goat Anti-Rabbit IgG (H + L) (Cell Signaling, 7074) or HRP-labeled Goat Anti-Mouse IgG (H + L) (Cell Signaling, 7076) for 1 h at room temperature. Finally, the chemiluminescence system was used for detection and imaging. The densitometry value of the bands was determined using ImageJ software (NIH, Bethesda, MD), and the relative protein expression was calculated.

2.6. Global proteomic analysis

The hPDLSCs were cultured in the tenogenic medium treated with 0.1 ng/ml IL-1β or 10 ng/ml TNF-α for 7 days. Total proteins were isolated from the collected cell samples. Subsequently, the LFQ-based proteomic analysis was conducted for the extracted proteins in each group as established procedures [33]. Differential expression analysis was performed by comparing the expression levels of the identified proteins in the hPDLSCs treated with IL-1β or TNF-α with those in the Ctrl group. The proteins with a P value < 0.05 were recognized as DEPs for Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses. The protein interaction network of differentially expressed proteins (DEPs) was obtained from the STRING, Cytoscape, and CytoHubba. BinGO analysis was conducted in the Cytoscape using the BinGO plugin. Other detailed analyses were described in *2.7 Bioinformatics analysis*. All proteomics data has been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the iProX partner repository (PXD056585).

2.7. Bioinformatics analysis

The RNA-seq or Microarray data for mice tendons (GSE181173) [27], rat tenocytes (GSE253913), and human tendons (GSE26051) [34] were obtained from the GEO database. In the RNA-seq data of mice tendons and rat tenocytes, the genes with P value < 0.05 and fold change >2 were used for GO and KEGG enrichment analyses. In the Microarray data of human tendons, the genes with P value < 0.05 and fold change >1.2 were used for GO and KEGG enrichment analyses. The GO and KEGG enrichment analyses were performed on the DAVID website. The network of enriched KEGG pathways was visualized in the Cytoscape using the GlueGO plugin. Other bioinformatic analyses like GSEA and Heatmap with cluster analysis were conducted on the OmicStudio Tools (https://www.omicstudio.cn/). The gene lists of "GO:0002367: cytokine production involving in immune response" and "GO:0007249: canonical NF-κB signal transduction" were obtained from the RGD and MGI databases. The gene list of teno-lineage differentiation was identified in a previous study [35].

2.8. SF microsphere preparation and TPCA-1 loading

SF microspheres were prepared using established methods [36]. Briefly, raw silk fibers were boiled in a 0.02 M Na_2CO_3 solution for 30 min and then washed with deionized water. The obtained fibroin was dried overnight at 37 °C in an oven and then dissolved in a lithium bromide solution. Then, the solution was transferred to a dialysis bag and dialyzed against deionized water for 2 days. Finally, the solution was centrifuged twice at 9000 rpm at 4 °C for 20 min, and then the supernatant was collected.

The 5 % (w/v) SF/polyvinyl alcohol (PVA) solution was prepared by mixing 5 % (w/v) SF and 5 % (w/v) PVA at a volume ratio of 1:4 for SF microsphere preparation. Subsequently, 14.87 μM TPCA-1 or 1 μg/ml RhoB (Macklin, Shanghai, China) was incorporated into the solution. According to our previous findings, 5 % SF microspheres had a loading efficiency of 67.27 % [32], and thus 14.87 μM TPCA-1 was encapsulated into SF microspheres. Then, after the mixed solution was sonicated, dried in the oven, and re-dissolved in water, SF microspheres were formed. The microspheres were collected through centrifugation and subsequently re-suspended in deionized water in an equivalent volume to SF to obtain microspheres suspension. The images of SF@RhoB microspheres were obtained using a light microscope and fluorescence microscopy. After being dried and sputter-coated with gold, the surface structure of SF microspheres was observed using a Zeiss EVO 18 SEM (Carl-Zeiss, Oberkochen, Germany).

2.9. Preparation of GelMA hydrogel delivery system

Pure 5 % (w/v) GelMA prepolymer solution was prepared by dissolving the freeze-dried GelMA (Jurassic, Haining, China) and 0.5 % lithium phenyl-2,4,6-trimethylbenzoylphosphinate in PBS. Then, the GelMA solution and SF@TPCA solution were prepared for the GelMA-SF hydrogel blend solution at a volume ratio of 3:1, respectively. Subsequently, the mixed solutions were crosslinked after UV irradiation (Wave Length: 365–370 nm) for 30 s. For the hPDLSCs-loaded GelMA-SF hydrogel, hPDLSCs were incorporated into GelMA precursor solution and subsequently mixed with SF or SF@TPCA microsphere solution at a volume ratio of 3:1 to fabricate the hPDLSCs-loaded GelMA-SF or GelMA-SF@TPCA hydrogel.

2.10. In vitro degradation

The wet weight of the hydrogels (M0) was determined at the beginning of the assay. Subsequently, scaffolds were incubated in PBS at 37 °C with continuous shaking. At the designated time-points, the wet weight of the scaffolds (M1) was measured. The remaining weight was calculated using the following equation:

Weight remaining (%) = M1/M0*100%

2.11. Controlled release profile

The hydrogels (300 μl in volume, n = 6 per group) were incubated in 3 ml PBS and placed them in a shaker with 100 rpm at 37 °C. At each designated time point, 300 μl supernatant was collected for examination, while 300 μl fresh PBS was simultaneously replenished. The release of RhoB over time was quantified using a Synergy H1 microplate reader (BioTek, Winooski, VT, USA) with excitation/emission wavelengths set at 550/620 nm.

2.12. Animal experiment

The study protocol was approved by the Animal Experimental Ethical Inspection Committee of Southeast University (approval no. 20210915091, 2024060100). In the rat patellar tendon window injury model, male SD rats (n = 3–8 rats per group) were used to create a defect of 4 mm × 1 mm (length × width) in the middle of the right patellar tendon under general anesthesia. The left leg of each rat served as the Ctrl group. After surgery, the incision was closed and the rats were allowed unrestricted cage activity. Rats were sacrificed for histological staining and evaluation at 1 week postoperatively. The rat patellar tendon window model was utilized and divided into two groups (n = 29 rats per group): hPDLSCs-loaded GelMA-SF (Ctrl group) and hPDLSCs-loaded GelMA-SF@TPCA (TPCA-1 group). For each defect, 20 μL of hydrogel precursor loaded with 5×10^5 hPDLSCs were injected into the wound site and then crosslinked using UV irradiation for 30 s. The skin was subsequently closed, and the rats were allowed for free cage activity after surgery. All the procedures were performed under sterile conditions. Specimens were harvested on 3 day and at 1, 2, and 4 weeks postoperation for evaluations. In the rat transected Achilles tendon model, the right Achilles tendons of male SD rats (n = 3 rats per group) were transversally transected under general anesthesia. After surgery, the rats were allowed unrestricted cage activity and sacrificed for histological staining at 4 weeks postoperatively.

2.13. Histology and immunohistochemistry

For the histological assessment, samples were collected and fixed in 4 % paraformaldehyde. Then, the samples were embedded and sectioned. Hematoxylin and eosin (H&E), Masson trichrome (MT), Safranin O-Fast Green staining (SO/FG), alcian blue, immunohistochemical (IHC), and IF staining were performed according to standard procedures. The repair effect of injured tendons was quantified by conducting histological scoring based on six parameters observed in H&E staining images, including fiber structure, fiber arrangement, nuclear roundness, vascularity, inflammation, and cell quantity as previously described [31]. The collagen density in the MT staining was calculated using ImageJ software (NIH, Bethesda, MD) for semi-quantitative analysis. IHC or IF staining was performed using anti-NF-κB polyclonal antibody (Proteintech, 10745-1-AP), anti-IL-1β polyclonal antibody (Servicebio, GB11113), anti-TNF-α polyclonal antibody (Servicebio, GB13452), anti-SOX9 monoclonal antibody (Proteintech, 67439-1-Ig), anti-OCN polyclonal antibody (Proteintech, 23418-1-AP), anti-iNOS polyclonal antibody (Affinity, AF0199), anti-CD68 monoclonal antibody (Proteintech, 66231-2-Ig), anti-CD206 polyclonal antibody (Proteintech, 18704-1-AP), anti-human nuclei monoclonal antibody (Sigma-Aldrich, MAB1281), anti-SCX polyclonal antibody (Abcam, ab58655), anti-TNMD polyclonal antibody (Abcam, ab203676), anti-MKX polyclonal antibody (Affinity, DF2430). To quantify the results, representative images were analyzed by ImageJ software (NIH, Bethesda, MD).

2.14. Gait analysis

Gait analysis was preformed to evaluate the motor functionality of repaired tendons (n = 5 rats per group) in rats at 2 weeks using the CatWalk XT system (Noldus, Netherlands). After adjusting to the environment, the rats were subjected to consecutive and compliant runs. A detailed introduction to the CatWalk XT system has been described in the previous study [37]. Various intensity parameters, including max contact max intensity, max contact mean intensity, max intensity, and mean intensity were analyzed.

2.15. Mechanical test

Mechanical test was conducted to investigate the mechanical strength of repaired patellar tendons in rats using an electric universal testing machine (UTM2502, Sunstest, Shenzhen, China). The machine was equipped with a 500-N sensor and the tendons were stretched at an elongation rate of 5 mm/min. The tensile modulus of repaired tendons at 4 weeks was determined by measuring the slope of strain-stress curves in the linear region. The max stress and max force were recorded at the

point of rupture.

2.16. Statistical analysis

All data are presented as means ± SEM. Cell experiments were repeated in cells from at least two individual donors and the results are representative of the repeated experiments. All statistical analyses were conducted using GraphPad Prism 9 software. Shapiro-Wilk test was employed to test the normality of quantitative data. The Student's t-test (for normal data) and Mann-Whitney test (for non-normal data) were used for the comparisons between two groups, and the ANOVA (for normal data) and Kruskal-Wallis (for non-normal data) test were employed for the comparisons among three groups, as indicated in the figure legends. P value of less than 0.05 was recognized as significant. Statistically significant differences were shown as *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

3. Results and discussions

3.1. Dysregulated cell differentiation occurs in an inflammatory niche of injured tendons with the activation of NF-κB signaling

To globally uncover the changed cellular processes and pathways after acute injures, we first analyzed the transcriptional profiling of mouse injured Achilles tendons (GSE181173) [27]. The Achilles tendon was partially transected (Injured group) in eight-week-old C57BL/6J male mice, and a sham surgery was conducted as the control (Ctrl group). All tendon samples were collected at 3 weeks after surgery for RNA sequence (RNA-seq). GO enrichment analysis was performed for the up-regulated differentially expressed genes (DEGs, $P < 0.05$, fold change >2) to discern the altered biological processes (BP) after tendon injuries. Notably, several BP terms associated with pathological cell differentiation were enriched to "skeletal system development", "cartilage development", "ossification", and "chondrocyte differentiation", indicating chondrogenesis and osteogenesis were enhanced in injured tendons (Fig. 1A). Besides, other tendinopathy-related cellular processes related to angiogenesis and extracellular matrix alteration including "extracellular matrix organization", "collagen fibril organization", "angiogenesis", and "collagen catabolic process" were observed (Fig. 1A). Among these changes, the dysregulated cell differentiation was one of the most important cellular responses in tendons after acute injury. Subsequently, gene set enrichment analysis (GSEA) was conducted on all identified genes to provide further clarification. The results indicated that the pathological cell differentiation towards cartilage and bone lineages was significantly promoted (Fig. 1B). To verify it, the chondrogenesis and osteogenesis of injured tendons were investigated in rat transected Achilles tendon model (Fig. S1) and rat patellar tendon injury model (Fig. 1C and D). SO/FG and alcian blue staining showed that chondrocyte-like cells and hyaline cartilage-like tissues were formed in the repaired Achilles tendon at 4 weeks after injury (Fig. S1). Consistently, we observed the formation of cartilage matrix and a great number of SOX9-and OCN-positive cells in the region of injured patellar tendons at 4 weeks after injury (Fig. 1C and D). These findings together revealed that the enhanced chondrogenic and osteogenic potentials in the early stage of acute tendon injury probably resulted in heterotopic chondrogenesis and ossification/calcification if not been properly controlled. For instance, Lui et al. investigated ectopic chondro-ossification in a tendon window injury model and found that 33 % of injured tendon samples showed ectopic chondrogenesis at 4 weeks and ectopic ossification with chondrocyte-like cells was observed in 50 % of samples at 12 weeks [38]. Lin et al. discovered that Achilles tenotomy induced cartilaginous matrix deposition in injured tendons within 3 weeks, which was accompanied by the up-regulated expressions of cartilage and bone-related genes [39]. The study of Asai et al. further investigated the critical tendon cell population that initiated ectopic chondrogenesis and osteogenesis in a model of transected

Achilles tendons [40]. They recognized that the recruited tendon progenitor cells in injured sites of tendons displayed stronger chondrogenic and osteogenic potentials while having a substantially decreased expression of tendon markers like SCX as compared to the cells isolated from normal tendons. The subcutaneous transplantation of tendon progenitor cells isolated from injured Achilles tendons (at 1 week after injury) induced cartilage-like cell formation and cartilage matrix deposition including COL2 and COL10 within 10 days. Taken together, these findings highlighted that the chondrogenic and osteogenic of stem cells could be initiated in injured tendons within 3 weeks after acute tendon injury, while its underlying mechanisms were still unclear.

Inflammation begins immediately after tendon injury, and a pro-inflammatory niche was found to impair tenogenic potential but enhance chondrogenic or osteogenic differentiation of tendon stem cells [26,27,41,42]. Thus, we subsequently investigated inflammation activation pathways in injured tendons with a focus on NF-κB signaling, which was reported to play a critical role in the progress of chronic and aging-related tendinopathy [28,29]. GSEA revealed that inflammatory responses, particularly acute inflammatory responses, were significantly activated in injured tendons as compared to the Ctrl (Fig. S2). A list of 248 genes annotated in "GO:0007249: canonical NF-κB signal transduction" was obtained from Mouse Genome Informatics (MGI) database (https://www.informatics.jax.org/), and we identified that 132 of 248 NF-κB cascade-related genes were significantly changed ($P < 0.05$), with 73 up-regulated and 59 down-regulated (Fig. 1E). Furthermore, the genes encoding NF-κB complex subunits (*Nfkb1*: 1.41-fold increase, $P < 0.001$; *Nfkb2*: 1.41-fold increase, $P = 0.002$; *Rel*: 1.29-fold increase, $P = 0.031$; *Rela*: 1.12-fold increase, $P = 0.026$; *Relb*: 1.19-fold increase, $P = 0.011$) and regulatory NF-κB proteins (*Bcl3*: 1.52-fold increase, $P = 0.031$; *Bcl10*: 1.27-fold increase, $P < 0.001$; *Ikbke*: 2.48 fold-increase, $P < 0.001$; *Nfkbie*: 2.05-fold increase, $P < 0.001$) were significantly up-regulated in Injured *vs.* Ctrl (Fig. 1F and G), indicating the activation of NF-κB signaling in injured tendons. Besides, GSEA revealed that regulation of NIK NF-κB signaling, a non-canonical NF-κB signaling that regulated immunity and inflammation [43], was also significantly enhanced (Fig. 1H). IHC staining for NF-κB was conducted to evaluate the activation of NF-κB signaling after acute tendon injury. The results revealed that the ratio of NF-κB-positive cells in injured tendons significantly increased on day 3, peaked at 1 week, and gradually decreased until 4 weeks after tendon injury, as compared to the control tendons (Fig. 1I), which confirmed the activation of NF-κB signaling in the early stage of acute tendon injury. It was reported that NF-κB signaling activation drove the secretion of pro-inflammatory cytokines such as IL-1β and TNF-α and thereby impaired the tenogenesis of endogenous TSPCs and disrupted tendon matrix homeostasis [4,7,44]. Consistently, the results revealed that injured tendons exhibited the significantly increased expression of genes encoding IL-1 and TNF superfamily cytokines, which could contribute to the pro-inflammatory environment of injured tendons (Fig. 1J, S3A, and S4). Then, we investigated the genes associated with "GO:0002367: cytokine production involving in immune response" and identified 77 dysregulated cytokines, with 53 up-regulated and 24 down-regulated (Fig. S3B). Among these cytokines, the expression of *Il1b* was substantially increased (Fig. S3B), and probably played a critical role in inflammation activation and the dysregulated differentiation of TSPCs as previously reported [4, 26]. KEGG enrichment analysis for the up-regulated DEGs ($P < 0.05$, fold change >2) and GSEA revealed that the infiltration and functional changes of various immune cells like neutrophils, macrophages, monocytes, and dendritic cells could be initiated (Fig. S5), which could partially result in the productions of chemokines and pro-inflammatory cytokines. To verify these results, we created an acute tendon injury with a defect of 4 mm × 1 mm for 1 week in rats. H&E staining showed that numerous inflammatory cells with round nuclei accumulated (as indicated by black arrows) in the tendon lesion sites with disorganized matrix formation (Fig. 1K). IHC staining revealed that injured tendons had a significantly increased IL-1β and TNF-α production compared to
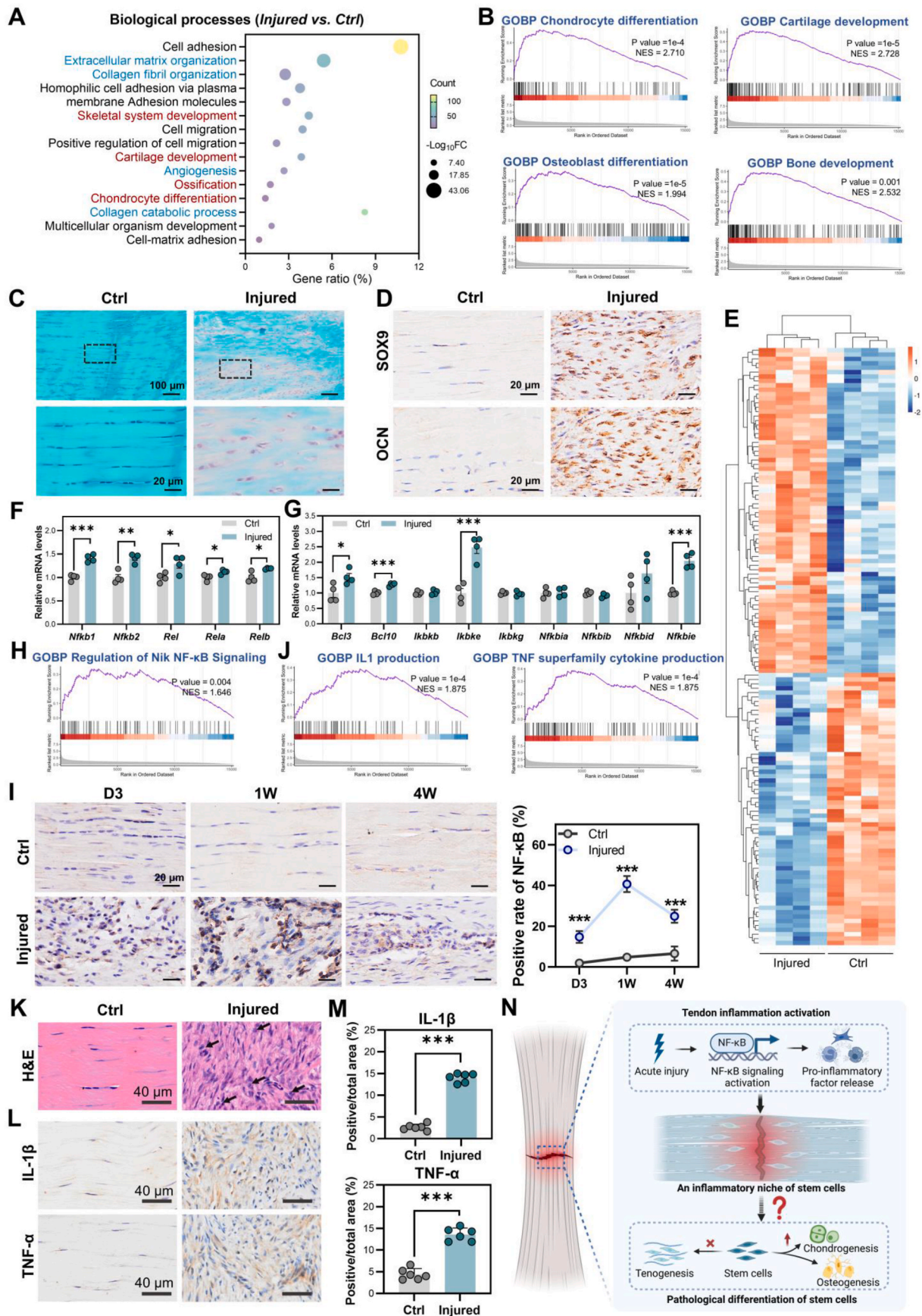
(caption on next page)

Fig. 1. **Dysregulated stem cell differentiation is associated with NF-κB signaling and pro-inflammatory cytokine secretions in injured tendons.** (A) Top GO terms of BP enriched from the up-regulated DEGs (Injured *vs.* Ctrl, fold change >2, P < 0.05) in mouse tendons from RNA-seq data (n = 4 biological replicates). The top 15 terms were screened and presented according to the smaller P value. (B) GSEA plots associated with chondrogenesis and osteogenesis of stem cells in mouse tendons (Injured *vs.* Ctrl). (C) Representative images of SO/FG staining in the Ctrl and injured patellar tendons at 4 weeks after injury. Low magnification: scale bars = 100 μm; high magnification: scale bars = 20 μm. (D) Representative images of IHC staining for SOX9 and OCN in the Ctrl and injured patellar tendons at 4 weeks after injury. Scale bars = 20 μm. (E) Heatmap of DEGs associated with NF-κB signaling in mouse tendons. (F–G) Expressions of NF-κB complex protein-encoding genes (F) and regulatory NF-κB protein-encoding genes (G) in the Ctrl and injured tendons in mice from RNA-seq data (n = 4 biological replicates). (H) GSEA plots associated with NF-κB signaling in mouse tendons (Injured *vs.* Ctrl). (I) Representative images and quantitative analysis of IHC staining for NF-κB in the Ctrl and injured tendons on different time points. (J) GSEA plots associated with IL-1 and TNF superfamily cytokine production in mouse tendons (Injured *vs.* Ctrl). (K) H&E staining of the Ctrl and injured tendons in rats at 1 week post-surgery. Black arrows indicated the cells with the characteristics of inflammatory cells. Scale bars = 40 μm. (L) Representative images of IHC staining for IL-1β and TNF-α of the Ctrl and injured tendons in rats. Scale bars = 40 μm. (M) Quantitative analysis of IHC staining for IL-1β and TNF-α. (N) Illustration of dysregulated stem cell differentiation in tendon inflammatory niche with the activation of NF-κB signaling after acute injury. The figure was generated with BioRender (https://biorender.com/). Results were shown as means ± SEM. n = 6 randomly-selected microscopic images for quantitative analysis for Fig. 1I and M. P values were determined by the Student's t-test (D, E, K). *p < 0.05, **p < 0.01, ***p < 0.001.

normal tendons (Fig. 1L-M), which was consistent with RNA-seq data (Fig. 1J). In general, these results highly suggested that NF-κB signaling was involved in the activation of tendon inflammation, which probably induced a pro-inflammatory environment that promoted the pathological differentiation of stem cells (Fig. 1N).

In addition, we also investigated the transcriptional profiling of human tendons with chronic tendinopathy (GSE26051) [34]. The grossly normal appearing tendons near the lesions were collected as the control tendons. GO enrichment analysis for the up-regulated DEGs (P < 0.05, fold change >1.2) revealed that osteoblast differentiation and ossification were enhanced in the tendinopathic tendons when compared to the Ctrl (Fig. S6A). Meanwhile, it was observed that most of the teno-lineage differentiation genes such as *MKX* (0.49-fold decrease, P = 0.037) and *TNMD* (0.33-fold decrease, P = 0.066) were down-regulated in the diseased tendons as compared to the Ctrl (Fig. S6B), indicating the tenogenic potentials of endogenous stem cells was possibly inhibited. Similarly, several genes associated with the NF-κB cascade including *NFKB1* (1.19-fold increase, P = 0.068) and *IKBIP* (1.68-fold increase, P = 0.022) (Figs. S6C–D) were enhanced. In general, the activation of NF-κB signaling in chronic tendinopathy was much weaker than that in acute tendon injury, which could partially be attributed to biopsy in near sites for two groups and the usage of corticosteroids to inhibit inflammation as described in this study [34]. Consistently, previous clinical studies have revealed that NF-κB signaling is involved in the activation of human tendon inflammation in the early- and intermediate-stages of tendinopathy but not in the advanced stage of tendinopathy [5,28]. These results suggested that NF-κB signaling participated in tendon inflammation activation after acute injuries, and targeting NF-κB signaling could be a promising intervention strategy to rescue the pro-inflammatory environment of acutely injured tendons and tailor a pro-tenogenic niche for stem cells to enhance tendon repair.

3.2. Inflammation activates tenocytes towards a pro-inflammatory phenotype that aggravates tendon niche by activating NF-κB signaling

Tenocytes, the primary cells of tendon tissue, play a pivotal role in the initiation and regulation of inflammation following acute tendon injuries. Upon injury, tenocytes respond by expressing cell-surface immune receptors that recognize damage-associated molecular patterns or pathogen-associated molecular patterns, leading to the production of cytokines and chemokines such as IL-1β, IL-6, and IL-8 [4]. These mediators orchestrate the recruitment of various immune cells, like neutrophils, macrophages, and T cells, which contribute to the removal of debris and pathogens. Besides, it was reported that tenocytes in response to the repeated inflammatory stimulus were activated into a pro-inflammatory phenotype and secreted more pro-inflammatory cytokines, which exacerbated tendon microenvironment and induced chronic inflammation if not properly resolved [4]. To better elucidate the cellular responses and pathways of tenocytes in the acute inflammatory environment, we investigated the transcriptome dataset of rat

tenocytes treated with IL-1β for 24 h (GSE253913). Various cellular processes of inflammation activation were significantly enriched from up-regulated DEGs (IL-1β *vs.* Ctrl, P < 0.05, fold change >2), including "inflammatory response", "cellular response to IL-1", "cytokine-mediated signaling pathway", "neutrophil chemotaxis", "cellular response to TNF", "positive regulation of IL-1β production", "positive regulation of TNF production", "chemokine-mediated signaling pathway", and "cellular response to IFN-γ", confirming the pro-inflammatory phenotype of tenocytes after IL-1β stimulation (Fig. 2A). KEGG enrichment analysis further revealed that multiple signaling pathways like "cytokine-cytokine receptor interaction", "TNF signaling pathway", "IL-17 signaling pathway", and "NF-κB signaling pathway" were activated (Fig. 2B). We further investigated the expression of NF-κB-associated genes from RNA-seq data. The transcripts of several NF-κB complex subunits (*Nfkb1*: 3.47-fold increase, P < 0.001; 1; *Nfkb2*: 2.72-fold increase, P < 0.001; *Relb*: 4.95-fold increase, P < 0.001) and regulatory NF-κB proteins (*Bcl3*: 42.04-fold increase, P < 0.001; *Ikbke*: 2.46-fold increase, P < 0.001; *Nfkbia*: 14.50-fold increase, P < 0.001; *Nfkbib*: 2.24-fold increase, P < 0.001; *Nfkbid*: 14.28-fold increase, P = 0.012; *Nfkbie*: 3.63-fold increase, P < 0.001; *Nfkbiz*: 36.93-fold increase, P < 0.001) were significantly up-regulated (Fig. 2C). Both TNF and IL-17 signaling pathway were upstream signaling pathway of NF-κB signaling cascade [45,46], highlighting the critical role of NF-κB signaling in inflammation activation in tenocytes. With the activation of NF-κB signaling in tenocytes, the expression of most tendon-related genes including tendon markers, *Scx* (0.44-fold decrease, P = 0.002) and *Eln* (0.14-fold decrease, P < 0.001), was significantly suppressed (Fig. 2D). In addition, we examined the expression of genes associated with "GO:0002367: cytokine production involving in immune response" from Rat Genome Database (RGD) database (https://ngdc.cncb.ac.cn/databasecommons/database/id/102) and found that 27 genes were significantly dysregulated, with 22 up-regulated and 5 down-regulated (Fig. 2E). Among these genes, the transcripts of pro-inflammatory factors (*Il1b*, *Il6*, and *Wnt5a*) and chemokines (*Ccl20*) was increased while those of anti-inflammatory regulators (*Gata3*, *Tgfb2*, and *Tgfb3*) were decreased (Fig. 2E). Taken together, in response to the pro-inflammatory stimulus, tenocytes could be activated into a pro-inflammatory phenotype with the activation of NF-κB signaling. The activated tenocytes secreted various pro-inflammatory cytokines and chemokines to recruit and activate innate and adaptive immune cells [47,48], which possibly exacerbated the local inflammatory microenvironment of endogenous and transplanted stem cells to inhibit tendon repair.

3.3. The inflammatory microenvironment inhibits the tenogenesis of stem cells and promotes collagen matrix degradation with the involvement of NF-κB signaling

As RNA-seq data suggested that tendon inflammation was activated after acute injuries and thereby impaired the tenogenic potentials of endogenous stem cells (Fig. 1N), we subsequently clarified the effects of the inflammatory environment on the tenogenesis of stem cells in
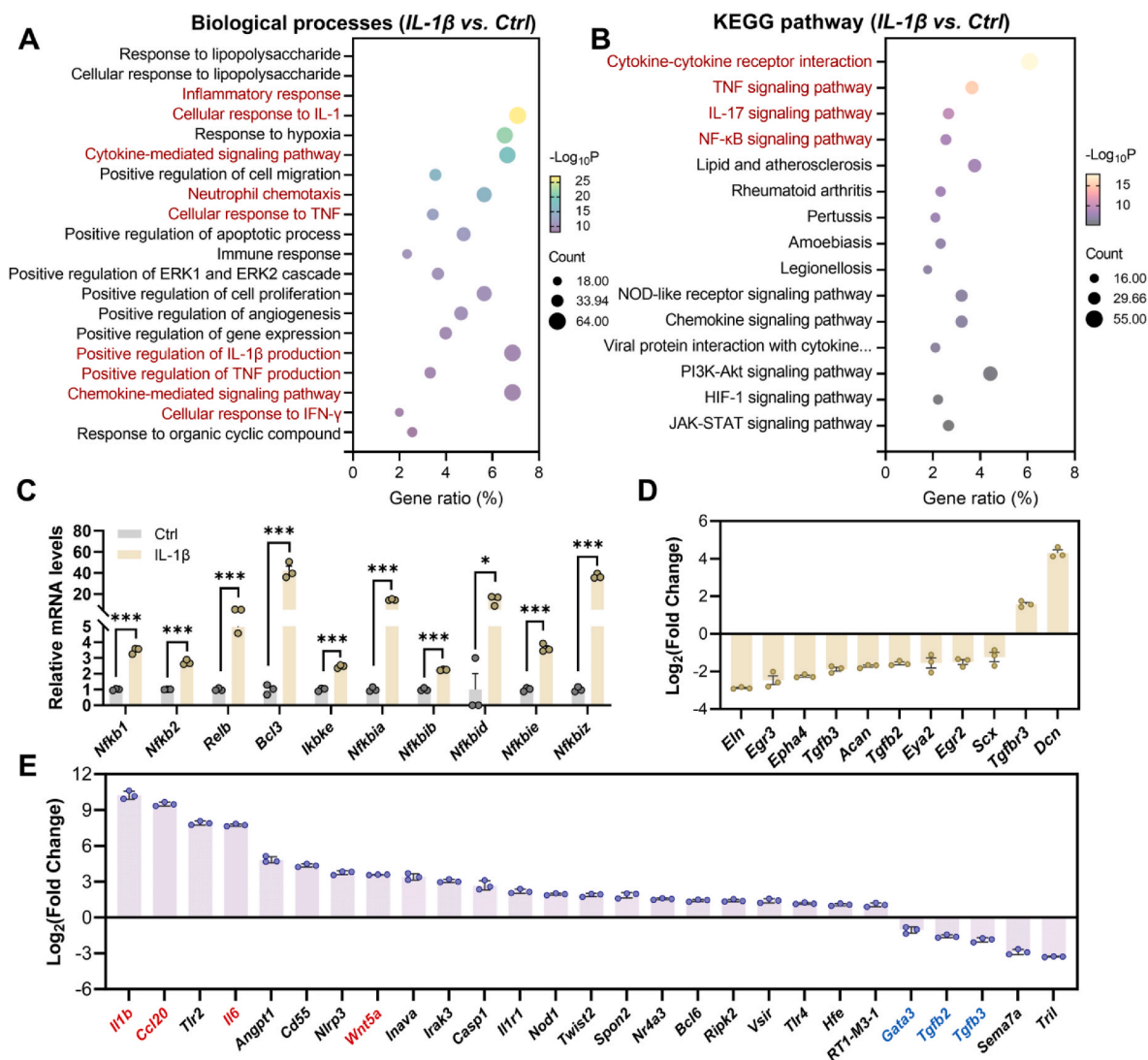
Fig. 2. Tenocytes are activated towards a pro-inflammatory phenotype after an inflammatory stimulus. (A) Top GO terms of BP enriched from the up-regulated DEGs (IL-1β *vs.* Ctrl, fold change >2, P < 0.05) in rat tenocytes from RNA-seq data (n = 3 biological replicates). The top 20 terms were screened and presented according to the smaller P value. The tenocytes were treated with or without 50 ng/ml IL-1β for 24 h respectively. (B) Top KEGG pathways enriched from the up-regulated DEGs (IL-1β *vs.* Ctrl, fold change >2, P < 0.05) in rat tenocytes from RNA-seq data. The top 15 terms were screened and presented according to the smaller P value. (C) Expressions of NF-κB complex protein-encoding genes and regulatory NF-κB protein-encoding genes in rat tenocytes of the Ctrl and IL-1β groups (n = 3 biological replicates). (D) Expression of DEGs (IL-1β *vs.* Ctrl) of tendon lineage (n = 3 biological replicates). (E) Expression of DEGs (IL-1β *vs.* Ctrl) encoding cytokines involved in immune responses (n = 3 biological replicates). The fold change was determined by calculating the ratio of each expression value in the IL-1β group to the average value of the Ctrl group (D and E). Results were shown as means ± SEM. P values were determined by the Student's t-test (C–E). *p < 0.05, ***p < 0.001.

different types, including hPDLSCs (the candidate stem cells for tendon repair), rTSPCs (the tendon-derived stem cells), and hBMSCs (the most commonly-used stem cells for tendon repair in clinical settings) (Fig. 3A), which had important implications for future stem cell therapy. IL-1β and TNF-α, two critical pro-inflammatory cytokines that were significantly up-regulated after tendon injuries and involved in inflammation activation (Fig. 1J-M) [4], were used to simulate tendon inflammatory microenvironment by using different doses. After IL-1β treatment for 3 and 7 days, the gene expressions of tenogenic markers (*SCX* and *TNMD*), tendon matrix (*COL1* and *COL14*), and matrix metalloproteinases (MMPs, *MMP1* and *MMP3*) in hPDLSCs were examined by qPCR. Notably, the transcriptional levels of *SCX*, *TNMD*, *COL1*, and *COL14* were significantly decreased while *MMP1* and *MMP3* expressions were substantially elevated in hPDLSCs with IL-1β stimulus on both days 3 and 7, regardless of the treatment concentrations (Fig. 3B–E). In addition, the lowest concentration of IL-1β (0.1 ng/ml) was sufficient to

effectively inhibit tenogenic differentiation and promote tendon extracellular matrix (ECM) degradation in hPDLSCs, as indicated by the down-regulated tendon markers (*SCX*: 0.44-fold decrease, P < 0.001; *TNMD*: 0.19-fold decrease, P = 0.093; *COL1*: 0.16-fold decrease, P < 0.001; *COL14*: 0.02-fold decrease, P < 0.001) and up-regulated MMPs (*MMP1*: 4.93-fold increase, P = 0.015; *MMP3*: 59.78-fold increase, P < 0.001) within 3 days (Fig. 3B–C). In contrast, all the concentrations of TNF-α could not change the gene expression of tendon markers and MMPs in hPDLSCs on day 3 (Fig. 3F and G), and 10 ng/ml TNF-α significantly inhibited the expressions of *SCX* (0.54-fold decrease, P = 0.033), *TNMD* (0.21-fold decrease, P = 0.035), *COL1* (0.56-fold decrease, P < 0.001), and *COL14* (0.52-fold decrease, P = 0.003), as well as increased *MMP1* (1.78-fold increase, P = 0.027) and *MMP3* (2.21-fold increase, P < 0.001) expressions on day 7 (Fig. 3H and I). Collectively, these results demonstrated that the suppressed tenogenic differentiation and the enhanced tendon matrix catabolism were induced by IL-1β and
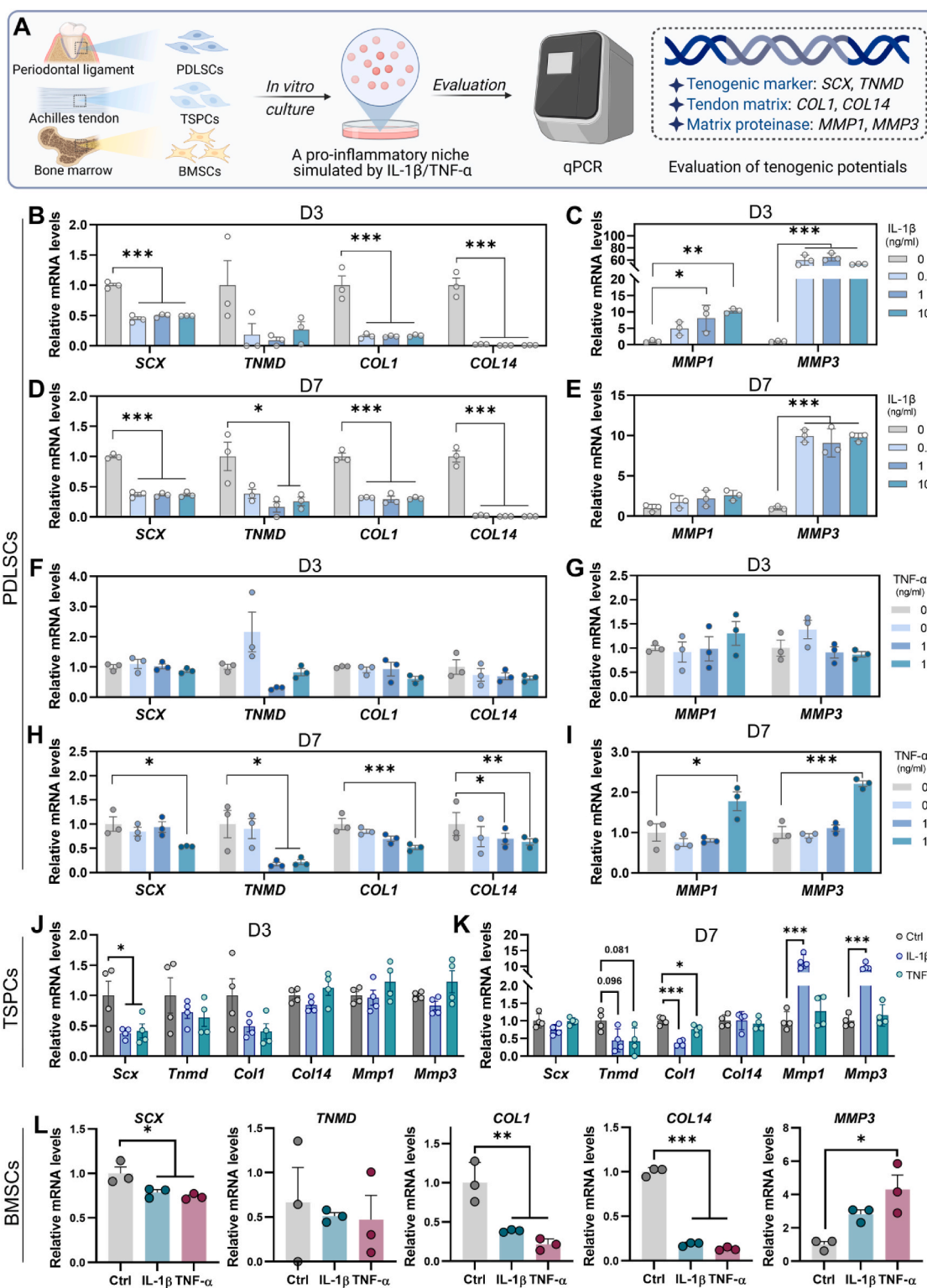
Fig. 3. The tenogenesis of stem cells is inhibited in the inflammatory environment simulated by IL-1β or TNF-α. (A) Schematic illustration of experiment procedures. The figure was generated with BioRender (https://biorender.com/). (B–C) Gene expression of tendon markers (B) and MMPs (C) in hPDLSCs treated with IL-1β for 3 days by qPCR (n = 3 experimental units). Each experimental unit represents an independent culture well. (D–E) Gene expression of tendon markers (D) and MMPs (E) in hPDLSCs treated with IL-1β for 7 days by qPCR (n = 3 experimental units). (F–G) Gene expression of tendon markers (F) and MMPs (G) in hPDLSCs treated with TNF-α for 3 days by qPCR (n = 3 experimental units). (H–I) Gene expression of tendon markers (H) and MMPs (I) in hPDLSCs treated with TNF-α for 7 days by qPCR (n = 3 experimental units). (J–K) Gene expression of tendon markers and MMPs in rTSPCs treated with IL-1β or TNF-α for 3 (J) or 7 (K) days (n = 4 experimental units) by qPCR. (L) Gene expression of tendon markers and MMPs (B) in hBMSCs treated with IL-1β or TNF-α for 7 days (n = 3 experimental units) by qPCR. Results were shown as means ± SEM. P values were determined by the ANOVA (B-K, L except for *MMP3*) and Kruskal-Waillis test (L for *MMP3*). *p < 0.05, **p < 0.01, ***p < 0.001.

TNF-α in hPDLSCs, and the effects of IL-1β was more notable than TNF-α.

Similarly, we found that 0.1 ng/ml IL-1β and 10 ng/ml TNF-α induced a significantly decreased *Scx* expression (an early tenogenic marker; 0.38-fold decease in IL-1β *vs.* Ctrl, P = 0.033; 0.41-fold decrease in TNF-α *vs.* Ctrl, P = 0.043) on day 3, and inhibited gene expressions of terminal tenogenic marker, *Tnmd* (0.44-fold decease in IL-1β *vs.* Ctrl, P

= 0.096; 0.42-fold decrease in TNF-α *vs.* Ctrl, P = 0.081) and tendon matrix, *Col1* (0.37-fold decase in IL-1β *vs.* Ctrl, P < 0.001; 0.76-fold decrease in TNF-α *vs.* Ctrl, P = 0.021) in rTSPCs on day 7 (Fig. 3J and K). The transcriptional expressions of *Mmp1* (11.46-fold increase, P < 0.001) and *Mmp3* (9.84-fold increase, P < 0.001) were substantially elevated in rTSPCs after IL-1β treatment for 7 days (Fig. 3K). In addition
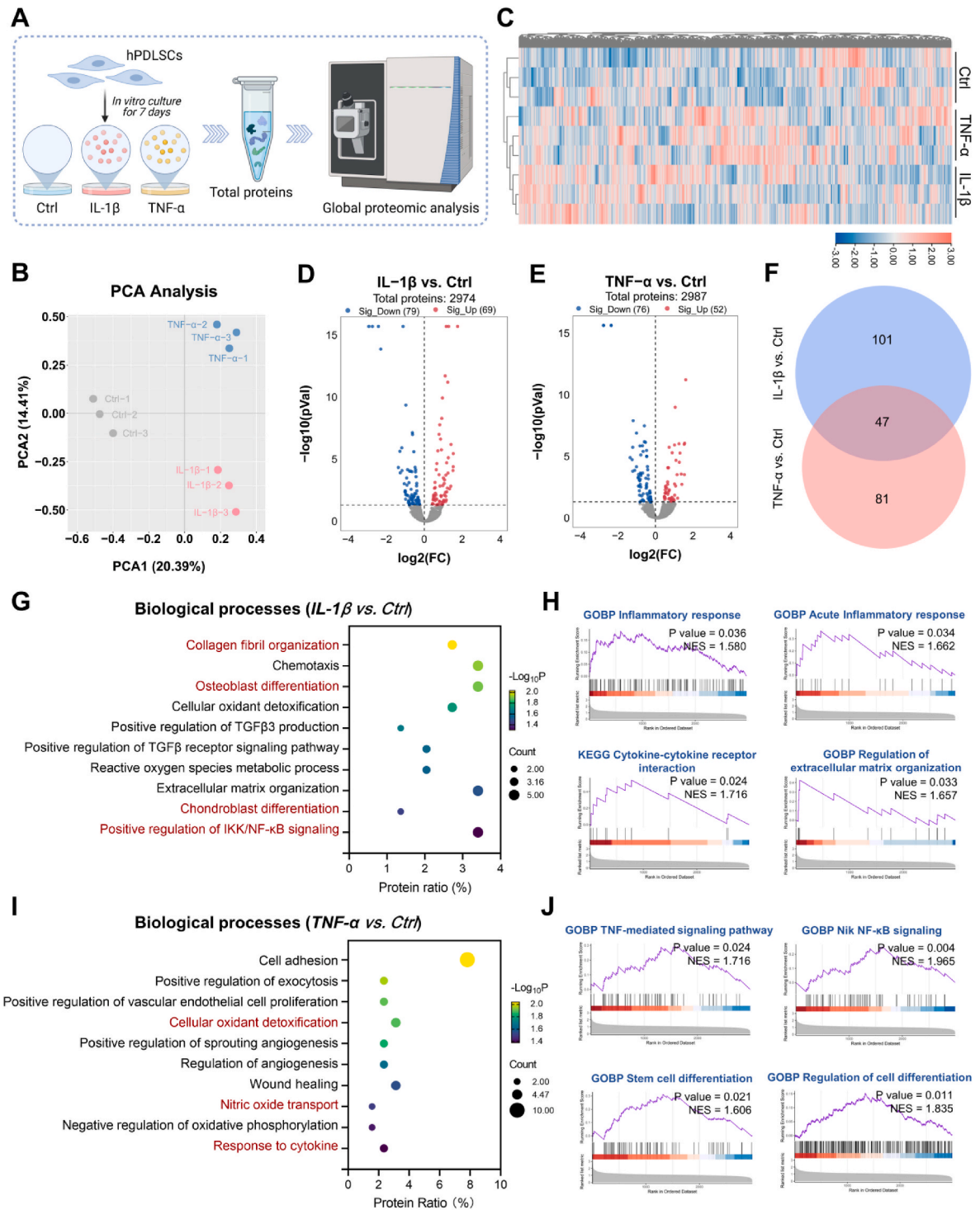
Fig. 4. Global proteomic analysis reveals the altered cellular processes and signaling pathways in hPDLSCs treated with IL-1β or TNF-α for 7 days. (A) Schematic illustration of experiment procedures. The figure was generated with BioRender (https://biorender.com/). (B) PCA of proteomic data of three groups (n = 3 experimental units). (C) Heatmap of all identified proteins by proteomic analysis. (D–E) Volcano plots of the identified proteins by proteomic analysis in IL-1β *vs.* Ctrl (D) and TNF-α *vs.* Ctrl (E). (F) Venn plot of DEPs in IL-1β *vs.* Ctrl and TNF-α *vs.* Ctrl. (G) The significantly enriched BP terms from all DEPs (IL-1β *vs.* Ctrl, P < 0.05). (H) GSEA plots associated with inflammation, cytokine-cytokine receptor interaction, and ECM organization (IL-1β *vs.* Ctrl). (I) The significantly enriched BP terms from all DEPs (TNF-α *vs.* Ctrl, P < 0.05). (J) GSEA plots associated with TNF signaling, NF-κB signaling, and stem cell differentiation (IL-1β *vs.* Ctrl).

to tendon and ligament-derived stem cells, we also investigated the influence of a pro-inflammatory stimulus on hBMSCs. The results revealed that the tenogenesis of hBMSCs was also notably inhibited by 0.1 ng/ml IL-1β and 10 ng/ml TNF-α on day 7, with significantly downregulated *SCX*, *COL1*, and *COL14* expressions, as well as upregulated *MMP3* expression (Fig. 3L). Interestingly, the expressions of tendon-related markers of different stem cells in similar conditions were grouped. The results revealed that PDLSCs, instead of BMSCs, showed similar expression patterns of tendon-related markers with TSPCs in response to pro-inflammatory stimulations (Fig. S7), confirming the high similarity in the functions of PDLSCs and TSPCs.

In general, the tenogenic potential of stem cells in different sources and types was markedly suppressed in the inflammatory environment, which could contribute to the dysregulated differentiation of endogenous or exogenous stem cells in injured tendons. This effect could partially explain the unsatisfied tendon healing after stem cell injections, as well as the mechanisms of heterotopic chondrogenesis, ossification, and calcification in chronic tendinopathy [6,27,42,49]. Besides, the dysregulated expression of MMPs in stem cells exposed to inflammatory stimulus could degrade tendon matrix including COL1, COL3, and COL14, which was also observed in human and mouse injured tendons (Fig. S8). Therefore, the manipulation of tendon inflammation environment could be a promising strategy to improve the therapeutic effects of stem cell delivery for tendon regeneration.

To clarify the underlying mechanisms of the impaired tenogenesis of hPDLSCs in the inflammatory environment, global proteomic analysis was performed for hPDLSCs treated with IL-1β and TNF-α (Fig. 4A). 0.1 ng/ml for IL-1β or 10 ng/ml for TNF-α was chosen in the following experiments since it effectively suppressed the tenogenesis but not proliferation of hPDLSCs (Fig. 3B–I and S9). After treated with IL-1β or TNF-α for 7 days, the total proteins of hPDLSCs were extracted for proteomic analysis. The primary component analysis (PCA) for proteomic data revealed that the three replicates of each group accumulated into a cluster (Fig. 4B), indicating the similarity of protein expression within each group and the notable difference in hPDLSCs among the three groups. Heatmap of the expression levels of all identified proteins displayed the differential expression patterns of hPDLSCs in the IL-1β and TNF-α groups as compared to the Ctrl group (Fig. 4C). Specifically, 148 DEPs (P < 0.05) were recognized in IL-1β *vs.* Ctrl, with 69 up-regulated and 79 down-regulated (Fig. 4D). When comparing the TNF-α and Ctrl groups (TNF-α *vs.* Ctrl), 52 of up-regulated and 76 down-regulated DEPs were recognized (Fig. 4E). 47 DEPs were shared in IL-1β *vs.* Ctrl and TNF-α *vs.* Ctrl, indicating several same cellular responses were induced in hPDLSCs after IL-1β or TNF-α stimulation (Fig. 4F).

Subsequently, functional analysis was performed to obtain insight into the changed protein expressions between groups. In IL-1β *vs.* Ctrl, GO enrichment analysis identified the altered cellular processes specific to "collagen fibril organization", "osteoblast differentiation", "chondroblast differentiation", and "positive regulation of IKK/NF-κB signaling" (Fig. 4G). GSEA revealed that IL-1β stimulation induced up-regulated acute inflammatory response, cytokine-cytokine receptor interaction, and ECM organization (Fig. 4H). We further analyzed the interaction network of all identified DEPs in IL-1β *vs.* Ctrl (Fig. S10A) and screened the top 20 proteins from the network (Fig. S10B), which could play more critical roles in the altered cellular processes in hPDLSCs after IL-1β stimulation. The results revealed that the proteins associated with inflammation including PTGS2, CXCL1, CXCL5, and ADAM17 were up-regulated in IL-1β *vs.* Ctrl (Fig. S10B), confirming the inflammation activation in hPDLSCs treated with IL-1β. Besides, the production of various collagens including COL1A2, COL6A1, COL6A2, and COL6A3 was decreased, with the up-regulated MMP3 and down-regulated TIMP3 (the inhibitor of MMP3) (Fig. S10B), which could contribute to the degradation of collagen matrixes. Collectively, these results confirmed the dysregulated differentiation and decreased collagen matrix expression of hPDLSCs in response to IL-1β stimulation, which was consistent with the results in mouse injured tendons (Fig. 1A

and B). It was worth noting that positive regulation of IKK/NF-κB signaling was also significantly enriched from the DEPs (IL-1β *vs.* Ctrl) (Fig. 4G and S11), suggesting the involvement of NF-κB signaling in the cellular processes mediated by IL-1β in hPDLSCs.

As for TNF-α *vs.* Ctrl, only a few BP terms associated with inflammation activation like "cellular oxidant detoxification", "nitric oxide transport", and "response to cytokine" were significantly enriched from the DEPs (Fig. 4I), which could be attributed to the weaker effects of TNF-α than IL-1β (Fig. 3). Despite it, GSEA for all identified proteins (TNF-α *vs.* Ctrl) revealed that the "TNF-mediated signaling pathway", "NIK NF-κB signaling", "stem cell differentiation", "regulation of stem cell differentiation" were activated in hPDLSCs after TNF-α stimulation (Fig. 4J). Similar to IL-1β, TNF-α induced the reduced expressions of multiple collagens including COL1A2, COL6A2, and COL6A3, as well as the suppressed TIMP3, which could result in decreased tendon matrix deposition (Fig. S12). Besides, SPARC, a protein of tendon lineage, was significantly down-regulated in hPDLSCs after TNF-α treatment (Fig. S12). Together, these results verified that the differentiation and collagen deposition of hPDLSCs were dysregulated in response to TNF-α stimulation, which also could be mediated by NF-κB signaling pathway.

3.4. TPCA-1 rescues the impaired tenogenesis of stem cells in the inflammatory environment by targeting IKKβ/NF-κB signaling pathway

As mentioned above, the proteomic analysis suggested that the NF-κB signaling pathway was probably involved in the dysregulated differentiation and collagen matrix deposition of hPDLSCs in the inflammatory environment. Thus, WB and IF staining were conducted to evaluate the activation of NF-κB signaling in hPDLSCs after 0.1 ng/ml IL-1β or 10 ng/ml TNF-α treatment for 30 min. The results revealed that pNF-κB level in the whole cell lysate was increased while IκBα level was decreased in hPDLSCs with IL-1β and TNF-α exposure (Fig. 5A). Additionally, accumulation of NF-κB in the nucleus was observed in hPDLSCs upon IL-1β and TNF-α treatments as compared to the Ctrl, as indicated by WB and IF staining (Fig. 5B–D). These results confirmed that the NF-κB signaling pathway in hPDLSCs was activated in the inflammatory environment simulated by IL-1β and TNF-α.

Subsequently, we further investigated whether the blocking of NF-κB signaling rescued the impaired tenogenesis of hPDLSCs when exposed to IL-1β or TNF-α. The proteomic results have revealed that IKK/NF-κB signaling is involved in the IL-1β-induced inhibition of tenogenesis in hPDLSCs (Fig. 4G and S11). The IKK complex is an essential fulcrum for NF-κB signaling, and the catalytic subunits of the IKK complex, consisting of IKKα and IKKβ, regulated the inflammation activation by targeting the IκBα:P50:P65 NF-κB complex [42]. TPCA-1 is a small molecule inhibitor of IKKβ that plays a critical role in NF-κB activation in response to inflammation. Our previous study demonstrated that TPCA-1 effectively suppressed the IL-1β-induced NF-κB signaling activation in keratocytes [32]. Thus, the different concentrations of TPCA-1 were used to inhibit IKKβ/NF-κB signaling in hPDLSCs upon IL-1β or TNF-α stimulation to examine its potentials to rescue tenogenesis in the inflammatory environment. Gradient TPCA-1 in the concentrations of 0, 0.1, 1, and 10 μM was added to treat the hPDLSCs with exposure to 0.1 ng/ml IL-1β for 3 days or 10 ng/ml TNF-α for 7 days. The results revealed that TPCA-1 substantially enhanced the transcriptional expressions of tendon markers, including *SCX*, *TNMD*, *COL1*, and *COL14*, while markedly reducing MMPs expressions (*MMP1* and *MMP3*) in a dose-dependent manner in hPDLSCs treated with IL-1β and TNF-α (Fig. 6A–D). In general, a higher concentration of TPCA-1 induced a better effect on rescuing the inhibited tenogenic differentiation of hPDLSCs after IL-1β and TNF-α treatments (Fig. 6). The similar roles of TPCA-1 in promoting tenogenesis were also observed in rTSPCs (Fig. S13). Together, these results demonstrated that NF-κB signaling activation played a critical role in inhibiting the tenogenesis of hPDLSCs upon pro-inflammatory stimulus, and the selective inhibition of IKKβ/NF-κB signaling by TPCA-1 effectively rescued it. It was worth
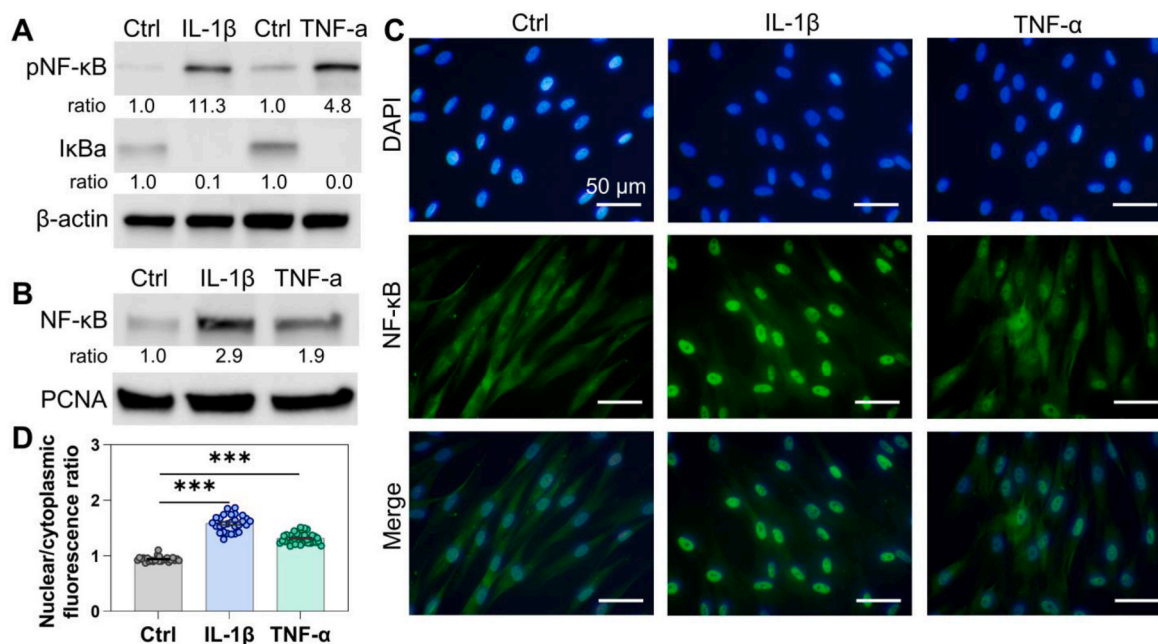
Fig. 5. NF-κB signaling pathway is activated in hPDLSCs after IL-1β or TNF-α treatment. (A) Immunoblots of indicated proteins in the whole cell extract of hPDLSCs after IL-1β or TNF-α treatment for 30 min. (B) Immunoblots of indicated proteins in the nucleus of hPDLSCs after IL-1β or TNF-α treatment for 30 min. (C) Representative images of IF staining for NF-κB in hPDLSCs after IL-1β or TNF-α treatment for 30 min. Scale bars = 50 μm. (D) Quantitative analysis of IF staining for NF-κB (n = 25–38 cells from 3 experimental units). Results were shown as means ± SEM. P values were determined by the Kruskal-Wallis test (D). ***p < 0.001.

noting that as the concentration of TPCA-1 increased, the proliferation of hPDLSCs was inhibited. The TPCA-1 in the concentration of 10 μM has slightly but significantly suppressed hPDLSCs proliferation (Fig. S14). Taking the roles of TPCA-1 in rescuing tenogenic differentiation and the inevitable loss of TPCA-1 *in vivo* into consideration, we believed that 10 μM TPCA-1 was an appropriate concentration and chose it to perform the following animal experiments.

Interestingly, the activation of NF-κB signaling was also involved in the inflammatory phenotype switch of tenocytes upon IL-1β stimulations (Fig. 2B and C), indicating that tenocytes could benefit from the administration of TPCA-1 and thereby induce inflammation resolution for tendon healing. Emerging evidence has demonstrated the feasibility of targeting IKKβ/NF-κB signaling to treat acute and chronic tendinopathy [28,29]. For instance, Abraham et al. demonstrated that the blocking of IKKβ in tendon stromal cells not only protected mice from chronic tendinopathy but also improved the outcomes of surgical repair for injured tendons [28]. Wang et al. reported that inhibition of IKKβ/NF-kB signaling using small interfering RNA rescued the senescence of TSPCs induced by aging-related inflammation [29]. Furthermore, previous study has reported that NF-κB-p65 was predominantly expressed in CD68⁻ nonmyeloid cells, indicating the IKKβ/NF-kB signaling inhibition by TPCA-1 could not influence the functions of immune cells to initiate normal inflammatory cascade to remove debris and pathogens after tendon injury [5,28]. In general, TPCA-1 is a promising candidate to rescue tendon inflammation and thereby tailors a pro-tenogenic niche for transplanted hPDLSCs to initiate tendon regeneration by targeting IKKβ/NF-kB signaling.

*3.5. Controlled release of TPCA-1 engineers a pro-tenogenic niche for transplanted hPDLSCs to enhance tendon regeneration*

Since only a few stem cells and drugs could remain *in situ* after *in vivo* injection, a hydrogel delivery system composed of GelMA hydrogel and SF microspheres was developed to enhance the therapeutic effects of hPDLSCs and TPCA-1. Tendon inflammation after acute injuries often lasts for one week [7,50]. Our previous work has developed SF nano-/microspheres as a delivery system for the controlled release of

small molecule drugs and found that the SF microsphere prepared by 5 % concentration of SF/PVA showed a rapid release of small molecular drug within 5 days [36]. Thus, this SF microsphere was employed to load and rapidly release TPCA-1 in the inflammatory phase of injured tendons. Owing to the compelling biocompatibility, degradability, and photocrosslinked properties, GelMA hydrogel was commonly used as a delivery platform for various cell types and bioactive factors [51]. Thus, GelMA hydrogel was introduced to maintain the transplanted hPDLSCs and TPCA-1-loaded SF microsphere (SF@TPCA) in tendon lesion sites and provided a water-abundant environment for hPDLSCs survival and tissue ingrowth. The preparation of the GelMA hydrogel containing SF@TPCA (GelMA-SF@TPCA) was illustrated in Fig. 7A. Scanning electron microscope (SEM) images revealed that the fabricated SF microspheres displayed a smooth surface and spherical shape (Fig. 7B). Dynamic light scattering (DLS) revealed the average hydrodynamic diameter of SF microspheres was 3.95 ± 0.81 μm (Fig. 7C). To easily monitor the loading of hydrophobic TPCA-1 (M.W. 279 g/mol), RhoB, a model molecule with similar hydrophobicity and molecule weight (479 g/mol) that emits red fluorescence after excitation, was encapsulated into SF microspheres (SF@RhoB). The images of RhoB-loaded SF microspheres obtained from the light and fluorescence microscopy were shown in Fig. 7D and E respectively, which confirmed the successful loading of RhoB into SF microspheres. Subsequently, SF@TPCA microspheres were integrated into the GelMA hydrogels (Fig. 7F), and the degradation and TPCA-1 release profiling of GelMA-SF@TPCA hydrogel were investigated. The results revealed that GelMA-SF@TPCA hydrogel almost undegraded within the first 3 days and underwent a fast degradation of ~90 % weight in the following 7 days (Fig. 7G), which would allow the transplanted stem cells to remain and subsequently migrate into injury site to initiate tendon repair. In addition, the release profiling of RhoB suggested that the loaded TPCA-1 in the hydrogel system could be sustainedly released and reach a peak concentration on day 5 to counteract the harsh inflammatory environment of injured tendons. (Fig. 7H). Collectively, these results indicated that the developed SF microsphere-incorporated GelMA hydrogel system supported the delivery of stem cells and TPCA-1 to initiate tendon repair.

We next investigated the effects of the hPDLSCs and TPCA-1 delivery
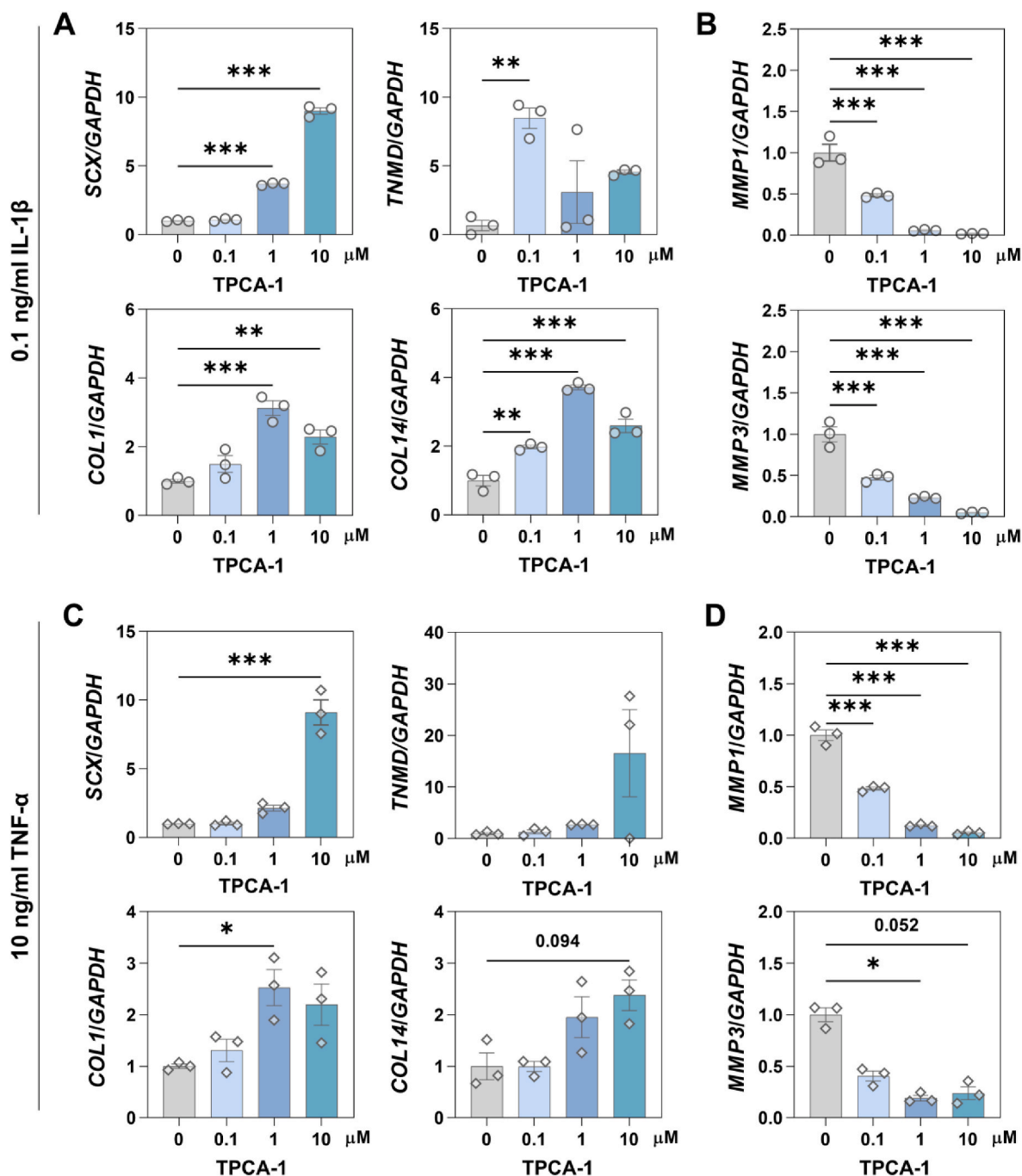
Fig. 6. TPCA-1 rescues the suppressed tenogenesis of hPDLSCs by IL-1β or TNF-α in a dose-dependent manner. (A–B) Gene expression of tendon markers (A) and MMPs (B) in hPDLSCs treated with TPCA-1 for 3 days in the presence of 0.1 ng/ml IL-1β stimulation by qPCR (n = 3 experimental units). (C–D) Gene expression of tendon markers (C) and MMPs (D) in hPDLSCs treated with TPCA-1 for 3 days in the presence of 10 ng/ml TNF-α stimulation by qPCR (n = 3 experimental units). Results were shown as means ± SEM. P values were determined by the ANOVA (A-B; C for *SCX*, *TNMD*, and *COL1*; D for *MMP1*) and Kruskal-Wallis test (C for *COL14*; D for *MMP3*). *p < 0.05, **p < 0.01, ***p < 0.001.

on inflammation and tendon repair in a rat acute tendon injury model [11]. In this model, a 4 mm × 1 mm window defect was created in the central part of the patellar tendons. The hPDLSCs, SF@TPCA microspheres, and GelMA precursor solutions were uniformly mixed and injected into tendon defects, and subsequently *in situ* gelation was induced upon UV irradiation for 30 s (TPCA-1 group). The rats treated with GelMA-SF hydrogels but without TPCA-1 loading were set as the Ctrl group. H&E staining and IHC staining for human nuclei (hNuclei) revealed that the hPDLSCs remained and survived in tendon defects at least for 2 weeks after *in vivo* transplantation, which allowed them to

initiate the regenerative processes (Fig. 8A and B). As shown in Fig. 8C, the repaired tendons in the Ctrl group suffered from a severer inflammation on days 3 and 7 (1W) compared to those of the TPCA-1 group. We further investigated the activation of NF-κB signaling and the expression of immune markers in injured tendons of different groups by IHC and IF staining. The results revealed that the positive rate of NF-κB in both groups showed a peak on day 7, while the TPCA-1 group showed a substantially lower NF-κB-positive ratio than the Ctrl group, indicating the activation of NF-κB signaling was effectively inhibited by the delivery of TPCA-1 (Fig. 8D). The CD68-positive macrophages in the Ctrl
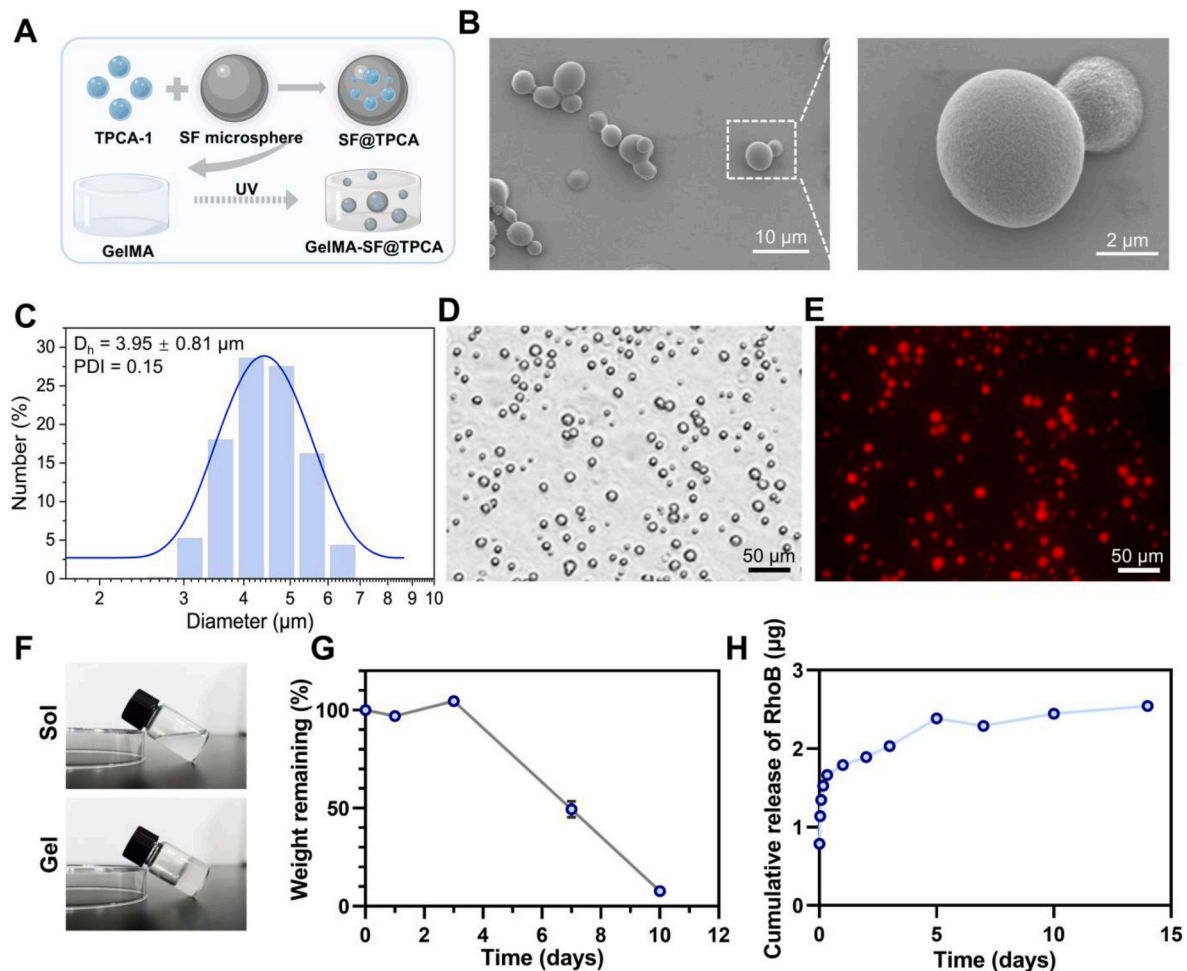
Fig. 7. GelMA-SF@TPCA hydrogel system supports a sustained release of TPCA-1 within 5 days. (A) Illustration of the fabrication of GelMA-SF@TPCA hydrogels. (B) SEM images of SF microspheres. Low magnification: scale bar = 10 μm; high magnification: scale bar = 2 μm. (C) DLS analysis of SF microspheres. (D) Light microscopic image of SF microspheres loaded with RhoB. Scale bar = 50 μm. (E) Fluorescent image of SF microspheres loaded with RhoB. Scale bar = 50 μm. (F) General view of GelMA-SF@TPCA hydrogel (sol-gel transition). (G) Degradation curve of GelMA-SF@TPCA hydrogels incubated in PBS at 37 °C. (H) Cumulative release curve of RhoB from GelMA-SF@RhoB hydrogels incubated in PBS at 37 °C. Results were shown as means ± SEM.

group showed a high iNOS expression but a low CD206 expression on day 3 (Fig. 8E). In contrast, the high CD206 but low iNOS expressions were observed in the CD68-positive macrophages in the TPCA-1 group (Fig. 8E). These findings indicated that the delivery of TPCA-1 promoted the switch of macrophages from pro-inflammatory phenotype into anti-inflammatory phenotype. IHC staining for IL-1β was conducted to evaluate the immune microenvironment of repaired tendons at 4 weeks after injury. The results showed that the expression of IL-1β in the TPCA-1 group was significantly decreased as compared to the Ctrl group. In general, these data supported the anti-inflammatory effects of TPCA-1, which could contribute to a suppressed inflammatory environment for the transplanted PDLSCs to initiate tendon repair.

Finally, the structure and function of repaired tendons in the Ctrl and TPCA-1 groups were evaluated. At 4 weeks, H&E and MT staining revealed that the repaired tendons in the Ctrl group showed notable inflammatory cell accumulation (as indicated by black arrows in H&E staining) and disorganized tendon matrix (Fig. 9A). In contrast, the tendon-like tissue with aligned collagen fibers and spindly nuclei was observed in the repaired tendons of the TPCA-1 group, without obvious inflammatory cell infiltration (Fig. 9A). Histological score for repaired tendons in fiber structure, fiber arrangement, rounding of the nuclei, inflammation, vascularity, and cell numbers was conducted from H&E staining images. The results showed that the scores for fiber structure, fiber arrangement, rounding of the nuclei, and inflammation in the

TPCA-1 group were significantly decreased compared to the Ctrl group (Fig. S15), indicating that the suppressed tendon inflammation and improved tendon matrix organization were mediated by TPCA-1 delivery. Generally, a more mature tendon tissue was formed in the TPCA-1 group than in the Ctrl group, as evidenced by the significantly lower total histological scores (3.13 *vs.* 5.94, P = 0.004) and higher collagen density (0.44 *vs.* 0.28, P = 0.048) (Fig. 9B and C). Finally, the early and terminal marker of tenogenic differentiation was evaluated in the repaired tendons at 4 weeks by IF staining. It was found that an early tenogenic marker, SCX, was expressed and exhibited similar expression levels in the TPCA-1 and Ctrl group, which suggested that the tenogenesis of hPDLSCs had been initiated at 4 weeks in both groups (Fig. 9D and F). However, TNMD, a terminal marker of tenogenesis, was extensively expressed in repaired tendons in the TPCA-1 group but less in the Ctrl group (Fig. 9E). Quantitative analysis revealed that the TPCA-1 group displayed a 1.88-fold increase (P < 0.001) in TNMD expression as compared to the Ctrl group (Fig. 9G). The expression of MKX, a transcription factor for tenogenic differentiation was also been investigated by IHC staining. The results revealed that the MKX expression was significantly upregulated in the TPCA-1 group as compared to the Ctrl group (Fig. 9H). These findings demonstrated that the tenogenic differentiation of hPDLSCs was enhanced in repaired tendons in the TPCA-1 group. Gait analysis and mechanical test were performed to evaluate the functions of repaired tendons at 2 or 4 weeks after injury. As shown
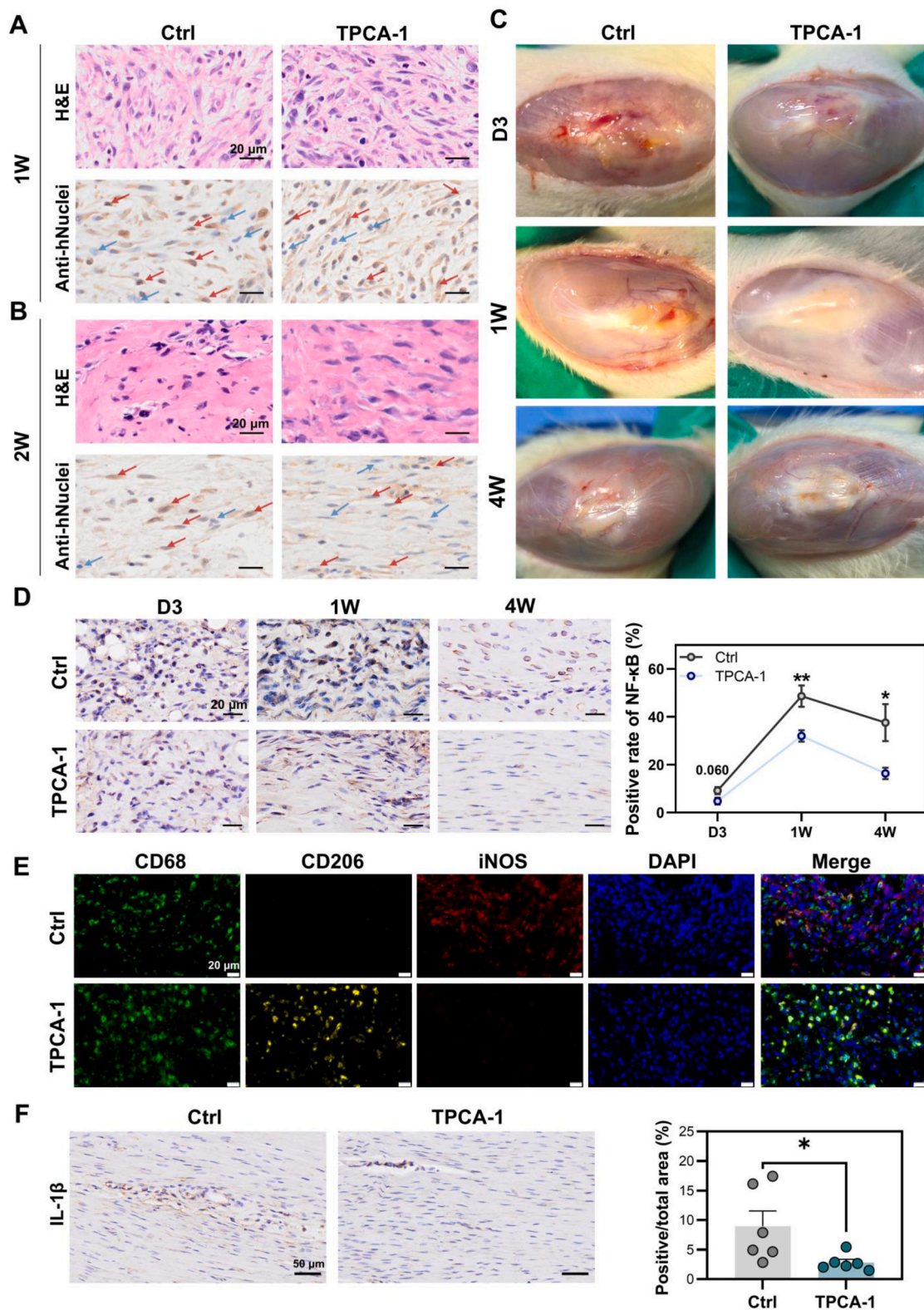
Fig. 8. The controlled release of TPCA-1 rescues the inflammatory tendon microenvironment after acute injury in rats. (A–B) Representative images of H&E staining and IHC staining for hNuclei in the Ctrl and TPCA-1 groups at 1 (A) and 2 (B) weeks post-surgery. The rats in the Ctrl and TPCA-1 groups were treated with hPDLSCs-loaded GelMA-SF and GelMA-SF@TPCA hydrogels, respectively. Red arrows indicated the hNuclei-positive stained hPDLSCs and blue arrows indicated the hNuclei-negative tendon resident cells. Scale bars = 20 μm. (C) Gross images of repaired tendons in the Ctrl and TPCA-1 groups at different time points. (D) Representative images and quantitative analysis of IHC staining for NF-κB on different time points (n = 6 randomly-selected microscopic images). Scale bars = 20 μm. (E) Representative images of IF staining for CD68 (macrophage marker), CD206 (anti-inflammatory marker), and iNOS (pro-inflammatory marker) on day 3 after injury. Scale bars = 20 μm. (F) Representative images and quantitative analysis of IHC staining for IL-1β at 4 weeks after injury (n = 6 randomly-selected microscopic images). Scale bars = 50 μm. Results were shown as means ± SEM. P values were determined by the Student's t-test. *p < 0.05, **p < 0.01.
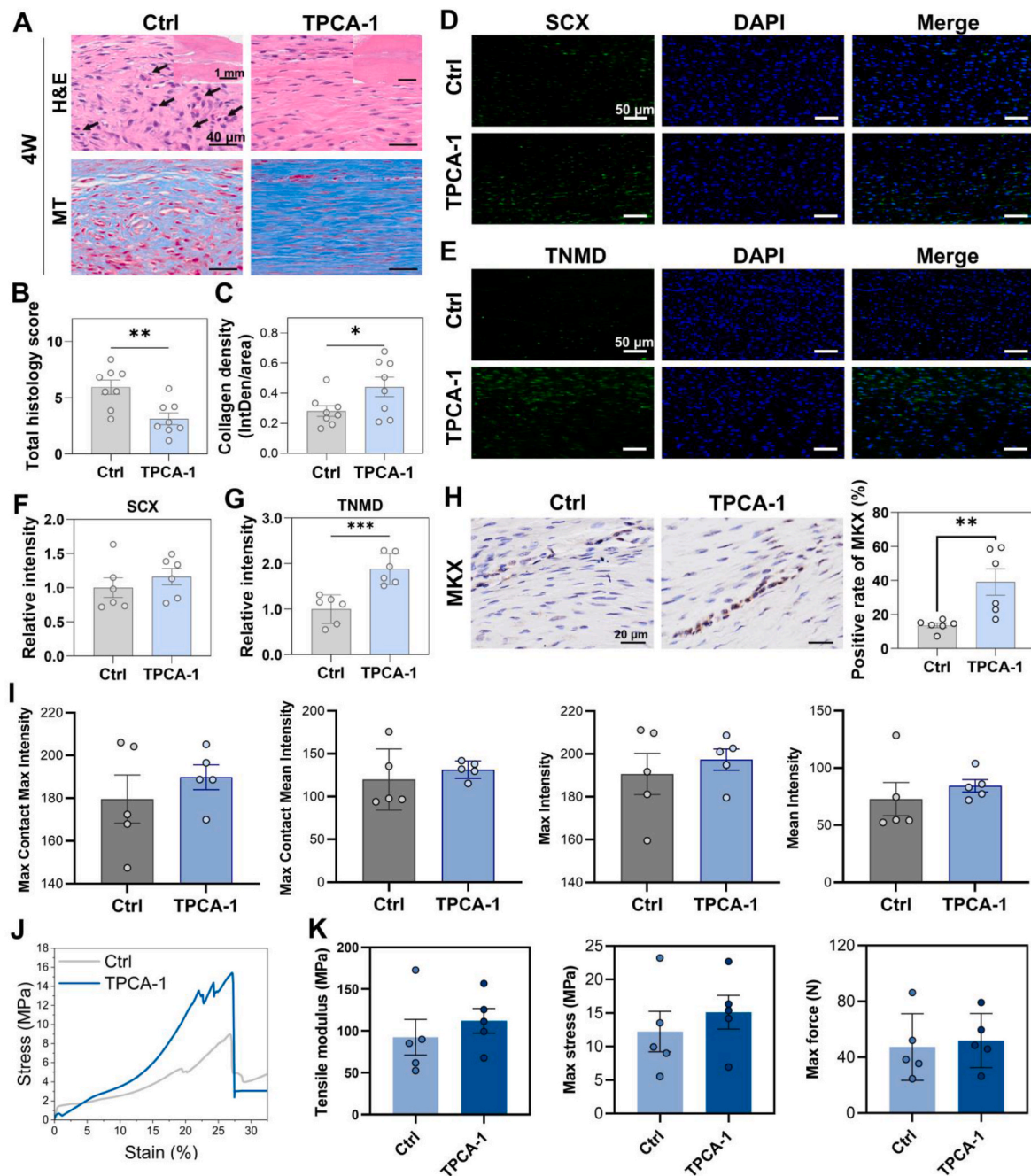
Fig. 9. The controlled release of TPCA-1 promotes tenogenic differentiation of hPDLSCs for tendon repair in rats. (A) H&E staining and MT staining of the repaired tendons in the Ctrl or TPCA-1 groups. Black arrows indicated the cells with the characteristics of inflammatory cells at 4 weeks. Low magnification: scale bars = 1 mm; high magnification: scale bars = 40 μm. (B) Total histology scores of repaired tendons in the Ctrl and TPCA-1 groups from H&E staining images at 4 weeks after injury (n = 8 biological replicates). A lower score indicates a more mature tendon tissue. (C) Quantitative analysis of collagen density in the repaired tendons of the Ctrl and TPCA-1 groups from MT staining images (n = 8 biological replicates). (D–E) Representative images of IF staining for SCX (D) and TNMD (E) in the repaired tendons of the Ctrl and TPCA-1 groups at 4 weeks after injury. Scale bars = 50 μm. (F–G) Quantitative analysis of IF staining for SCX (F) and TNMD (G). (H) Representative images and quantitative analysis of IHC staining for MKX at 4 weeks after injury. Scale bars = 20 μm. (I) Quantitative analysis of intensity parameters via gait analysis at 2 weeks after injury (n = 5 biological replicates). (J) Typical strain-stress curves of repaired tendons. (K) Tensile modulus, max stress, and max force of repaired tendons at 4 weeks after injury (n = 5 biological replicates). Results were shown as means ± SEM. n = 6 randomly-selected microscopic images for quantitative analysis for Fig. 9F–H. P values were determined by the Student's t-test (D–F) and Mann-Whiteney test (G). *p < 0.05, **p < 0.01, ***p < 0.001.

in Fig. 9I, the rats in the TPCA group exhibited higher values of intensity parameters, including max contact max intensity, max contact mean intensity, max intensity, and mean intensity than those of the Ctrl group, although no significant difference between groups was recognized. Similarly, we found that the tensile modulus, max stress, and max force of repaired tendons in the TPCA-1 group were slightly increased compared to the Ctrl group. These data supported that the delivery of TPCA-1 could improve the functional recovery of injured tendons. Since only partial injury of the patellar tendon was created (i.e., the main mechanical strength of tendons remained) and the time point for functional evaluation was relatively short, the significant difference between the Ctrl and TPCA-1 groups was not been recognized. In addition, SO/FG

and IHC staining for SOX9 and OCN were conducted to evaluate chondrogenesis and osteogenesis in the repaired tendons at 4 weeks after injury. The results displayed that ectopic cartilage with a mass of SOX9- and OCN-positive cells could be observed in the Ctrl group but not in the TPCA-1 group (Fig. S16), indicating the effects of TPCA-1 on dampening pathological chondrogenic and osteogenic differentiation in injured tendons. Collectively, these results demonstrated that the IKKβ/NF-kB signaling inhibition via the sustained release of TPCA-1 engineered a suitable stem cell niche for transplanted hPDLSCs, thereby substantially improving their therapeutic effects on tendon repair, as indicated by the suppressed inflammation, enhanced tenogenesis, organized collagen matrix, and improved motor functions and mechanical strength.

In the last two decades, stem cells with the capacity of self-renewal, multi-lineage differentiation, and inflammatory modulation have been identified in tendon tissue and showed a critical role in tendon development and homeostasis. The supplementation of stem cells emerged as a promising strategy to promote tendon regeneration. In clinical practice, hBMSCs and hADSCs have been investigated for treating tendon disorders, and positive results on pain relief and function improvement have been reported, which probably are attributed to the paracrine functions of stem cells [9,10,13]. However, the therapeutic effects of stem cells were far from the expected, which could be primarily caused by the unexploited stem cell differentiation towards tendon lineage [6]. Increasing clinical investigations have demonstrated that activation of inflammation by the release of various pro-inflammatory cytokines is present in early tendinopathy [5,27,28,52], which induces an inflammatory microenvironment that impairs tenogenesis [26,41]. Although the immunomodulatory functions of stem cells were recognized, it seemed to be insufficient for the survival and tenogenic differentiation of transplanted stem cells against tendon inflammation to initiate tendon regenerative processes, as evidenced by the lasting pain and swelling of lesion sites after stem cell injection in several failed clinical trials [6,12,53].

Previous scientific studies have demonstrated that recipient immune cells, especially pro-inflammatory T lymphocytes, induced the apoptosis of exogenous BMSCs and suppressed their ability to mediate bone regeneration, which was associated with the enhanced TNF-α signaling [54,55]. Moreover, the local administration of aspirin or indomethacin, a non-steroidal anti-inflammatory drug, was found to substantially promote BMSCs-based bone regeneration [54,55]. In injured tendons and ligaments, an inflammatory niche was demonstrated to induce pathological differentiation of endogenous stem cells towards chondrogenesis and osteogenesis, usually resulting in ectopic cartilage formation, ossification, and calcification (Fig. 1C–D and S1) [27,42,56]. Ectopic cartilage and bone formation were also found at the implantation site after the *in vivo* administration of exogenous stem cells for tendon repair (Fig. S16) [18,19]. Consistently, our results revealed that the undesired chondrogenic and osteogenic differentiation of TSPCs could be initiated within 4 weeks after acute tendon injury with inflammation activation (Fig. 1A–D and S1). Therefore, the manipulation of the inflammatory environment is a promising approach to promote tendon healing via tailoring a pro-tenogenic niche of transplanted stem cells.

Previous studies have revealed that NF-κB signaling was activated in the early and intermediate stages of tendinopathy [5,28], and the inhibition of IKKβ/NF-κB signaling pathway was found to protect tendons from overuse-induced tendinopathy, improve the outcomes of surgical repair in injured tendons, and rescue TSPCs senescence caused by aging-related inflammation [28,29]. These findings indicated that NF-κB signaling was a critical molecular target to rescue tendon inflammation. Our results revealed that NF-κB signaling was involved in inflammation activation in mouse tendons after acute injury, which resulted in dysregulated stem cell differentiation (Fig. 1). Moreover, the activation of NF-κB signaling was also observed in the tenocytes and hPDLSCs exposed to an inflammatory environment simulated by IL-1β or TNF-α (Fig. 2B and C and 5), where the tendon-related gene expressions were

substantially decreased (Figs. 2D and 3). The selective inhibition of IKKβ/NF-κB signaling by TPCA-1 was found to rescue the suppressed tenogenesis of hPDLSCs and rTSPCs in the inflammatory niche (Fig. 6 and S13). All these findings highly indicated that the delivery of TPCA-1 could promote tendon inflammation resolution and engineer a pro-tenogenic niche for exogenous stem cells by targeting the IKKβ/NF-κB signaling pathway.

In addition, the improper cell source could be another factor that hindered tendon repair after *in vivo* stem cell transplantation. Compared to hBMSCs and hADSCs, hPDLSCs showed a higher potential to differentiate into tendon lineage and thereby initiate tendon repair [21]. Moreover, periodontal ligaments, the source of hPDLSCs, could be easily obtained from the surgically extracted wisdom teeth and other health teeth from orthognathic surgery. These teeth were usually trashed as medical waste. The construction of a "teeth bank" will turn these trashed teeth into treasure, and provide enough hPDLSCs for regenerative medicine in tendon repair. Similar to hBMSCs and hADSCs, hPDLSCs display low immunogenicity and high immunomodulatory functions [57,58], indicating that the allogeneic transplantation of hPDLSCs is practicable. Besides, we and other groups have confirmed the promising role of hPDLSCs transplantation in promoting tendon repair [11,21,59]. Thus, hPDLSCs are alternative stem cells to improve outcomes of tendon repair, showing great potential for future clinical applications.

In clinical practice, low retention of drugs or stem cells in tendon lesions after *in vivo* injection was found to decrease their therapeutic effects [6,12]. To overcome this issue, Chen et al. constructed a collagen hybrid peptide (CHP)-modified poly(lactic-co-glycolic acid) (PLGA) nanoparticle system to deliver rapamycin by targeting pathological collagens, which was found to effectively prevent heterotopic ossification in tendons [42]. Wang et al. used gold nanoclusters (AuNCs) to load IKKβ small interfering RNA (siRNA) for the effective inhibition of IKKβ/NF-κB signaling *in vivo*, and showed the intra-articular IKKβ siRNA delivery using AuNCs rescued the senescence of TSPCs and promoted tendon healing in degenerative tendinopathy [29]. To improve the efficiency of hPDLSCs and TPCA-1 after *in vivo* transplantation, a SF microsphere-incorporated GelMA hydrogel system was developed in this study. Our results revealed that the developed hydrogel system could maintain the transplanted hPDLSCs in the lesion sites (Fig. 8A) and supported a sustained TPCA-1 release for 5 days (Fig. 7H). Notably, the delivery of TPCA-1 effectively inhibited tendon inflammation and engineered a suitable niche for the tenogenesis of hPDLSCs by targeting IKKβ/NF-κB signaling (Fig. 10), thereby leading to mature tendon tissue formation with substantial collagen deposition, aligned fiber organization, and increased mechanical strength (Figs. 8 and 9). These findings suggested that a well-designed delivery system for cells and/or drugs is essential to further improve the therapeutic effects of stem cells in future tendon treatments.

In general, this study first recognized that the pro-inflammatory niche impaired the tenogenesis of endogenous or exogenous stem cells by activating NF-κB signaling after acute tendon injury, and proposed the delivery of TPCA-1 to engineer a pro-tenogenic niche for transplanted stem cells, especially hPDLSCs, to initiate tendon regeneration by targeting IKKβ/NF-κB signaling. This study also has limitations. To mention, 1) we only evaluated the roles of NF-κB signaling in dysregulating stem cell differentiation towards tendon lineages according to previous findings, while other inflammation-related signaling pathway like WNT signaling was not investigated; 2) since we focused on evaluating the effect of NF-κB signaling inhibition on rescuing the tenogenic potentials of stem cells, its influence on the functions of other tendon resident cells, particularly tenocytes was not known, which is needed to be comprehensively investigated in the future.

4. Conclusions

In conclusion, this study integrated multiple transcriptional datasets of injured tendons and inflammation-activated tenocytes. It revealed the
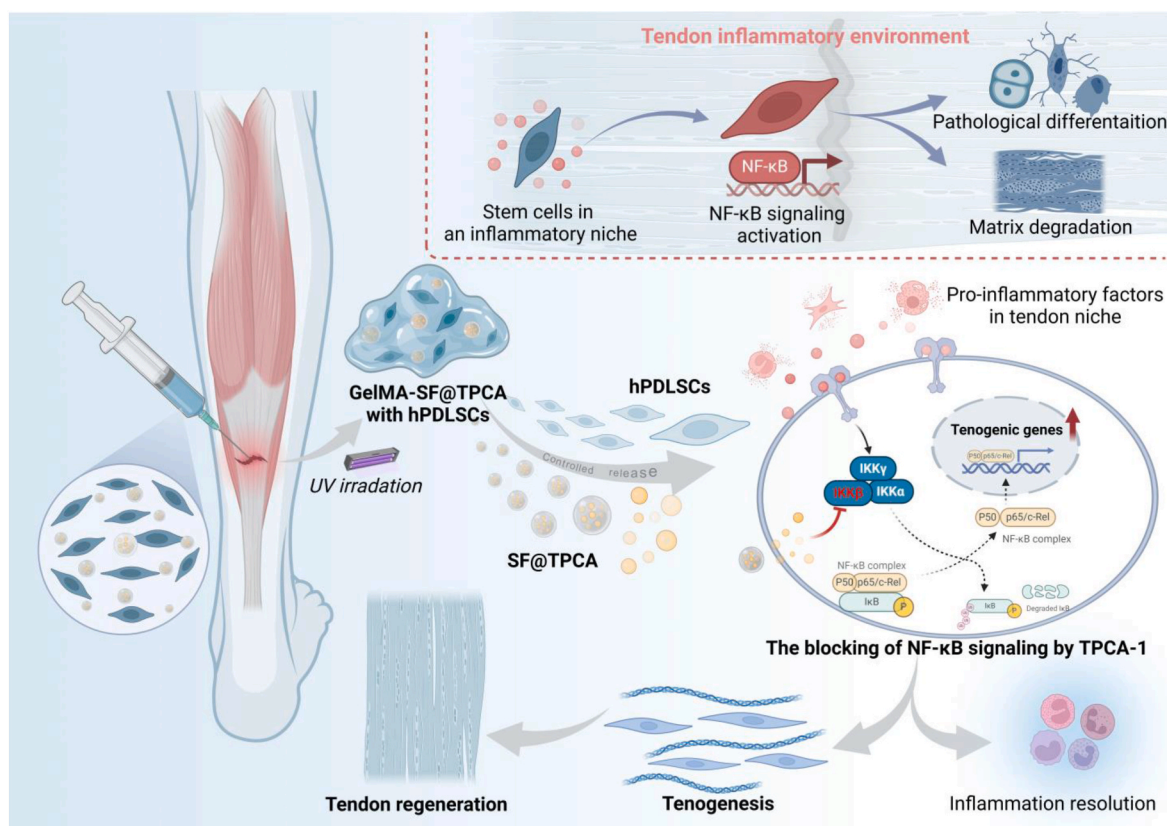
Fig. 10. Schematic illustration of TPCA-1 delivery engineering a pro-tenogenic niche of transplanted hPDLSCs for tendon regeneration by targeting IKKβ/NF-κB signaling. The schematic figure was generated with BioRender (https://biorender.com/).

involvement of NF-κB signaling activation in tendon inflammation and the release of pro-inflammatory factors following acute injuries, thus contributing to the establishment of a harsh inflammatory microenvironment. The tendon inflammatory niche significantly inhibited tenogenesis of both endogenous and transplanted stem cells and resulted in pathological differentiation of stem cells. However, this detrimental effect could be rescued by selective inhibition of IKKβ/NF-κB signaling using TPCA-1. Furthermore, the controlled release of TPCA-1 by a SF microsphere-incorporated GelMA hydrogel system promoted inflammation resolution and engineered a pro-tenogenic niche for transplanted hPDLSCs to initiate tenogenesis for enhanced tendon regeneration. This study uncovered the cellular processes and pathways in tendons after acute injury and confirmed the critical roles of NF-κB signaling in the dysregulated stem cell differentiation, which added insights into the pathological mechanism underlying tendon injury. Besides, the delivery of hPDLSCs and TPCA-1 using the developed hydrogel system could be a potential strategy to improve the therapeutic outcomes of stem cell transplantation for tendon repair in future clinical practice.

CRediT authorship contribution statement

Jialin Chen: Writing – review & editing, Resources, Funding acquisition, Conceptualization, Data curation, Project administration. **Renwang Sheng:** Writing – original draft, Methodology, Investigation, Formal analysis, Visualization, Writing – review & editing. **Qingyun Mo:** Methodology, Investigation, Formal analysis, Data curation, Validation, Writing – original draft. **Ludvig J. Backman:** Resources, Methodology, Investigation, Validation, Writing – review & editing. **Zhiyuan Lu:** Investigation. **Qiuzi Long:** Investigation. **Zhixuan Chen:** Investigation. **Zhicheng Cao:** Investigation. **Yanan Zhang:** Investigation. **Chuanquan Liu:** Investigation. **Haotian Zheng:** Investigation. **Yu Qi:** Investigation. **Mumin Cao:** Investigation. **Yunfeng Rui:** Supervision, Resources, Funding acquisition, Conceptualization. **Wei Zhang:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Data availability

All study data are included in the article and/or Supplementary information.

Ethics approval and consent to participate

All animal protocols in this study were approved by the Animal Experimental Ethical Inspection Committee of Southeast University (approval no. 20210915091, 2024060100).

Declaration of competing interest

The authors declare no conflict of interest.

Acknowledgement

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioactmat.2024.10.016.

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