Hyaluronic acid-chitosan nanoparticles encoding CrmA attenuate interleukin-1β induced inflammation in synoviocytes *in vitro*

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Abstract. Osteoarthritis (OA) is a common degenerative joint disease characterized by inflammation of synoviocytes and degradation of cartilage. In the present study, hyaluronic acid/chitosan (HA/CS) nanoparticles were used as a vehicle for gene therapy of OA, and the cytokine response modifier A (CrmA) pDNA was proposed as the target gene. The HA/CS/pCrmA nanoparticles were prepared and the characteristics of the nanoparticles were examined. The nanoparticles were spherical, and the smallest size was obtained with the HA:CS weight ratio of 1:4. The release analysis exhibited a constant release over 29 days. The pDNA was completely combined with HA/CS nanoparticles and the HA/CS nanoparticles protected pDNA from degradation. Subsequently, rat synoviocytes were transfected with HA/CS/pDNA nanoparticles, and the results demonstrated that the HA/CS nanoparticles were able to improve the transfection capacity of pDNA. The cytotoxicity of the HA/CS/pDNA nanoparticles was additionally detected using a MTS assay to ensure that the HA/CS nanoparticle was a safe carrier. To additionally investigate the effects of HA/CS/pCrmA nanoparticles on synoviocytes in OA, the MMP-3 and MMP-13 gene expression levels were detected at the gene and protein expression levels. These results indicated that the HA/CS/pCrmA nanoparticles attenuated interleukin-1β-mediated inflammation in synoviocytes. It was concluded that the HA/CS/pCrmA nanoparticles may provide a novel approach to the treatment of OA.

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Introduction

Osteoarthritis (OA) is a common degenerative joint disease characterized by the degradation and destruction of cartilage (1,2). Although the details of the pathological mechanism of OA remain unclear, accumulating evidence had suggested that inflammation serves an important role in its development. Notably, the inflammation of the synovium is frequently involved in the occurrence and development of this disease (3). The synovium is widely distributed in the cartilage cavity and is in direct contact with articular cartilage. Synovial fluid provides nutrients for exchange with articular cartilage. However, synovial inflammation may secrete certain cytokines, including interleukin-1β (IL-1β), tumor necrosis factor-α and nitric oxide (4). IL-1β is one of the crucial inflammatory cytokines, which has been demonstrated to active the nuclear factor-kB signaling pathway to release inflammatory mediators, including prostaglandin E2 and matrix metalloproteinases (MMPs) (5). MMP-3 and MMP-13, zinc-containing and calcium-dependent proteinases, are frequently expressed in synoviocytes and chondrocytes in response to inflammatory cytokines, which collectively degrade all components of the extracellular matrix (ECM) and serve crucial roles in the degenerative changes of cartilage matrix in OA (6,7). Additionally, these mediators may induce the aging and apoptosis of chondrocytes, and damage to the articular cartilage (8). Therefore, attenuation of the inflammation response in synoviocytes may have a protective effect on the metabolism of chondrocytes, thereby providing a promising treatment for OA.

At present, gene therapy has been regarded as a promising method of curing diseases. Gene therapy involves the delivery of a therapeutic gene into target cells to modulate their function and subsequently treat the disease. However, a key issue in the therapeutic application of this technology is that vectors are required for the safe and efficient delivery of plasmid DNA (pDNA). Although viral gene vectors have high transfection efficiency, their poor biological safety and highly immunogenic properties restrict their application (9). By contrast, non-viral gene vectors have been widely studied due to their clinical safety (10,11).

As a non-viral vector for gene delivery, chitosan (CS) offers certain advantageous properties, including non-toxicity, biodegradability and good biocompatibility (12).

Nanoparticles composed of CS and a plasmid encoding the IL-1 receptor antagonist have been demonstrated to markedly attenuate the severity of histologic cartilage lesions (13). However, at present, its low transfection efficiency limits the application of CS. Previously, it has been suggested that the transfection efficiency of CS vectors may be improved by combining CS with cationic or anionic biopolymers, prior to the addition of DNA (14,15). Hyaluronic acid (HA), a biocompatible anionic biopolymer, has been used in a wide array of clinical applications (16). It has been indicated that HA/CS/pDNA nanoparticles were suitable for cell transfection via endocytosis and exhibited a higher transfection efficiency compared with either CS/pDNA nanoparticles or naked plasmid DNA (17). Lu *et al* (18) suggested that HA/CS/pDNA nanoparticles encoding transforming growth factor β1, with diameters of 100-300 nm, may promote chondrocyte adhesion, proliferation and synthesis of the ECM.

In our previous study, HA/CS microspheres were demonstrated to be a safe carrier for the controlled release of drugs, due to their good biocompatibility, biodegradabilty and high stability (19). It was hypothesized that HA/CS nanoparticles may additionally be suitable as gene carriers to deliver the therapeutic gene into synoviocytes. The cytokine response modifier A (CrmA) is a caspase inhibitor that broadly inhibits the activity of a number of caspases and IL-1β converting enzyme proteases, and subsequently attenuates IL-1β induced inflammation and apoptosis in chondrocytes of OA (20,21), which suggested that CrmA pDNA may be a potential target gene.

In the present study, HA/CS/pCrmA nanoparticles were constructed as a gene delivery system. The characterization, safety, transfection efficiency and cytotoxicity of the complex was additionally measured. It was hypothesized that the HA/CS/pCrmA nanoparticles may have protective effects against inflammation in synoviocytes from an *in vitro* OA model, and the strategy provides a potential approach to the treatment of OA.

Materials and methods

Preparation of HA/CS/pDNA nanoparticles. The plasmid DNA (Guangzhou Fulengen Co., Ltd., Guangzhou, China) contained the enhanced green fluorescent protein expression vector (pEGFP) encoding a cytomegalovirus enhancer inserted upstream and the sequence of CrmA (pEGFP-CrmA). The plasmid was propagated in *Escherichia coli* cells, and subsequently isolated and purified. Following this, the absorption ratio of the plasmid at λ=260 and 280 nm was measured to ensure the concentration and purity. The empty plasmid control comprised pEGFP only (naked pDNA).

The HA/CS nanoparticles were produced as described previously (20). CA (molecular weight=5 kDa; deacetylation degree: 90%), and chitosanase were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Sodium hyaluronate (molecular weight=35 kDa) was purchased from Freda Biochem Co., Ltd. (Jinan, China). HA was dissolved in ultra-pure water at 1% (w/w, pH 5.5), and CS was dissolved in acetic acid to obtain a CS solution at 2% (w/w, pH 5.5). Then, these two solutions were filtered separately through a 0.22 μm membrane, and then combined at a rate of 600 x g for 1 h at room temperature to obtain a stable HA/CS solution. A well-mixed solution comprising 100 ml paraffin oil and 1 g Span 80 (Sigma-Aldrich; Merck KGaA) was combined at 300 x g for 1 h at room temperature. Subsequently, 6 ml HA-CS solution was gradually added into this paraffin-Span 80 solution at a speed of 1 ml/min. The reaction system was mixed at the 300 x g for an additional 2 h at room temperature, following which 10 ml sodium tripolyphosphate solution (10% w/w) was added. Then, the reaction system was mixed at the 300 x g for an additional 1 h at room temperature. Following centrifugation at a speed of 300 x g for 10 min at room temperature and removal of the supernatant, HA/CS nanoparticles were collected. The nanoparticles were washed with 100% alcohol and 100% acetone three times to completely remove the residual paraffin oil and Span 80. Different HA:CS weight ratio (1:1, 1:2, 1:3, 1:4, 1:5, 1:6, 1:7, 1:8) nanoparticles were prepared using the method described above.

A total of 12.5 μg/ml pDNA was added into the HA/CS nanoparticles and mixed by the vortex agitator for 5 sec; subsequently, the mixture was maintained for 1 h at 37˚C to form HA/CS/pDNA nanoparticles completely. The prepared HA/CS/pDNA nanoparticles were subsequently freeze-dried for subsequent study.

Characterization of HA/CS/pDNA nanoparticles. The lyophilized HA/CS/pDNA nanoparticles were dissolved in distilled water at room temperature to obtain a homogeneous solution. Subsequently, the solution was dropped onto a glass slide and dried at 37˚C. Subsequent to sputter-coating of the slide with gold, the morphology of HA/CS/pDNA nanoparticles was observed by scanning electron microscope (SEM; JSM-6330; JEOL, Ltd., Tokyo, Japan).

Electrophoresis assay. In order to detect whether pDNA was stably retained within the HA/CS nanoparticles, the HA/CS/pDNA nanoparticles or naked pDNA were dissolved in PBS, and then the samples were analyzed on a 1% agarose gel at 80 V for 45 min. Furthermore, the nanoparticles were subsequently incubated with 4 μg/ml DNase I at 37˚C for 30 min, following which the complexes were treated with EDTA for 15 min to stop the reaction. To value the effect of chitosan on the protection of pDNA, the complexes were additionally incubated with 2.78 μg/μl chitosanase for an additional 12 h at 37˚C, followed by analysis using an 1% agarose gel at 80 V for 45 min at room temperature. Finally, the gels were stained with ethidium bromide for 90 min at room temperature and imaged visually using a GDS-8000 (UVP, LLC, Phoenix, AZ, USA).

Release assay of pCrmA from HA/CS nanoparticles in vitro. To obtain an improved understanding of the release kinetics of the pCrmA from HA/CS nanoparticles, the amount of pCrmA was evaluated on days 0, 3, 5, 7, 9, 11, 14, 17, 19, 21, 23, 25, 27 and 29. The HA/CS/pCrmA nanoparticles were incubated in PBS solution (pH 7.4) in a shaker bath at 50 x g at 37˚C under sterile conditions. Following centrifugation of the samples at 4,000 x g at 37˚C for 10 min, supernatants were collected and replaced with equal volumes of fresh sterile PBS solution (pH 7.4) following each sampling. Finally, the amount

of pCrmA released was measured spectrophotometrically at 260 nm.

Cell culture and transfection. Specific pathogen-free male Sprague-Dawley rats (4 weeks old; weighing 80-100 g) were purchased from the Laboratory Animal Center of Wuhan University (Wuhan, China). The procedures involving animals were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals published by The National Institutes of Health (Bethesda, MD, USA) and were approved by the Wuhan University Animal Care and Use Committee (approval no. 2017-0208). The rats were housed under standard conditions (room temperature: 18-22˚C; humidity: 40-60%, 12:12-h dark-light cycle) and allowed free access to chow and distilled water. Subsequent to acclimating for 1 week, the rats were sacrificed by anesthesia with 5% isoflurane. The synovial tissue was isolated and sliced into small pieces, which were digested with 0.25% trypsin for 2 h and subsequently digested with 0.2% collagenase II at 37˚C for 1 h. Following centrifugation at 300 x g for 5 min at room temperature, synoviocytes were collected from the supernatants. Subsequently, the cells were cultured in DMEM/F12 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and 1% antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin; Sigma-Aldrich; Merck KGaA) and incubated at 37˚C with 5% CO_2 in a humidified atmosphere. After 4 days of culturing, once the cells had reached 80% confluence in the first passage, they were used in the subsequent experiments.

The synoviocytes were resuspended in 6-wells plates and the density of the cells was adjusted to $2x10^5$ cells/well. The appropriate HA/CS/pDNA nanoparticles were mixed throughly in PBS for 20 min at 37˚C. The complexes were diluted with DMEM/F12 medium with 10% FBS and 1% antibiotics. Subsequently, the cells were washed twice with PBS and 1.5 ml DMEM/F12 medium with 10% FBS containing 40 μg/ml HA/CS/pEGFP or HA/CS/pCrmA was added to each well. After incubation at 37˚C under 5% CO_2 for 72 h, the nanoparticles were removed and the cells were subsequently collected. The synoviocytes transfected with HA/CS/pEGFP were used as a control, and the protein expression of CrmA was measured by western blot analysis.

Protein extraction was performed using radioimmunoprecipitation lysis buffer (cat. no. P0013B) and concentration was measured by a bicinchoninic protein assay kit (cat. no. P0010; both from Beyotime Institute of Biotechnology, Haimen, China). A total of 50 μg protein per lane was separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane. Following blocking with 5% non-fat dry milk in TBS-0.05% Tween for 2 h at room temperature, the membrane was incubated at 4˚C overnight with primary antibodies, including anti-CrmA (cat. no. 556427; 1:500; BD Pharmingen, San Diego, CA, USA) and anti-β-actin (cat. no. BM0627; 1:3,000; Wuhan Boster Biological Technology, Ltd., Wuhan, China). Subsequently, the membrane was washed and incubated with a horseradish peroxidase-conjugated secondary antibody (cat. no. BA1050; 1:5,000; Wuhan Boster Biological Technology, Ltd.) at 37˚C for 2 h. The protein bands were visualized using an enhanced chemiluminescence system (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Cell viability. When the first passage cells reached 80% confluence, the medium was changed to DMEM/F12 and the cells were incubated at 37˚C for an additional 5 h. The synoviocytes were divided into two groups: One group was cultured with HA/CS/pCrmA nanoparticles at different concentrations (0, 5, 10, 20, 40, 80 and 160 μg/ml); and the other group was treated with HA/CS/pEGPF nanoparticles.

Following transfection and incubation for 72 h, cell viability was valued by colorimetric MTS assay according to the manufacturer's protocol. The culture medium was replaced by 1.5 ml DMEM/F12 containing 10 μl MTS. Following incubation at 37˚C with 5% CO_2 for 4 h, the absorbance was measured at 490 nm.

Synoviocytes culture and IL-1β treatments. Primary synoviocytes were cultured with IL-1β to generate the OA model, and then divided into four groups: Blank; control; HA/CS/pEGFP; and HA/CS/pCrmA groups. The blank group was cultured with medium only, and the control group was treated with 10 ng/ml IL-1β. In the presence of IL-1β, the HA/CS/pEGFP and HA/CS/pCrmA groups were treated with 40 μg/ml HA/CS/pEGFP and 40 μg/ml HA/CS/pCrmA nanoparticles, respectively. Following incubation for 72 h, the synoviocytes were collected and used in the subsequent experiments.

Effects of HA/CS/pCrmA nanoparticles on the expression of MMP genes. To investigate the effects of HA/CS/pDNA on the synoviocytes, the expression levels of MMP-3 and MMP-13 genes were detected by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Following transfection and incubation for 72 h, total RNA was extracted from synoviocytes using TRIzol® reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. RNA concentration and purity were determined by a spectrophotometer, and the RNA was reverse transcribed into cDNA using the PrimeScript® RT Master Mix kit (Takara Biotechnology Co., Ltd., Dalian, China) and amplified by PCR. The qPCR assay was performed using SYBR Prime Ex Taq II kit (Invitrogen; Thermo Fisher Scientific, Inc.) on an iCycler iQ Real-Time PCR detection system (Takara Biotechnology Co., Ltd.) under the following conditions: Initial pre-denaturation at 95˚C for 30 sec, then 40 cycles of denaturation at 95˚C for 5 sec, annealing condition at 60˚C for 30 sec and final extension at 72˚C for 30 sec. The specific PCR products were confirmed by melting curve analysis and the target genes were normalized to the expression of GAPDH using the $2^{-\Delta\Delta Cq}$ relative quantification method (22). The forward/reverse primers used were: MMP-3 forward, 5'-GGC CATCTCTTCCTTCAG-3' and reverse, 5'-GTCACTTTC TTTGCATTTGG-3'; MMP-13 forward, 5'-TTCGGCTTA GAGGTGACAGG-3' and reverse, 5'-ACTCTTGCCGGTGTA GGTGT-3'; GAPDH forward, 5'-TGTCGTGGAGTCTACTG GTG-3' and reverse, 5'-GCATTGCTGACAATCTTGAG-3'. GAPDH was used as a normalization control.

Immunohistochemical (IHC) analysis. IHC staining was used to qualitatively detect protein expression levels of the MMP-3 and

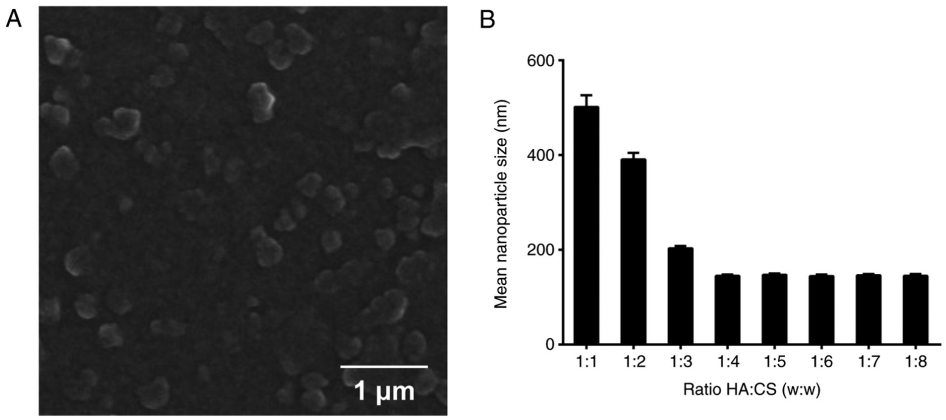

Figure 1. Characterization of HA/CS nanoparticles. (A) Morphologies of the HA/CS/pDNA nanoparticles were observed by scanning electron microscopy. The result indicated that nanoparticles were spherical with diameters between 100-300 nm. (B) Association between the weight ratio of HA to CS and the size of the nanoparticles. There was a significant decrease in nanoparticle size with the increasing amount of CS from 1:1 to 1:4; however, no marked alterations were observed between the weight ratios from 1:4 to 1:8. The smallest nanoparticle size was obtained with the HA:CS weight ratio of 1:4. HA, hyaluronic acid; CS, chitosan.

MMP-13 genes. Following transfection and incubation for 72 h, synovial cells were seeded on a 24-well plate with a slide in each well at a density of $2x10^4$ cells/well. The synoviocytes were fixed in 4% paraformaldehyde for 20 min at 25˚C, washed with PBS and subsequently incubated with primary antibodies against MMP-3 (cat. no. ab52915; 1:200) and MMP-13 (cat. no. ab75606; 1:200; both Abcam, Cambridge, UK) overnight at 4˚C. Following washing with PBS three times, the slides were incubated with secondary antibody labeled with horseradish peroxidase (cat. no. ab205718; 1:5,000; Abcam) at 37˚C for 30 min. Finally, diaminobenzidine was used as the chromogen. The negative control was cells stained without primary antibodies. The images were visualized and captured using the light Nikon H550S Photo Imaging System (Nikon Corporation, Tokyo, Japan; magnifcation, x400). A total of four positively-stained fields from each group were analyzed using Image Pro Plus software 6.0 (Media Cybernetics, Inc., Rockville, MD, USA).

Statistical analysis. All data are expressed as the mean ± standard deviation and analyzed using SPSS software, version 19.0 (IBM Corp., Armonk, NY, USA). Comparison of cell viability was analyzed by unpaired Student's t-test and the gene and protein expression levels of MMP3 and MMP13 among groups were analyzed by one-way analysis of variance followed by a post-hoc Bonferroni test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Characterization of HA/CS/pDNA nanoparticles. HA/CS/pDNA nanoparticles were prepared and the morphologies of the HA/CS/pDNA nanoparticles were observed by SEM. The SEM micrographs indicated that the majority of the HA/CS/pDNA nanoparticles were spherical, with a diameter between 100-300 nm (Fig. 1A), which was consistent with our recent study (23). In order to identify the association between the weight ratio of HA to CS and the size of the nanoparticles, nanoparticles with different weight HA:CS ratios were additionally prepared. As indicated in Fig. 1B, the nanoparticle size exhibited a significant decrease with the weight ratios from 1:1 to 1:4; however, no marked alterations between the weight ratios from 1:4 to 1:8 were observed. The smallest size was obtained with the HA:CS

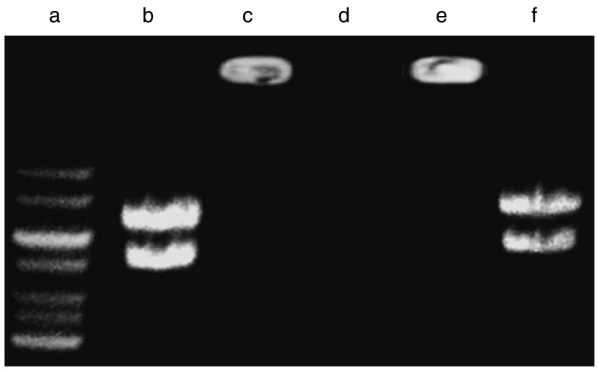

Figure 2. Electrophoresis assay of HA/CS/pCrmA nanoparticles. Lane a, DNA maker; lane b, naked pCrmA; lane c, HA/CS/pCrmA nanoparticles; lane d, naked pCrmA digested by DNase I; lane e, HA/CS/pCrmA nanoparticles digested by DNase I; lane f, HA/CS/pCrmA nanoparticles digested by chitosanase. HA, hyaluronic acid; CS, chitosan; CrmA, cytokine response modifier A.

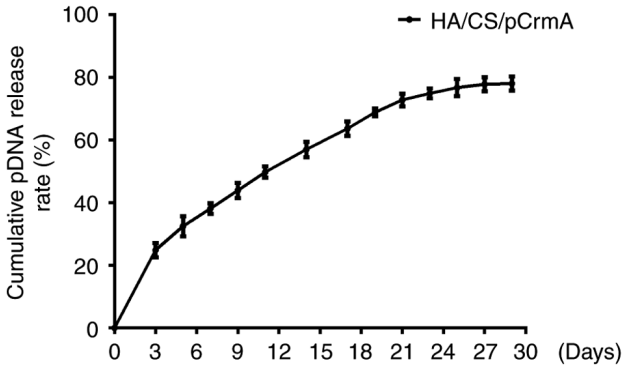

Figure 3. DNA release assay of HA/CS/pCrmA. The release curve exhibited a particularly high initial release rate of ~25% in the first 3 days. Subsequently, a slow release from 37-70% at a constant speed was observed. The pDNA release slowed and leveled off at day 29 at ~77%. HA, hyaluronic acid; CS, chitosan; CrmA, cytokine response modifier A.

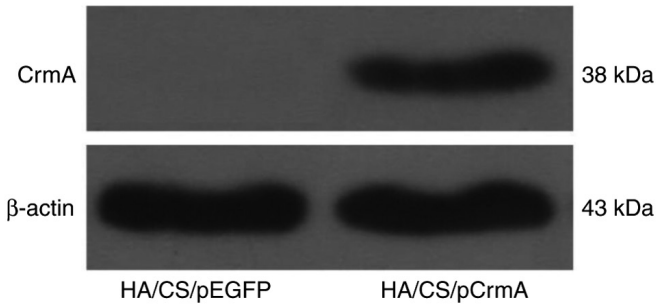

Figure 4. Transfection efficiency of HA/CS/pDNA nanoparticles *in vitro*. Western blot analysis was performed to detect the expression of CrmA by transfected synoviocytes. The marked expression of CrmA in synoviocytes cultured with HA/CS/pCrmA nanoparticles was visually observed. HA, hyaluronic acid; CS, chitosan; CrmA, cytokine response modifier A.

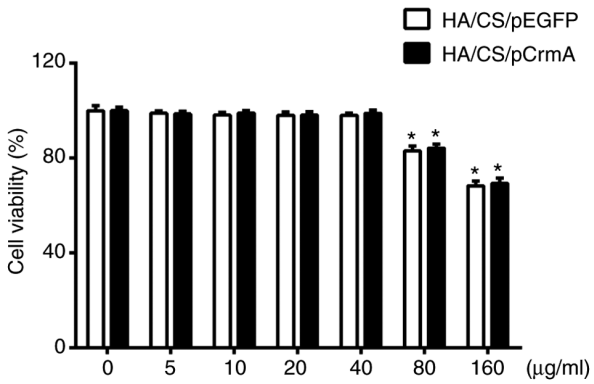

Figure 5. Cell viability. Following transfection and incubation with different concentrations of nanoparticles, cell viability was measured by colorimetric MTS assay. *P<0.05 vs. 0 μg/ml. HA, hyaluronic acid; CS, chitosan; CrmA, cytokine response modifier A; EGFP, enhanced green fluorescent protein.

weight ratio of 1:4. Therefore, HA/CS/pDNA particles at the weight of HA:CS of 1:4 was used in subsequent studies.

Electrophoresis assay. The capability of pDNA retainment and protection of the pDNA against degradation by the nanoparticles was examined by gel electrophoresis. As indicated in Fig. 2, compared with the naked pDNA (lane b), the migration of pDNA in the agarose gel in the HA/CS/pDNA samples was inhibited (lane c), which indicated that the pDNA had completely combined with HA/CS nanoparticles. Following incubation with DNase I, the naked pDNA was digested (lane d), while the pDNA from HA/CS nanoparticles remained intact (lane e). However, following digestion with chitosanase, the pDNA was released from the nanoparticles and the intensity of the DNA bands was similar to that of the control (lane f). Therefore, it may be concluded that HA/CS nanoparticles protected the pDNA from degradation by DNase I.

DNA release study in vitro. The *in vitro* release profiles of pDNA from the HA/CS nanoparticles in PBS are presented in Fig. 3. The release curve exhibited a marked increase in the release rate (~25%) in the first 3 days. Subsequently, a slow release from 37-70% at a constant rate was observed in the subsequent days. The pDNA release rate slowed and leveled off on days 25, 27 and 29, at ~77%.

Transfection efficacy of HA/CS/pDNA nanoparticles in vitro. The synoviocytes were transfected with HA/CS/pDNA nanoparticles, and then the protein expression levels of the target genes were visually detected by western blot analysis. The data presented in Fig. 4 indicated that CrmA protein was expressed in the HA/CS/pCrmA group. The expression of CrmA in the HA/CS/pEGFP group was hardly detectable. These data suggested that the HA/CS nanoparticles were able to transfect the synoviocytes efficiently.

Cell viability. The cytotoxicity of the HA/CS/pDNA nanoparticles was assessed by MTS assay (Fig. 5). When the synoviocytes were co-cultured with HA/CS/pDNA nanoparticles (concentration ≤40 μg/ml), the nanoparticles exhibited good cytocompatibility in the two experimental groups (cell viability >95%). There was no difference in the viability of synoviocytes between the HA/CS/pCrmA and HA/CS/pEGFP groups as the concentration of HA/CS/pDNA nanoparticles increased to 40 μg/ml. When the concentration of nanoparticles increased to 80 and 160 μg/ml, the cell viability exhibited a significant decrease in the two groups (P<0.05). Therefore, it was concluded that the HA/CS nanoparticle was a safe carrier when the concentration was ≤40 μg/ml. To ensure optimum performance of the nanoparticles, nanoparticles with a concentration of 40 μg/ml were selected for all subsequent experiments.

Effects of HA/CS/pCrmA nanoparticles on the expression of MMPs. To additionally investigate the effects of HA/CS/pCrmA nanoparticles on synoviocytes of OA, the expression levels of the MMP-3 and MMP-13 genes at the gene and protein level were detected. As demonstrated in Fig. 6, the control group exhibited significantly increased gene expression levels of MMPs compared with the blank group (P<0.05), indicating that IL-1β may lead to inflammation and the increased production of MMPs. The gene expression levels of MMPs in HA/CS/pEGFP and HA/CS/pCrmA group were decreased compared with the control group (P<0.05). However, the HA/CS/pCrmA group exhibited a significantly decreased expression level of MMPs compared with the HA/CS/pEGFP group (P<0.05).

IHC staining was used to assess the protein expression levels of the MMPs. Fig. 7 indicated that the synoviocytes in the control group were markedly stained compared with that in the blank group (P<0.05), suggesting increased expression levels of MMPs in the control group. In the treatment groups, weakened expression levels of MMPs were observed compared with that in control group; in particular, the synoviocytes cultured with HA/CS/pCrmA nanoparticles exhibited weaker expression of MMPs. These results indicated that HA/CS/CrmA nanoparticles may attenuate IL-1β mediated inflammation in synoviocytes.

Discussion

In the present study, HA/CS nanoparticles were used as vectors to deliver plasmid DNA into synoviocytes in a controlled manner. HA/CS/pCrmA nanoparticles were prepared and the appropriate HA:CS weight ratio of 1:4 was identified and selected for improved transfection efficiency. The synovio-

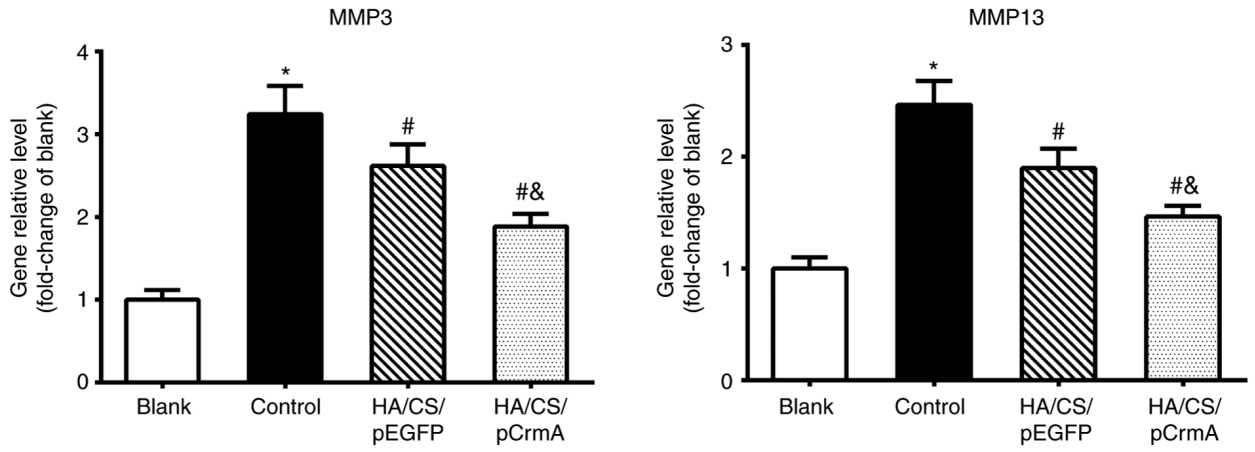

Figure 6. Effects of HA/CS/pCrmA nanoparticles on the mRNA expression of MMPs. The mRNA expression levels of MMPs were analyzed following transfection and incubation with HA/CS/pCrmA nanoparticles in the presence of IL-1β. Data are presented as the mean ± standard deviation (n=4). *P<0.05 vs. blank group; $^{\#}$P<0.05 vs. control group; $^{\&}$P<0.05 vs. HA/CS/pEGFP group. HA, hyaluronic acid; CS, chitosan; CrmA, cytokine response modifier A; EGFP, enhanced green fluorescent protein; MMP, matrix metalloproteinase; IL-1β, interleukin 1β.

cytes were then transfected with nanoparticles and the results indicated that HA/CS/pCrmA nanoparticles were a safe carrier and may decrease the expression level of MMPs genes in synoviocytes.

To ensure the safety and efficiency of gene delivery, biomaterial is preferred compared with virus vectors for preparation of transfection systems. CS is widely used to fabricate carriers and tissue engineering material (24-27). It is a cationic polymer and interacts with negatively-charged cellular membranes (28). However, the single use of CS, due to its poor water solubility, has limited its application (24). HA, as an anionic polymer, is an additional biomaterial with clinical applications (29-31). HA is able to combine with cluster of differentiation 44 (CD44), which is known as a cell surface receptor and widely expressed in a variety of cell membranes (32). In the present study, HA was associated with CS through electrostatic interaction and the HA/CS nanoparticles were formed, as previously described (33). The results indicated that there was a significant decrease in nanoparticle size with the increasing amount of CS, and the smallest size was obtained with a HA:CS weight ratio of 1:4. CS has been demonstrated to interact with pDNA through electrostatic/hydrogen bonding (34). As the amount of CS increased, the nanoparticle size became smaller and the surface charge became more positive (35), thereby contributing to their internalization into synoviocytes and improvement of transfection efficiency (36). Conversely, the conjunction of HA may additionally improve the cell adhesion ability and transfection efficiency (37). HA is able to bind to the CD44 receptors of synoviocytes to improve internalization rate and loosen the interaction between CS and pDNA (38,39), leading to the easy release of the loaded gene following internalization, thereby facilitating gene transfection and expression. Therefore, HA/CS/pDNA nanoparticles at the weight of HA:CS of 1:4 were used in the present study. Subsequently, the capability of the nanoparticles to retain and protect the pDNA from degradation was identified using gel electrophoresis. The results indicated that the migration of pDNA in the agarose gel was completely inhibited in the HA/CS/pCrmA group, suggesting that the pDNA had completely combined with HA/CS nanoparticles. This is consistent with the results of our previous studies (19,20).

The *in vitro* release assay demonstrated that the pDNA release rate leveled off at day 29, and the release rate measured ~77%, demonstrating the long-term transfection and highly efficient release capacity of the complex. The nanoparticles exhibited a sustained release of pDNA over 4 weeks, with a particularly high initial release rate in the first week. These release kinetics, in particular the high level of gene expression in the initial stages, would confer an advantage in the treatment of OA. The expression level of CrmA is an additional key characteristic of the nanoparticles. In the present study, western blot analysis was used to detect the expression level of CrmA in transfected synoviocytes. As expected, the obvious expression of CrmA in synoviocytes cultured with HA/CS/pCrmA nanoparticles was visually observed. This indicates that the HA/CS/pCrmA nanoparticles had successfully transfected the synoviocytes. The MTS assay indicated that the HA/CS/pDNA nanoparticles exhibited low cytotoxicity when cultured with synoviocytes at the concentrations of 5, 10, 20 and 40 μg/ml, while the nanoparticles at high concentrations (80 and 160 μg/ml) exhibited significant levels of cytotoxicity. This result indicated that HA/CS nanoparticles, at appropriate concentrations, possessed good cytocompatibility. In summary, HA/CS/pDNA nanoparticles may be used in a gene delivery system with considerable efficacy and low toxicity.

IL-1β serves a key role in the pathogenesis and progression of OA (40). It may stimulate chondrocytes and synoviocytes to secrete proteinases leading to cartilage destruction (41), and inhibit the synthesis of collagen type II (42), which is the primary component of the ECM in articular cartilage. Therefore, IL-1β may be a promising target for the treatment of OA. CrmA is a natural inhibitor of caspase-1 (43), which has great specificity for cleaving the precursor pro-IL-1β, thereby decreasing the secretion of IL-1β (44). In the present study, the IL-1β-induced synoviocytes were considered to represent an *in vitro* OA model. To additionally investigate the therapeutic potential of HA/CS/pCrmA nanoparticles on synoviocytes of OA, the expression levels of the osteogenic genes MMP-3 and MMP-13 were detected. As demonstrated in the results, the HA/CS/pEGFP and HA/CS/pCrmA groups exhibited greater downregulated expression levels of MMPs compared with the controls. In addition, the

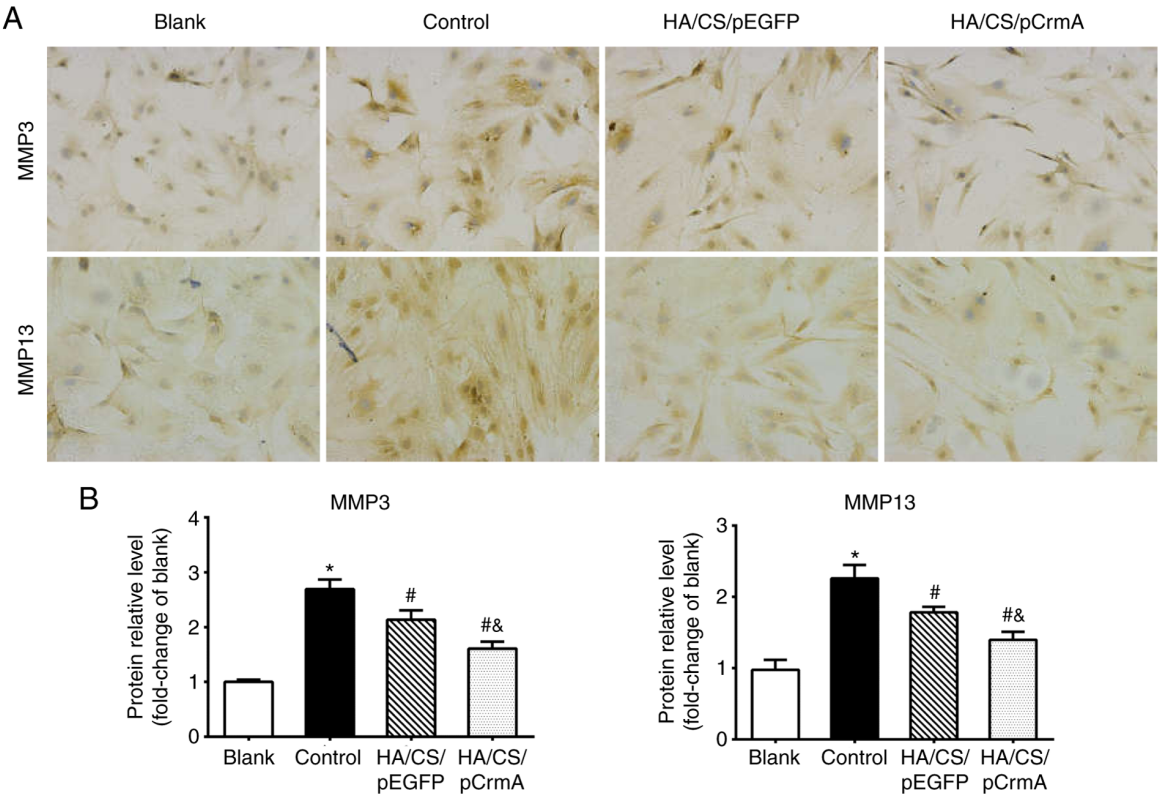

Figure 7. Effects of HA/CS/pCrmA nanoparticles on the protein expression of MMPs. (A) Representative pictures of immunohistochemical staining were used to assess the protein levels of the MMPs (magnification, x400). (B) Protein expression levels of MMPs (n=4). *P<0.05 vs. blank group; #P<0.05 vs. control group; &P<0.05 vs. HA/CS/pEGFP group. HA, hyaluronic acid; CS, chitosan; CrmA, cytokine response modifier A; EGFP, enhanced green fluorescent protein; MMP, matrix metalloproteinase.

HA/CS/pCrmA nanoparticles decreased the expression level of MMPs genes significantly compared with the HA/CS/pEGFP nanoparticles, indicating that the inflammation of synoviocytes was attenuated by HA/CS/pCrmA nanoparticles. HA and CS have been widely used in clinical settings. HA injection has been used in the treatment of OA (45), and CS is structurally similar to glycosaminoglycans (46), which are important components of the ECM. It was concluded that the use of HA/CS nanoparticles may have beneficial effects on synoviocytes; concomitantly, CrmA exhibited great potential in inhibiting the inflammatory response in synoviocytes of OA. As aforementioned, the suppression of inflammatory cytokines activity may be an important mechanism of attenuating IL-1β-induced inflammation in synoviocytes.

In the present study, HA/CS nanoparticles were successfully constructed as vectors for the delivery of CrmA into synoviocytes. As expected, the HA/CS/pCrmA nanoparticles exhibited good safety and biocompatibility, and conferred protection against inflammation in synoviocytes induced by IL-1β *in vitro*. Furthermore, HA/CS/pCrmA nanoparticles have additionally been indicated to serve protective effects on the cartilage damage and synovial inflammation in a rat anterior cruciate ligament transaction model of OA *in vivo* in our recent study (23). Therefore, the HA/CS/ pCrmA nanoparticles may present a potential novel approach for the gene therapy of OA in a clinical setting.

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Availability of data and materials

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

Authors' contributions

BQ, X-FX and P-HZ conceived and designed the experiment. R-HD, G-QX and X-FS performed the experiments. G-QX acquired the reagents and materials. XS analyzed the data. X-FX and P-HZ wrote the manuscript. All authors approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was approved by the Institutional Animal Care and Use Committee of Wuhan University (approval no. 2017-0208).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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