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NF-κBp65-specific siRNA inhibits expression of genes of COX-2, NOS-2 and MMP-9 in rat IL-1β-induced and TNF-α-induced chondrocytes

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Summary

Objective: Small interfering RNA (siRNA) triggers RNA interference in mammalian somatic cells. Nuclear factor κB (NF-κB) is a transcription factor that is implicated in inflammation and immune activation. This study was to use NF-κBp65-specific siRNA to inhibit the expression of genes of cyclooxygenase-2 (COX-2), nitric oxide synthase-2 (NOS-2) and matrix metalloproteinase-9 (MMP-9), which is paralleled with the initiation and progression of cartilage lesions in osteoarthritis (OA) model, in induced chondrocytes, and therefore to explore a new gene therapy for OA.

Methods: Western blot and reverse transcriptase polymerase chain reaction (RT-PCR) were performed to optimize the silencing effects of NF-κBp65-specific siRNA in cultured rat chondrocytes, and then to determine the expression of COX-2, NOS-2 and MMP-9 in induced chondrocytes. The activation of NF-κB was determined by electrophoretic mobility shift assay (EMSA). Western blot and RT-PCR were subjected to densitometric analysis and then band intensities were also determined.

Results: The NF-κBp65-specific siRNA inhibited the expression of NF-κBp65 and activation of NF-κB, reducing significantly the expression of COX-2, NOS-2 and MMP-9 induced by interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) in cultured chondrocytes.

Conclusions: NF-κBp65-specific siRNA can inhibit the expression of COX-2, NOS-2 and MMP-9 in IL-1β-induced and TNF-α-induced chondrocytes. This suggests that NF-κBp65-specific siRNA has potential to be a useful, preventive and therapeutic agent for OA at early stages.

Key words: siRNA, NF-κBp65, Articular chondrocytes, COX-2, NOS-2, MMP-9, IL-1β, TNF-α.

Introduction

Osteoarthritis (OA) is a degenerative joint disease affecting a large population, and results in significant morbidity and disability. It is strongly associated with aging and sport injury. Although excessive or traumatic joint loading coupled with genetic predisposition is considered to be an initiative factor in OA[1], the etiology of OA is poorly understood, except that several cytokines and enzymes in human OA have been reported. Studies have shown that interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), synthesized locally by synovial cells and chondrocytes, are pivotal driving forces in inducing and sustaining cartilage damage in arthritis due to their ability of suppressing the balance between excessive cartilage destruction and cartilage repair process[2–4]. They can inhibit proteoglycan anabolism and enhance the degradation of extracellular matrix components through matrix metalloproteinases' (MMPs) activation[5,6]. Activated MMPs can cleave most of the components of cartilage extracellular matrix including type II collagen and aggrecan. IL-1β and TNF-α can also mediate their effects through cyclooxygenase-2 (COX-2) induction and nitric oxide synthase-2 (NOS-2) expression, which produce the inflammatory mediators prostaglandins and nitric oxide (NO) that are responsible for the pain and swelling of the inflamed joint[7–11]. Furthermore, NO interferes with chondrocyte function, resulting in loss of cartilage matrix through MMPs' activation, induction of apoptosis or inhibition of proteoglycan and type II collagen synthesis[12–15]. The mediated induction of COX-2 produces high levels of prostaglandin E_2 (PGE_2), which mediates cartilage resorption by decreasing proliferation of chondrocytes, enhancing MMPs' activity, and inhibiting aggrecan synthesis in chondrocytes[16]. Dumond *et al.*[17] studied in rat early experimental OA and demonstrated that the expression of typical factors such as COX-2, NOS-2 and MMPs is paralleled with the initiation and progression of cartilage lesions in OA model.

Ideally, successful treatment of OA would not only reduce or eliminate joint pain, but also retard or reverse the loss of articular cartilage. In this respect, present treatment is unsatisfactory. For example, nonsteroidal anti-inflammatory drugs, the most commonly used agents in the management of arthritis, improve the quality of life by diminishing pain and inflammation; intra-articular corticosteroids and hyaluronan are useful with acutely inflamed painful joints[18,19]; and joint lavage has been shown to improve symptoms of knee OA[20]. But all these frequently cause adverse side effects and do not modify the course of the degenerative process. However, gene therapy offers one way to obviate this limitation. One target gene in the treatment of arthritis is nuclear factor κB (NF-κB).

NF-κB is a transcription factor that is implicated in inflammation and immune activation[21–23], activated by oxidants and cytokines[24–27] such as IL-1β and TNF-α, which play

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important roles in the initiation and development of OA. A total of five subunits that form dimers have been identified in mammalian cells, that is, RelA (p65), RelB, c-Rel, p50, and p52. The most common and best characterized form of NF-κB is the RelA−p50 heterodimer. Each dimer combination exhibits differences in DNA-binding affinity and transactivation potential, and its DNA-binding activity is important for many cytokine-inducible responses. NF-κB is retained in the cytoplasm through heterodimerization with IκB proteins that mask the NF-κB nuclear location signal. IL-1β and TNF-α trigger the phosphorylation and degradation of IκB, thus permitting the entry of p65/p50 into the nucleus, where p65/p50 activates the transcription of various genes, and two of the many genes are NOS-2[28,29] and COX-2[30,31]. p65 has shown to be a key active subunit in NF-κB transcription in several cell types[32–35]. Since NF-κBp65 plays a major role in the expression of key inflammatory cytokines that involve in the pathogenesis of arthritis, efficient methods are needed to interrupt its action.

In the present study, we used small interfering RNA (siRNA), which is able to trigger RNA interference in mammalian somatic cells[36,37], to inhibit the expression of p65 in cultured IL-1β-induced and TNF-α-induced chondrocytes, so as to explore the inhibitive effects in transcription activation of NF-κB, and further observe the expression of MMPs, NOS-2, and COX-2 in both levels of mRNAs and proteins. Our results provided evidences that NF-κBp65-specific siRNA can effectively inhibit the transcription activation of NF-κB and the expression of MMP-9, NOS-2 and COX-2 in induced chondrocytes.

Materials and methods

siRNA TEMPLATE DESIGN

Ambion's siRNA target design online tool was utilized to choose five sequences (Table I) for target rat NF-κBp65 mRNA. BLAST searches were conducted on all sequences to ensure gene specificity. All template oligonucleotides were synthesized by AuGCT Biotechnology Co., Ltd. (Beijing, PR China). All siRNA duplexes were synthesized with the silencer siRNA construction kit (Ambion, Austin, TX) according to the manufacturer's protocol. The positive (anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) siRNA) and negative control (scrambled siRNA) siRNAs were purchased from Ambion. The procedure overview was: In separate reactions, two template oligonucleotides were hybridized to a T7 Promoter Primer and extended by the Klenow fragment of DNA polymerase to create double-stranded siRNA transcription templates. The sense and antisense siRNA templates were transcribed by T7 RNA polymerase and the resulting RNA transcripts were hybridized to create dsRNA. The leader sequences were removed by digesting the dsRNA with a single-stranded specific ribonuclease, and the DNA template was removed at the same time by a deoxyribonuclease. The resulting siRNA was purified by glass fiber filter binding and elution which removed excessive nucleotides, short oligomers, proteins, and salts in the reaction.

CHONDROCYTES' ISOLATION AND CULTURE

Articular chondrocytes were isolated from femoral heads and knees of Sprague Dawley male rats (200−250 g, Experimental Animal Center of Peking University Health Science Center, Beijing, PR China) under aseptic conditions. The primary chondrocytes were obtained by enzymatic dispersal of cartilage as previously described[38]. Cells were seeded into Costar 24-well plates (Costar, Corning, NY) at a final density of 1×10^5 cells/well in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT) and cultured for 3 days under normal growth conditions. Experiments were performed with the first or second passage cells. In each experiment, monolayer cells were made quiescent for 24 h in DMEM medium without serum, and then incubated in 1% FBS with or without the transfection complexes for 4 h as described below, after which they were processed for the various analyses.

siRNA TRANSFECTION AND CYTOKINE INDUCTION

Three days postseeding and quiescent for 24 h, NF-κBp65-specific siRNA was transfected into chondrocyte monolayers using the siPORT™ Lipid (Ambion) according to the manufacturer's protocol. After 4 h of transfection, 2 ml fresh medium containing 10% FBS was added to the plates. According to different experiments, chondrocytes were stimulated with IL-1β (Sigma, St. Louis, MO) or TNF-α (Sigma) at a concentration of 10 ng/ml, which were optimized formerly (data not shown), in 1 ml complete medium for 24 h or 1 h at indicated time points, then the cells were harvested from three independent wells and used for the following analyses. This procedure was performed at least three times. The positive and negative controls were analyzed in parallel.

SELECTION OF OPTIMAL siRNA AND OPTIMIZATION OF TRANSFECTION CONDITION

The effects of silencing were determined by Western blot and reverse transcription polymerase chain reaction (RT-PCR) analyses. The starting amount for transfection

Table I
siRNA template sequences

Sequence range (GC%)	siRNA oligonucleotide template	
siRNA$^{150-170}$ (42.9)	Antisense	5′-AAGCACAGATACCACTAAGACCCTGTCTC-3′
	Sense	5′-AAGTCTTAGTGGTATCTGTGCCCTGTCTC-3′
siRNA$^{665-685}$ (33.3)	Antisense	5′-AAGACATTGAGGTGTATTTCACCTGTCTC-3′
	Sense	5′-AATGAAATACACCTCAATGTCCCTGTCTC-3′
siRNA$^{740-760}$ (47.6)	Antisense	5′-AAGTGGCCATTGTGTTCCGAACCTGTCTC-3′
	Sense	5′-AATTCGGAACACAATGGCCACCCTGTCTC-3′
siRNA$^{909-929}$ (47.6)	Antisense	5′-AAGGACCTACGAGACCTTCAACCTGTCTC-3′
	Sense	5′-AATTGAAGGTCTCGTAGGTCCCCTGTCTC-3′
siRNA$^{928-948}$ (38.1)	Antisense	5′-AAGAGCATCATGAAGAAGAGTCCTGTCTC-3′
	Sense	5′-AAACTCTTCTTCATGATGCTCCCTGTCTC-3′

of siRNA was 0.3 μg/ml and the ratio of siRNA to transfection reagent was 1:1 according to the manufacturer's recommendations. To optimize the contribution of siRNA concentration and siRNA/transfection reagent ratio to transfection condition, concentrations of siRNA were varied between 0.3 μg/ml and 2.4 μg/ml, siRNA and siRNA/transfection reagent ratios were varied between 1:1 and 1:10. A time-course experiment was performed at 0 h, 24 h, 48 h, 72 h, 96 h and 120 h after transfection and 24 h stimulation with IL-1β. Finally, the optimized concentration was determined at 1.2 μg/ml and the ratio 1:1, the time point after transfection was 72 h.

NUCLEAR PROTEIN ISOLATION AND ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

EMSA was used to study the effect of siRNA on NF-κB activation. Nuclear proteins of chondrocytes were isolated using NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Pierce, Rockford, IL) according to the manufacturer's protocol. The concentration of the proteins in the samples was measured using the BCA Protein Assay Kit (Pierce) and their assay protocol. The oligonucleotides for the NF-κB consensus sequence were 5′-AGTTGAGGG GACTTTCCCAGGC-3′ and 3′-TCAACTCCCCTGAAAGGG TCCG-5′ which were end-labeled with biotin (synthesized by Beijing AuGCT Biotechnology Co., Ltd.). Complementary oligonucleotides were annealed in 10 mmol/l Tris–Cl, pH 8.0, 1 mmol/l EDTA, pH 8.0, and 0.1 mol/l NaCl (STE buffer) by slow cooling to room temperature in boiling water. Nuclear extracts (5 μg) were added in 20 μl of binding reactions and incubated for 20 min at room temperature. EMSA were performed according to the manufacturer's protocol of LightShift® Chemiluminescent EMSA Kit (Pierce). To establish the specificity of the reaction, negative controls without cell extracts and competition assays with a 200-fold excess of unlabeled oligonucleotide were performed. In competition assays, the corresponding unlabeled probe was added to the reaction mixture 10 min before the addition of the labeled probe.

mRNA EXPRESSION ANALYSIS

Total mRNA was isolated from cultured chondrocytes with Trizol (Invitrogen, Carlsbad, CA). Isolated RNA was reverse transcribed and then amplified with a commercial kit (Access RT-PCR system®, Promega, Madison, WI) according to the manufacturer's protocol. The specific primers, which were designed with software of Primer Premier 5, used for RT-PCR, temperature of annealing (Ta) and cycles are shown in Table II. Total RNAs (2 μg) were reverse transcribed for 45 min at 54 °C, and PCR amplification was then performed in a volume of 25 μl. The house-keeping gene GAPDH was amplified as an internal control for normalization. PCR products were separated on 1.5% agarose gels and visualized by ethidium bromide staining. DNA bands were visualized under UV light, photographed and quantitated by densitometric analysis.

WESTERN BLOT ANALYSIS

Total proteins were extracted from the cultured chondrocyte monolayers with Trizol (Invitrogen) on the indicated days, and protein concentrations were determined by the BCA method. Western blot analysis was performed as previously described[39]. The blots were immersed in 20 ml of blocking buffer (5% skimmed milk and 1‰ Tween-20 in Tris-buffered saline (TBST)) for 2 h at room temperature and subsequently incubated overnight at 4 °C with the following primary antibodies: polyclonal anti-NF-κBp65 (1/500), anti-COX-2 (1/400), anti-MMP-9 (1/500), monoclonal anti-NOS-2 (1/400) and anti-GAPDH (1/5000), followed by a secondary IgG (1/10,000) conjugated to horseradish peroxidase, then were washed three times for 15 min each in 20 ml of TBST with gentle shaking. Visualization of the immunocomplexes was conducted with the Luminol reagent kit according to the manufacturer's specifications. The blots imaged by autoradiography were quantified by densitometry. All reagents were from Santa Cruz (Santa Cruz, CA), except for anti-GAPDH (Ambion).

STATISTICAL ANALYSIS

Densitometric results, expressed as arbitrary units (AU) as *n*-fold over control are expressed as the mean ± standard error of the mean (S.E.M.). Comparisons between two groups were made by Student's *t* test, comparisons among multiple groups were made by one-way analysis of variance (ANOVA) and least significant difference (LSD) *post hoc* multiple comparison tests, at a significance level of $P < 0.01$.

Results

THE IDENTIFICATION OF SYNTHESIZED siRNA BY *IN VITRO* TRANSCRIPTION

The degree of purification and the concentration of siRNA used for transfection are critical to the success of gene silencing experiment. Transfecting too much siRNA causes

Table II
Specific primers used for RT-PCR

Gene		Primer sequence	Size (bp)	Ta* (°C)	Cycles
NF-κBp65	Forward	5′-TCACCAAAGACCCACCTCACCG-3′	243	60	35
	Reverse	5′-GGACCGCATTCAAGTCATAGTCCC-3′			
COX-2	Forward	5′-GTGGGATGACGAGCGACTG-3′	288	57	28
	Reverse	5′-CCGTGTTCAAGGAGGATGG-3′			
NOS-2	Forward	5′-TTCAGATCCCGAAACGCTACAC-3′	308	60	30
	Reverse	5′-ACAATCCACAACTCGCTCCAAG-3′			
MMP-9	Forward	5′-CCTGCGTATTTCCATTCATC-3′	498	58	34
	Reverse	5′-GCCTTGGGTCAGGTTTAGAG-3′			
GAPDH	Forward	5′-ATCATCTCCGCCCCTTCTGC-3′	437	57	25
	Reverse	5′-GCCTGCTTCACCACCTTCTT-3′			

*Ta: temperature of annealing.

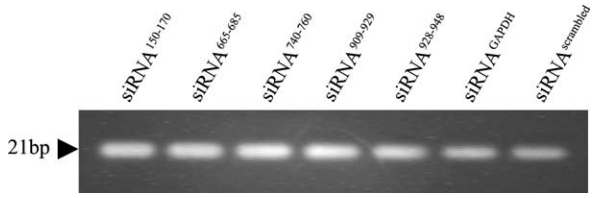

Fig. 1. Seven synthesized siRNAs were analyzed by electrophoresis on 2% agarose gel and ethidium bromide staining. The single band was present at 21 bp.

nonspecific reductions in gene expression and toxicity to the transfected cells, while transfecting too little siRNA does not change the target gene, and poor purification can impair the effect of gene silencing. When we measured the absorbance of siRNA sample at 260 nm with the UV spectrophotometer to assess the concentration of the siRNA preparation, concentrations of seven siRNAs were 537 μg/ml, 539 μg/ml, 571 μg/ml, 585 μg/ml, 554 μg/ml, 532 μg/ml, and 521 μg/ml, respectively. The degree of purification of siRNA was assessed by gel electrophoresis on 2% agarose in 90 mmol/l Tris−Cl, 90 mmol/l boric acid, and 2 mmol/l EDTA (TBE) that showed a single band at 21 bp (Fig. 1). These siRNAs were diluted to 300 μg/ml with 10 mmol/l Tris−Cl, pH 8.0, and 1 mmol/l EDTA, pH 8.0 (TE) and stored at −80°C.

OPTIMIZATION OF THE siRNA TRANSFECTION

The silencing effect of siRNA varied with the sequence of siRNA, the total siRNA content for transfection and the siRNA/transfection reagent ratio. Of the siRNAs tested, the silencing of NF-κBp65 was achieved at 48 h posttransfection with siRNA$^{928-948}$-based RNA with 1.2 μg/ml siRNA and transfection reagent/siRNA ratio of 1:1, the expression of NF-κBp65 was reduced to 71.69% at the level of mRNA and to 74.82% at the level of protein (Fig. 2). The obvious silencing of NF-κBp65 at the level of mRNA was started from the concentration of 1.2 μg/ml; and at the level of protein it was started from the concentration of 0.6 μg/ml (Fig. 3). The fact of no reduction in NF-κBp65 mRNA levels at 0.6 μg/ml but a decrease in protein levels may suggest post-translational regulation of p65 level[40]. A time-course experiment was performed at multiple time points after transfection, the expression of NF-κBp65 significantly decreased at 48 h and kept silencing effect for 48 h (Fig. 4). These optimal schemes were used in subsequent experiments.

THE EFFECTS OF NF-κBP65-SPECIFIC siRNA$^{928-948}$ ON THE ACTIVATION OF NF-κB

On EMSA, exposure to IL-1β or TNF-α significantly enhanced NF-κB binding in comparison with normal chondrocytes, and the effect of TNF-α (lane 6) was greater than that of IL-1β (lane 4) (Fig. 5). However, siRNA$^{928-948}$-preincubated IL-1β-stimulated or TNF-α-stimulated chondrocytes showed a significant inhibition of NF-κB binding (lanes 5 and 7), but not reducing to the level of normal chondrocytes. This binding reaction was specific since unlabeled NF-κB probe prevented the formation of the complexes with biotin-labeled NF-κB probe (lane 3).

NF-κBP65-SPECIFIC siRNA$^{928-948}$ INHIBITED THE EXPRESSION OF COX-2, NOS-2 AND MMP-9 IN CHONDROCYTES INDUCED BY IL-1β

In cultured chondrocytes, IL-1β remarkably increased the expression of COX-2, NOS-2 and MMP-9, but pretransfection of siRNA$^{928-948}$ reduced the expression of COX-2, NOS-2 and MMP-9 to 45.12%, 31.26% and 52.28%, respectively, at the level of mRNA and to 42.31%, 29.49% and 50.42%, respectively, at the level of protein induced by IL-1β + lipid, and did not reduce to the levels of normal chondrocytes. There was no difference between IL-1β group and IL-1β + lipid group, suggesting that transfection agent had no effect on the expression of these genes. Among groups of positive control, negative control and IL-1β + lipid, there also was no significant difference, which indicated that

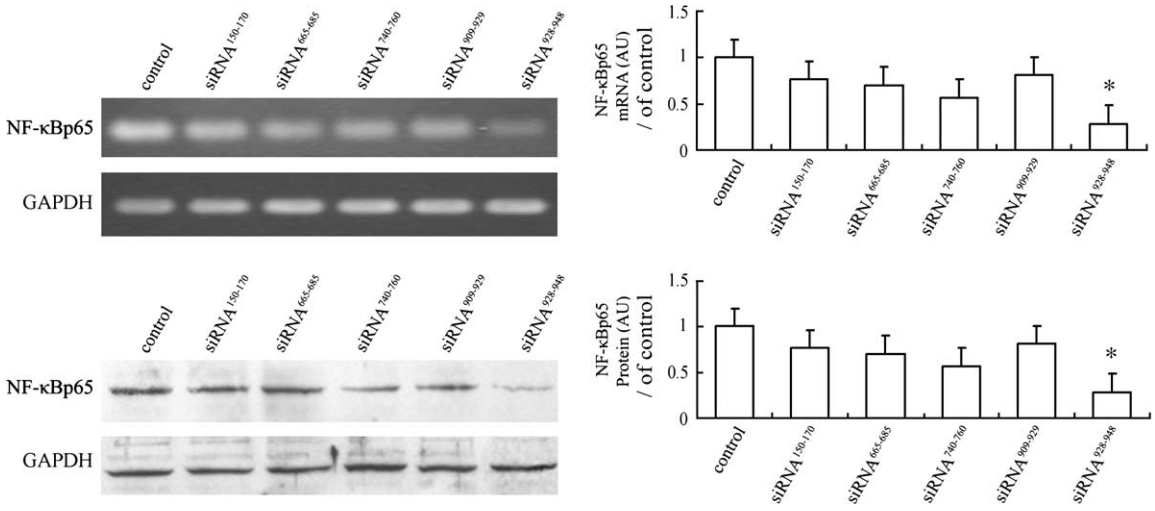

Fig. 2. Different RNAi activities induced by different siRNAs. Rat chondrocytes were transfected with different siRNAs at a concentration of 1.2 μg/ml for 48 h, and then stimulated with IL-1β (10 ng/ml) for 24 h, the expression of NF-κBp65 was assessed at levels of mRNA and protein by RT-PCR and Western blot. The densitometric quantification of NF-κBp65 was normalized to GAPDH. Upper panel: Representative RT-PCR of NF-κBp65 and GAPDH expression, graph shows the mean ± S.E.M. of the three independent experiments. Lower panel: Corresponding Western blot analysis for both NF-κBp65 and GAPDH, and graph shows NF-κBp65 at protein levels. *$P < 0.01$ vs control.

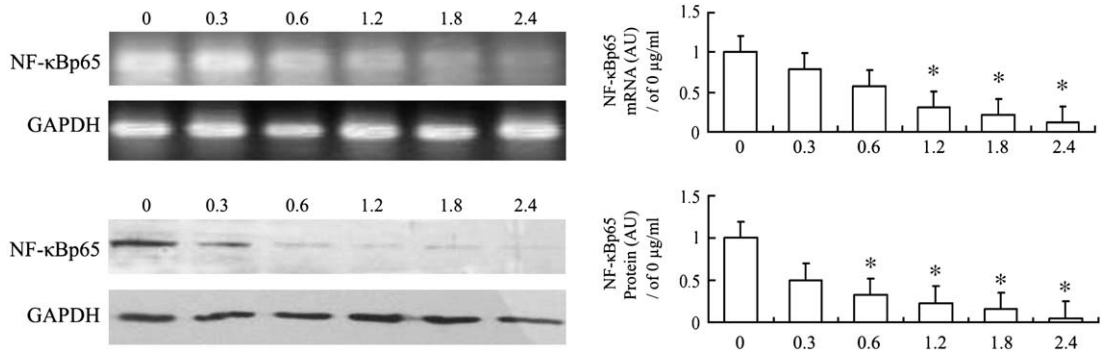

Fig. 3. The silencing effect of siRNA$^{928-948}$ on the expression of NF-κBp65 at different concentrations (μg/ml). The rat chondrocytes were transfected with siRNA$^{928-948}$ at concentrations of 0 μg/ml, 0.3 μg/ml, 0.6 μg/ml, 1.2 μg/ml, 1.8 μg/ml and 2.4 μg/ml for 48 h, and then stimulated with IL-1β (10 ng/ml) for 24 h, the expression of NF-κBp65 was assessed at levels of mRNA and protein by RT-PCR and Western blot. The densitometric quantification of NF-κBp65 was normalized to GAPDH. Upper panel: Representative RT-PCR of NF-κBp65 and GAPDH expression, graph shows the mean ± S.E.M. of the three independent experiments. Lower panel: Corresponding Western blot analysis for both NF-κBp65 and GAPDH, and graph shows NF-κBp65 at protein levels. *$P < 0.01$ vs control.

the silencing effect of siRNA$^{928-948}$ was specific (Fig. 6). At the same time, transfection of siRNA^{GAPDH} reduced the expression of GAPDH to 21.39% at the level of mRNA and to 19.87% at the level of protein (Fig. 6), which could be regarded as the transfection efficiency of siRNA by transfection agent. This also confirmed the selectivity of the NF-κBp65-specific siRNA and the viability of the system.

NF-κBP65-SPECIFIC siIRNA$^{928-948}$ INHIBITED THE EXPRESSION OF COX-2, NOS-2 AND MMP-9 IN CHONDROCYTES INDUCED BY TNF-α

In cultured chondrocytes, TNF-α also remarkably increased the expression of COX-2, NOS-2 and MMP-9, but when we pretransfected siRNA$^{928-948}$ into chondrocytes, the expression of COX-2, NOS-2 and MMP-9 was reduced to 30.19%, 21.32% and 44.98%, respectively, at the level of mRNA, and 26.91%, 19.85% and 48.26%, respectively, at the level of protein induced by TNF-α + lipid. And the expression of NOS-2 was the most markedly decreased. There was no significant difference between TNF-α group and TNF-α + lipid group, and among groups of positive control, negative control and IL-1β + lipid (Fig. 7).

Discussion

Because the pathogenesis of OA remains elusive, it remains incurable despite the existence of an extensive medical and surgical armamentarium. In recent years, gene therapy targeting cytokines offers a new hope to OA treatment, and IL-1β and TNF-α are considered to be the principal inflammatory cytokines[41,42] and are thus natural targets for therapy for OA. Therefore the use of biological agents that block the activity of these cytokines is the current focus[43]. These agents include such molecules as TNF-α soluble receptor (TNF-sR)[44], IL-1 receptor antagonists (IL-1Ra)[45,46], anti-inflammatory cytokines[47–49] and inhibitors of catabolic enzyme[50]. As there are many proinflammatory cytokines, oxidants and other factors exerting action in initiation and development of OA, it is hard to get complete therapeutic effects in blocking the activity of one or two cytokines. So we attempted to inhibit signal pathway of NF-κB, which is an important mediator of cellular responsiveness to immunogenic, stress and mitogenic signals leading to the induction of a number of genes encoding for cytokines, cell adhesion molecules, immunoreceptors, and oxidative stress-related enzymes[51], to explore a new approach of gene therapy on OA.

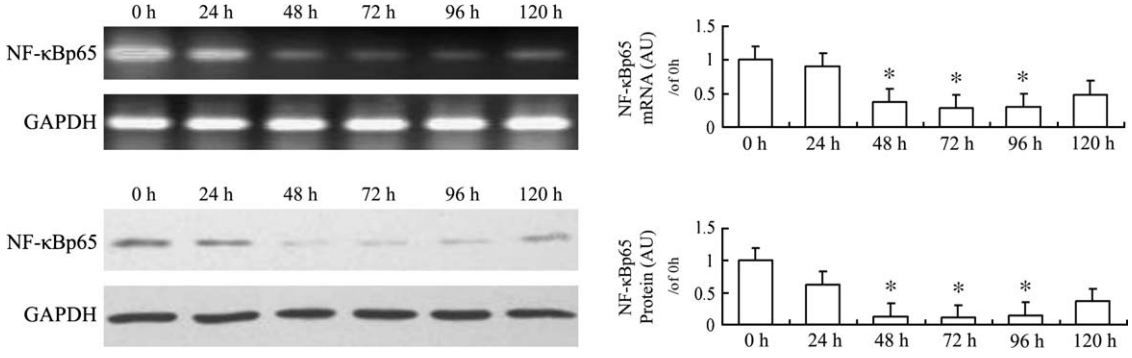

Fig. 4. siRNA$^{928-948}$ silencing of NF-κBp65 over time. The rat chondrocytes were transfected with siRNA$^{928-948}$ at a concentration of 1.2 μg/ml for 0 h, 24 h, 48 h, 72 h, 96 h and 120 h. Before harvest, the cells all were stimulated by IL-1β (10 ng/ml) for 24 h except for the cells that were transfected for 24 h, and stimulated for 18 h. The expression of NF-κBp65 was assessed by RT-PCR and Western blot, the expression of NF-κBp65 significantly decreased at 48 h and kept silencing effect for 48 h, and then increased at 120 h. The densitometric quantification of NF-κBp65 was normalized to GAPDH. Upper panel: Representative RT-PCR of NF-κBp65 and GAPDH expression, graph shows the mean ± S.E.M. of the three independent experiments. Lower panel: Corresponding Western blot analysis for both NF-κBp65 and GAPDH, and graph shows NF-κBp65 at protein levels. *$P < 0.01$ vs control.

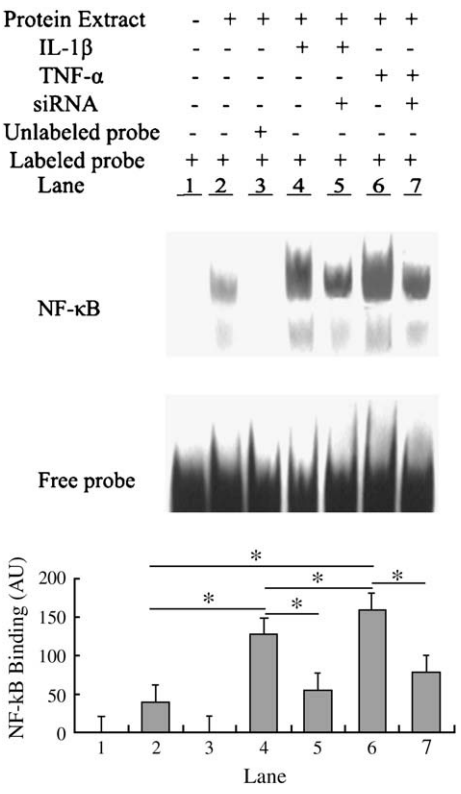

Fig. 5. The effects of IL-1β, TNF-α and siRNA$^{928-948}$ on NF-κB DNA binding in rat chondrocytes. Cells were preincubated for 72 h with siRNA$^{928-948}$ at 1.2 μg/ml, and then stimulated with IL-1β (10 ng/ml), and TNF-α (10 ng/ml), respectively, for 1 h. Upper panel: Representative autoradiogram of the three different experiments with similar results is shown. The specificity of the reaction was established using competition assays with a 200-fold excess of unlabeled probe (lane 3). The positions of the specific NF-κB complexes are indicated. Lower panel: Results of the densitometric analysis for the specific NF-κB binding are shown (mean ± S.E.M.). Asterisks (*) denote values which differ at $P < 0.01$.

NF-κB is one of the principal proinflammatory transcription factors[52]. Several methods have been used to inhibit the function of NF-κB ranging from specific small molecule inhibitors to nucleotide-based approaches such as antisense oligonucleotides[53], decoy DNA[54], and dominant negative mutant IκB in an adenoviral construct[78]. In the present study, we utilized siRNAs to knockdown p65 subunit and inhibited the function of NF-κB. The rationale for choosing p65 but not other potential NF-κB subunits was because p65, which contains a C-terminal transactivation domain in addition to the N-terminal Rel-homology domain, is the most common transcriptionally active subunit of NF-κB[32–35], and there are some relevant reports which targeted at p65 to study the effects of NF-κB in different cells[53,55–58]. So we synthesized NF-κBp65-specific siRNA by *in vitro* transcription, screened out one efficacious siRNA in cultured rat chondrocytes, and used it to carry out the subsequent experiment.

Posttranscription gene silencing (PTGS) and RNAi are terms describing the specific suppression of genes by complementary dsRNA[59]. Although the mechanism by which dsRNA suppresses gene expression is not entirely understood, experimental data provide important insights. In non-mammalian systems such as *Drosophila*, it appears that longer dsRNA is processed into 21–23 nt dsRNA (called small interfering RNA or siRNA) by an enzyme containing RNase III motifs[60–64]. The siRNA apparently then acts as a guide sequence within a multicomponent nuclease complex to target complementary mRNA for degradation[65]. However, mammalian cells have a potent antiviral response pathway that induces global changes in gene expression when dsRNA molecules longer than 30 nt are introduced into cells[66,67]. The antiviral response makes it difficult to distinguish target-specific effects of long dsRNA from the general antiviral response. Whereas, siRNA, comprising 21-mer dsRNAs, do not trigger the antiviral response, making it possible to perform gene silencing experiments in mammalian cells[36,37,68,69]. So we used 21-mer siRNA to perform experiments in rat chondrocytes.

Research at Ambion has found that typically more than half of randomly designed siRNAs provide at least a 50% reduction in target mRNA levels and approximately one of four siRNAs provides a 75–95% reduction[69]. It might be that some regions of mRNA may be either highly structured or bounded by regulatory protein, and difference in internal configuration of siRNA may also be one reason[70]. Our study indicated that one siRNA$^{928-948}$ could significantly reduce expression of p65 at levels of mRNA and protein, and others had no significant silencing effects.

Cells transfected with effective siRNA exhibit a reduction in the amount of the targeted mRNA and the protein that it encoded. To assess whether siRNA-mediated gene silencing occurred, levels of target RNA and target protein can be monitored. In our study, we examined NF-κBp65 protein level by Western blot and mRNA level by RT-PCR and found that expression of NF-κBp65 was obviously less in siRNA-transfected cells than in mock-transfected cells on both levels. In the subsequent experiment, both methods indicated good knockdown by the positive control siRNA^{GAPDH}, further indicating that the system worked well and with good silencing effects. These data suggested that the NF-κBp65-specific siRNA could bring action in primary cultured chondrocytes and silenced the expression of p65. Olaf *et al.*[55] utilized siRNA against the NF-κBp65 to significantly reduce the p65 protein, then to significantly suppress the secretion of IL-8 from BEAS-2B cells (The BEAS-2B cell line was derived from a human bronchial epithelial tumor cell that was transformed with Ad12-SV40 construct.) in a cellular model of TNF-α induced inflammation. Roopashree *et al.*[56] utilized siRNA PCR products targeting p65 cDNA to induce up to 92% reduction in hemagglutitin (HA)-p65 protein levels, a six-fold decrease in NF-κB dependent luciferase activity, and resulted in 70% reduction in p65 protein levels and blocked 13-hydroperoxy-octadecadienoic acid (13-HPODE)-induced expression of both monocyte chemoattractant protein-1 (MCP-1) and TNF-α genes. In our study, the expression of NF-κBp65 was reduced by 71.69% and 74.82% at levels of mRNA and protein, respectively, and inhibited induced expression of COX-2, NOS-2, and MMP-9. Although RNAi cannot replace a gene knockout experiment in which both alleles are deleted cleanly from the genome, it produces hypomorphic mutants that are extremely useful for understanding gene functions, and also plays an important role in therapeutic application for a number of diseases[71]. Zhou *et al.*[72] reported that siRNA silencing of p16^{INK4a} decreased at 24 h after transfection of siRNA and reached its maximum at 72 h. Our study here indicated reaching maximum at 48 h, and incomplete recovery at 120 h, which might be related to the concentration of siRNA as a result of different dilutions and types of cells[59,66].

To elucidate the role of p65 in the bioactivity of chondrocytes in OA model *in vitro*, siRNA was used to decrease NF-κBp65 expression in the study. First of all, IL-1β and

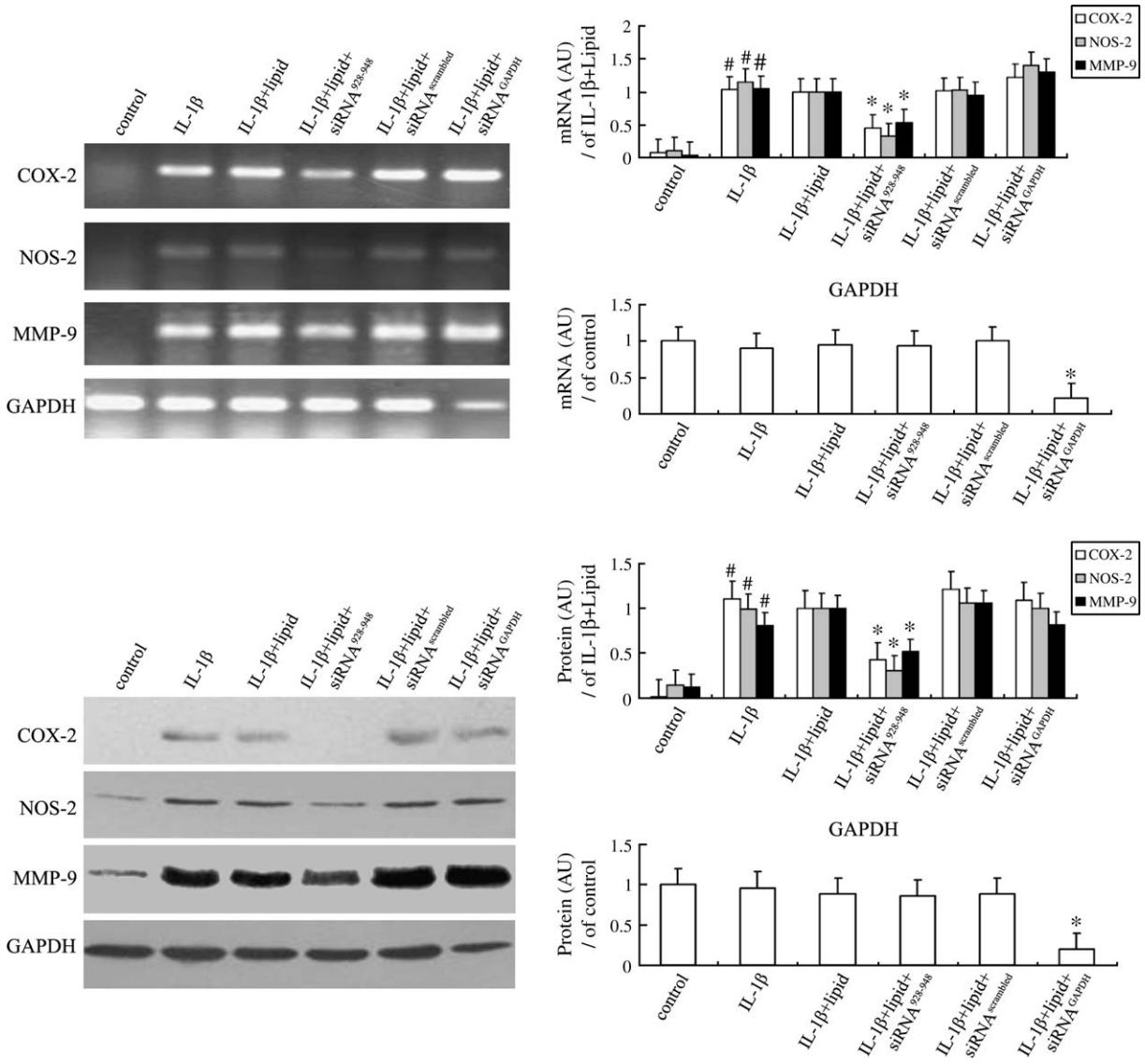

Fig. 6. The effect of siRNA$^{928-948}$ on the expression of COX-2, NOS-2, and MMP-9 induced by IL-1β and of siRNA^{GAPDH} on the expression of GAPDH. Rat chondrocytes were incubated with siRNA$^{928-948}$ for 48 h and stimulated with IL-1β for 24 h, all controls were performed in parallel. IL-1β significantly enhanced the expression of COX-2, NOS-2 and MMP-9 (#$P < 0.01$ vs control), and siRNA$^{928-948}$ could reduce the enhancement (*$P < 0.01$ vs corresponding values for IL-1β + lipid treated cells). Similarly, the positive control, siRNA^{GAPDH}, leads to significant reductions in the GAPDH expression (*$P < 0.01$ vs control). The densitometric quantification of COX-2, NOS-2 and MMP-9 was normalized to GAPDH. Upper panel: Representative RT-PCR of COX-2, NOS-2, MMP-9 and GAPDH expression. Graphs show the mean ± S.E.M. of the three independent experiments. Lower panel: Corresponding Western blot analysis for COX-2, NOS-2, MMP-9 and GAPDH, and graphs show protein levels.

TNF-α were found to markedly increase the activation of NF-κB and the expression of COX-2, NOS-2, and MMP-9 at levels of mRNA and protein, and after the silencing of NF-κBp65 gene expression, the signal pathway of activation of NF-κB was disturbed, and we found that the expression of NF-κB-dependent genes (such as COX-2 and NOS-2) and of downstream gene (MMP-9) was repressed. This indicated that p65 played crucial role in mediating expression of these genes. Since the expression of typical factors such as COX-2, NOS-2 and MMPs is paralleled with the initiation and progression of cartilage lesions[17], these results seem particularly important that p65 subunit is linked to joint cartilage destruction in OA model.

We all know that chondrocytes are targets of TNF-α and IL-1β[42], which exert catabolic function through specific cell surface receptors. TNF-α binds to TNF-α type 1 receptor (TNF-R1), activates TNF receptor-associated factor-2 (TRAF-2), IL-1, IL-1R1, and TRAF-6[73]. Then, TRAF-2 and TRAF-6 interact with mitogen-activated protein kinase kinase kinase (MAP3K) called NF-κB-inducing kinase (NIK). NIK is able to phosphorylate IκB kinase B (IKK-B) and IκB isoforms are rapidly phosphorylated which leads to IκB degradation. Therefore, distinct upstream signaling pathways induced by different cytokines can still lead to a similar effect[51]. In our study, the effects in silencing of NF-κBp65 to inhibit the expression of COX-2, NOS-2 and MMP-9 in chondrocytes induced by IL-1β were almost paralleled with those by TNF-α. The difference between cells induced by TNF-α and by IL-1β was that the former is more obvious than the latter, although there is no significant difference. The reason is that there are other transcriptional factors, including signal transducer and activator of

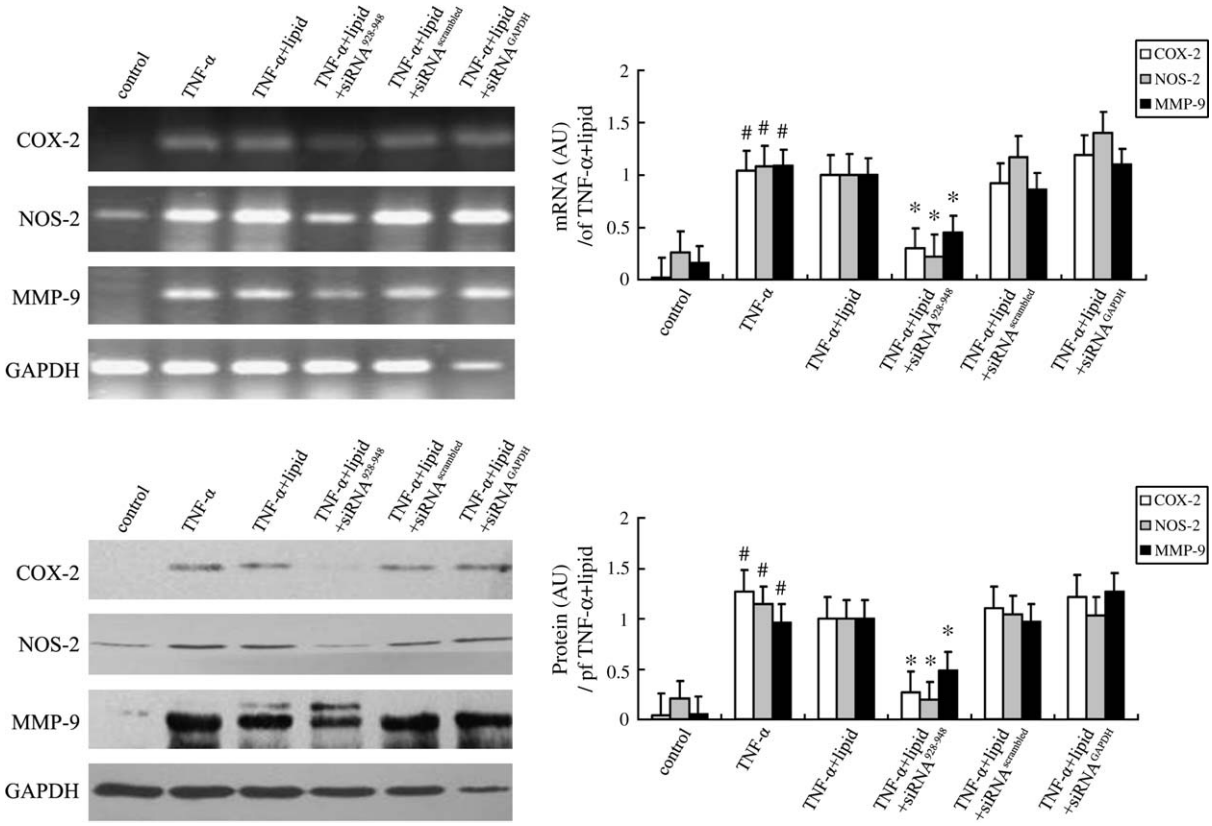

Fig. 7. The effect of siRNA$^{928-948}$ on the expression of COX-2, NOS-2 and MMP-9 induced by TNF-α in chondrocytes. Rat chondrocytes were incubated with siRNA$^{928-948}$ for 48 h and stimulated with TNF-α for 24 h, all controls were performed in parallel. TNF-α significantly enhanced expression of COX-2, NOS-2 and MMP-9 (#$P < 0.01$ vs control), and siRNA$^{928-948}$ could reduce the enhancement (*$P < 0.01$ vs corresponding values for TNF-α + lipid treated cells) at the levels of mRNA and protein. The densitometric quantification of COX-2, NOS-2 and MMP-9 was normalized to GAPDH. Upper panel: Representative RT-PCR images of COX-2, NOS-2, MMP-9 and GAPDH expression, and graph shows the mean ± S.E.M. of the three independent experiments. Lower panel: Corresponding Western blot analysis for COX-2, NOS-2, MMP-9 and GAPDH, and graph shows protein levels.

transcription (STAT)-1 and activator protein (AP)-1[74–76], participating in mediating inflammatory factors in IL-1β-induced chondrocytes. As COX-2 and NOS-2 being dependent on NF-κB[2,77] and MMP-9 being downstream factor of COX-2 and NOS-2[13,16], the decreased levels of COX-2 and NOS-2 are greater than that of MMP-9.

Since NF-κB is a known cell survival signal for most cells, we realize that chronic and long term reduction of basal NF-κB could lead to cell death or apoptosis. However, we believe that this could still be an effective anti-inflammatory intervention under disease and stimulated conditions. Importantly, the technology of siRNA which is a gene knockdown, not a gene knockout, can only decrease the increased levels of expression of catabolic genes in the early phase of OA, and cannot completely block conduction of the signal of NF-κB. So we conclude that although the precise mechanism of siRNA remains to be determined, it is likely that NF-κBp65-specific siRNA could be developed as a powerful approach to prevent induction of mediators of the degeneration of cartilage in OA *in vitro*. Further studies on *in vivo* effect of NF-κBp65-specific siRNA on OA in animal model are needed.

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