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Identifying the Regulative Role of NF-κB Binding Sites Within Promoter Region of Human Matrix Metalloproteinase 9 (*mmp-9*) by TNF-α Induction

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Abstract Matrix metalloproteinase 9 (MMP-9), a member of MMP family, is involved in many physiological processes, including cardiovascular disease (CVD). Tumor necrosis factor-α (TNF-α) is considered a cytokine with pleiotropic biological capabilities and leads to the process of CVD when TNF-α is abnormally released and stimulates MMP-9 expression and activation. In this study, we investigated the molecular mechanism of TNF-α-regulated MMP-9 expression. The experimental results confirm that TNF-α could upregulate MMP-9 expression in heart myoblast H9c2 cells of rat. To evaluate the MMP-9 regulation at transcriptional level, a DNA fragment of 2.2 kb (−2168/+18) of human *mmp-9* promoter region was cloned and constructed in a vector of luciferase reporter gene. The 2.2-kb sequences were identified as having three candidate nuclear factor-κ B (NF-κB) binding sites: NF-κB I (−1418/−1409), NF-κB II (−626/−617), and NF-κB III (−353/−345). A series of reporter vectors with the mutated NF-κB sites of *mmp-9* promoter sequences were constructed and transfected into H9c2 cells. The results show that the NF-κB II binding site (−626/−617) within the promoter region of *mmp-9* plays a key role in upregulation of *mmp-9* expression by TNF-α induction. In addition, we also first identified that the NF-κB I, similar to c-Rel, might be one of the NF-κB families to regulate *mmp-9* expression.

Keywords Matrix metalloproteinases 9 (MMP-9) · Nuclear factor-κ B (NF-κB) · Tumor necrosis factor-α (TNF-α) · Transcription factor · Cell line H9c2

Introduction

Matrix metalloproteinases (MMPs), a family of zinc-dependent proteinases, are essential for some physiological and pathological processes, such as embryonic development [1], tissue

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remodeling and vascular smooth muscle cell migration [2], wound repair [3], cardiovascular diseases (CVD) [4], rheumatoid arthritis [5], and cancer invasion and metastasis [6]. MMPs share certain biochemical properties, and the expression of MMPs is regulated by a variety of factors, including cytokines, growth factors, chemical agents, and physical stress [7–10]. Because of the decomposition of gelatin, matrix metalloproteinase 9 (MMP-9), known as 92-kDa gelatinase B [11]. Proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), can upregulate the macrophage to express MMP-9 [12–14]. TNF-α can significantly increase in some CVD, such as congestive heart failure, myocardial infarction, atherosclerosis, and ventricular hypertrophy, are associated with the increase in MMP-9 expression [15–17]. Previous reports showed that some of regulatory domains existed in the 670 bp promoter region of *mmp-9*, including activator protein 1 (AP-1), activator protein 2 (AP-2), specificity of protein 1 (SP-1), and nuclear factor κ B (NF-κB) [18]. Mutation or deletion of the NF-κB, AP-1, and SP1 motifs located at 600, 79, and 558 nucleotides upstream of the transcriptional start site of *mmp-9*, respectively, could reduce or abolish the ability of TNF-α to stimulate the *mmp-9* expression in osteosarcoma and hepatoma cells [18–20].

NF-κB is a family of transcription factor and the family members include RelA (p65), RelB, c-Rel, NF-κB1 (p50), and NF-κB2 (p52), respectively [21]. NF-κB subunits form homo- and heterodimers: The most prominent one is p65/p50 heterodimer, and it binds to the decametric consensus sequence GGGRNNTYCC [22]. NF-κB is rapidly activated in response to a variety of inflammatory and other stimuli that lead to degradation of inhibitor IκB. NF-κB is a key transcription factor that regulates inflammatory processes [23]. It is additionally activated by cellular stresses such as lipopolysaccharide, reactive oxygen species, and proinflammatory cytokines, including TNF-α and IL-1β [16]. NF-κB is involved in many regulations of MMP genes [24]. The expression of human *mmp-9*, varied in different cells in the presence of TNF-α or NF-κB transcription factor, plays an important role in regulating the gene expression [25].

In this study, a DNA fragment of 2.2 kb (−2168/+18) of human *mmp-9* was cloned. We identified three NF-κB binding sites within the promoter region of *mmp-9* and revealed the upregulated role of the NF-κB binding sites associated with TNF-α stimulation.

Methods

Cloning the Promoter Region of Human mmp-9

Based on a databank of NCBI (NG_011468), the human *mmp-9* promoter region including intron 1 sequences was amplified with high fidelity *Taq* polymerase (*Ex Taq*, Takara) PCR via two synthesized primers. One primer recognized the upstream of the *mmp-9* 5′ flanking sequences with *Mlu*I site (h5MMP9-2.2Mlu, 5′-CGACGCGTCGAGTTCTGGGCTTGAA CAC-3′, *Mlu*I site shown by underline), while the other reverse and complementary primer recognized the sequences at intron I of the *mmp-9* (h3MMP9-Ex1, 5′-GTGAGGGCA GAGGTGTCTGACTGC-3′). The DNA fragment with a size of 2.2 kb was amplified, sequenced, and then cloned into pCR2.1 TA vector (Invitrogen) to generate a clone designated phMMP9-2.2K.

The expression vector phMMP9-2.2K/luc with a promoter of *mmp-9* was constructed by inserting the *Sac*I/*Xho*I restricted fragment, hMMP-9 (2.2 kb), from phMMP9-2.2K into the promoterless luciferase expression vector pGL-3/Basic (Promega).

Site-Directed Mutagenesis of NF-κB Binding Sites in mmp-9 Expression Vector

The NF-κB binding site on *mmp-9* promoter (−2168~+1) was analyzed with TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCH.html) and MOTIF Search (http://motif.genome.jp) programs. The 2.2-kb sequences were identified as having three candidate NF-κB binding sites: NF-κB I (−1418/−1409), NF-κB II (−626/−617) and NF-κB III (−353/−345). Site-directed mutagenesis of the NF-κB binding sites by PCR method was performed according to QuikChange® Primer Design Program and Protocol (http://www.stratagene.com/sdmdesigner/default.aspx) [26]. The binding site of NF-κB I was changed from A[GG]TTTTTCC to A[AA]TTTTTCC; NF-κB II from T[GG]AATTCCC to T[TT]AATTCCC, and NF-κB III from GG[GGG]ATCC to GG[TTT]ATCC. The RN NF-κB I binging site was created by replacing NF-κB I with NF-κB II (Table 1). NF-κB multiple mutation vectors NF-κB I/II, NF-κB I/III, NF-κB II/III, and NF-κB I/II/III were set up followed by site-directed mutagenesis. The mutant vectors were constructed by carried luciferase expression gene.

Cell Culture and Gene Transfection

Rat embryonic cardiomyoblast cell line H9c2 (BCRC 60096; Bioresource Collection and Research Centre, Taiwan) was used for the gene transfection and expression studies. H9c2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) with 10 % fetal bovine serum (FBS; Invitrogen), and cells were maintained at 37 °C in an incubator in a humidified atmosphere with 5 % CO_2.

Transient transfection was carried out according to our published protocol [27, 28] with some minor modifications. Briefly, H9c2 cells were seeded at 1×10^5 cells per well in 24-well dishes (Nunc) 18 h prior to DNA transfection. Immediately before DNA transfection, the cells were rinsed with Dulbecco's phosphate-buffered saline (Invitrogen) and replenished with fresh serum-free culture media. For each well, we added 0.5 mL of complete medium with serum and antibiotics freshly before DNA transfection. One microgram of DNA was diluted into 50 μL of serum-free DMEM and diluted 3.0 μL of PolyJet™ reagent (SignaGen Laboratories) into another 50 μL of serum-free DMEM. Diluted PolyJet™ reagent was added to the diluted DNA solution, and the solution was immediately mixed and spanned

Table 1 The sequences of NF-κB binding sites on human *mmp-9* promoter region (−2168~+1) modified by site-directed mutagenesis

Binding site		Sequence		
NF-κB I	original	-1418	AGGTTTTTCC	-1409
Mut I	mutant	-1418	A[AA]TTTTTCC	-1409
NF-κB I	original	-1418	AGGTTTTTCC	-1409
Mut I RN	mutant (renature)	-1418	AGG[AA]TT[C]CC	-1409
NF-κB II	original	-626	TGGAATTCCC	-617
Mut II	mutant	-626	T[TT]AATTCCC	-617
NF-κB III	original	-353	GGGGGATCC	-345
Mut III	mutant	-353	GG[TTT]ATCC	-345

The boxed areas showed the nucleotide (A,T,C,G) change

down briefly the incubated mixture for 15 min at room temperature. An amount of 100 μL PolyJet™/DNA mixture was added onto the medium in each well and homogenized the mixture. Cells were incubated for 12 h with the transfection medium and then replaced with a fresh medium containing 10 % FBS.

Gelatin Zymography

Endogenous MMP-9 expression of H9c2 cells was detected by gelatin zymography via 20 ng/mL TNF-α treated for 24, 48, and 72 h. Gelatin zymography was performed based on our previous reports [29, 30]. Briefly, conditioned medium and cell lysates were electrophoresed in a polyacrylamide gel containing 0.1 % gelatin and 10 % SDS. The gel was then washed at room temperature for 2 h with 2.5 % Triton X-100, and flash medium was changed every half hour. Subsequently, the gel was immersed at 37 °C for 24 h in development buffer (5 mM $CaCl_2$, 50 mM Tris–HCl, 0.02 % NaN_3, pH7.5). The gel was stained with 0.2 % Coomassie Blue and distained with distain buffer (methanol/acetic acid/water=4.5:1:4.5). Proteolysis was detected as a white zone in a dark blue field.

mmp-9 Expression by TNF-α Induction

H9c2 cells were harvested at 80–90 % of confluence, seeded at 1×10^5 cells per well in 24-well dishes the day before transfection. After DNA transfection, TNF-α (ProSpec-Tany TechnoGene) and genistein (Sigma-Aldrich) at design concentrations were added into the medium. Gene expression was measured after 48 or 72 h of incubation.

Dual Luciferase Reporter Assay System

Mutations of *mmp-9* promoter genes expression were performed with the dual luciferase reporter assay system (Promega) with slight modifications. All reagents were prepared as described by the manufacturer. Briefly, the NF-κB mutation expression vectors and *Renilla* luciferase control vector pGL4.74/hRlu/TK (Promega) were cotransfected into H9c2 cells in a ratio of 50:1 by the polyethylenimine transfection method mentioned above. After 72 h of transfection and drug treatment, the cells were lysated and collected into 1.5 mL tube. A 10-μL suspend aliquot was used for luminescence measurement (Mithras LB 940, Berthold Technologies) by the program for dual luciferase detection. Firefly luciferase reagent II (LARII) (100 μL) was added to each sample of 96-well plate with a 10-s equilibration time and measurement of luminescence with a 10-s integration time, followed by the addition of 100 μL of the *Renilla* luciferase reagent and firefly quenching (Stop & Glo), 10-s equilibration time, and measurement of luminescence with a 10-s integration time. The data are represented as the ratio of firefly to *Renilla* luciferase activity (Fluc/Rluc).

Results

The human *mmp-9* 2.2 kb promoter region and the luciferase expression vector phMMP9-2.2K/luc are shown in Fig. 1. It encompassed the sequences from –2168 to +18 bp of the human *mmp-9* 5′ flanking region to drive the luciferase gene open reading frame. The predicted transcription regions, including NF-κB, Ap-1, and Sp-1 binding sites on *mmp-9* promoter (-2168∼+1), were analyzed by the TFSEARCH and MOTIF Search programs. NF-κB II (−626/−617) and NF-κB III (−353/−345) were confirmed to be a binding site of NF-κB. NF-κB I (−1418/−1409), similar to c-Rel with 85 % identical, was a suspect binding site of NF-κB and needs to be confirmed.

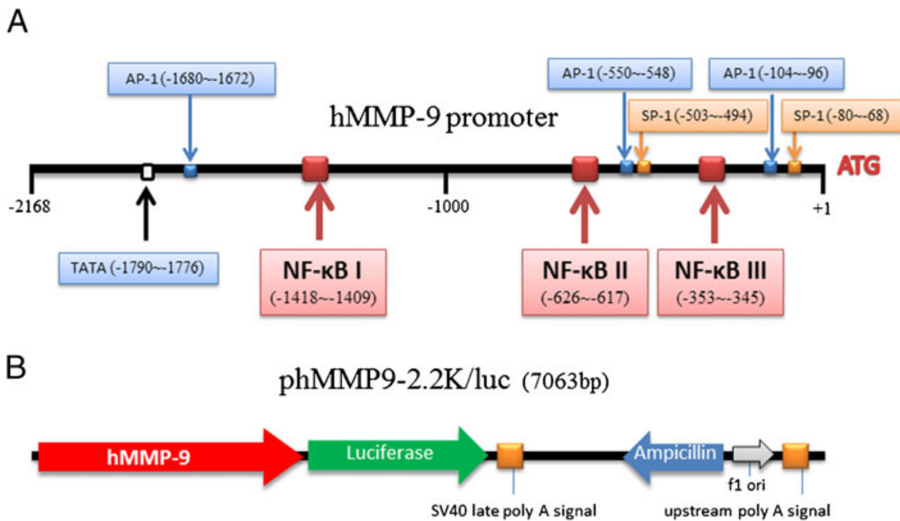


Fig. 1 Schematic map of the NF-κB binding sites on the promoter region of human *mmp-9* (**a**) and construction of expression vector phMMP9-2.2/luc (**b**). The NF-κB binding sites on the human *mmp-9* promoter (−2168∼+1) were analyzed by TFSEARCH and MOTIF Search programs. Three predicted NF-κB binding sites include NF-κB I (−1418/−1409), NF-κB II (−626/−617), and NF-κBIII (−349/−340). *AP-1* Activator protein 1, *NF-κB* nuclear factor-κ B, *SP-1* specificity of protein 1, *TATA* TATA box

mmp-9 expression induced by TNF-α was investigated. Different concentrations of TNF-α (0, 10, 20, 50, and 100 ng/mL) were added into phMMP9-2.2K/luc transfected H9c2 cells, and the luminescence was measured after 48 h of TNF-α treatment. The result shows that *mmp-9* expression was inducted according to the concentrations of TNF-α increased. The relative luciferase activity accrued beginning at 10 ng/mL and increased obviously at 20, 50, and 100 ng/mL of TNF-α treatment ($p<0.05$) (Fig. 2). From the results, we demonstrated that the *mmp-9* could be induced by TNF-α induction.

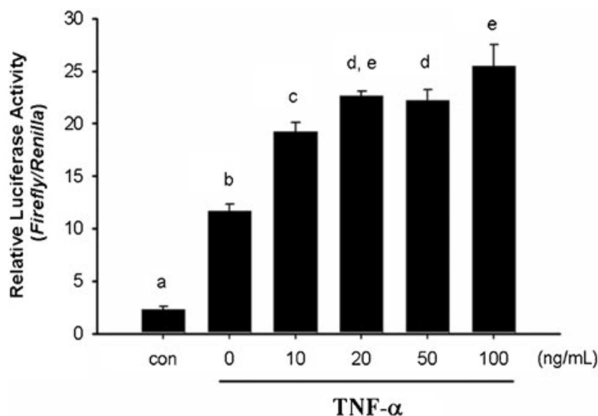


Fig. 2 The expression activity of 2.2 kb human *mmp-9* promoter by TNF-α induction. H9c2 cells were transiently transfected with phMM9-2.2 k/luc and then treated with various concentration of TNF-α for 48 h. "con" represents the H9c2 cells without gene transfection. Values are mean±SD from three independent experiments. *Different letters* (*a–e*) indicate that the values were significantly different from all other groups ($p<0.05$)

Endogenous MMP-9 protein expression of rat H9c2 cells was detected by zymography analysis through 20 ng/mL TNF-α treatment for 24, 48, and 72 h. The results show that endogenous MMP-9 protein of H9c2 cells significantly increased according to the time increased by TNF-α induction (Fig. 3a). On the contrary, in the control group without TNF-α induction, endogenous MMP-9 performance in the H9c2 cells was not detected or was very weak after 72 h of TNF-α treatment. The MMP-2 protein was continuously expressed with or without TNF-α induction.

Plasmid phMMP9-2.2/luc carrying the *mmp-9* promoter was transfected into H9c2 cells, sustained induction by 20 ng/mL TNF-α for 12, 24, 48, and 72 h, and then was assayed by luciferase analysis. The results show that exogenous human *mmp-9* promoter activity increased along with time increment. The luminescence activity rose obviously after 48 h of TNF-α induction and showed significant different compared with those of the control group ($p<0.01$) (Fig. 3b).

Side-directed mutagenesis of NF-κB binding sites NF-κB I, NF-κB II, and NF-κB III on the *mmp-9* promoter was performed and used to test the promoter activity by DNA transfection into H9c2 cells. The results show that the NF-κB II mutant clone lost its expression at TNF-α (20 ng/mL) induction, and the promoter activity decreased to 23 %, even lower than treatment without TNF-α induction (control group) (Mut II in Fig. 4). In NF-κB I and NF-κB III mutant clones, *mmp-9* promoter activity

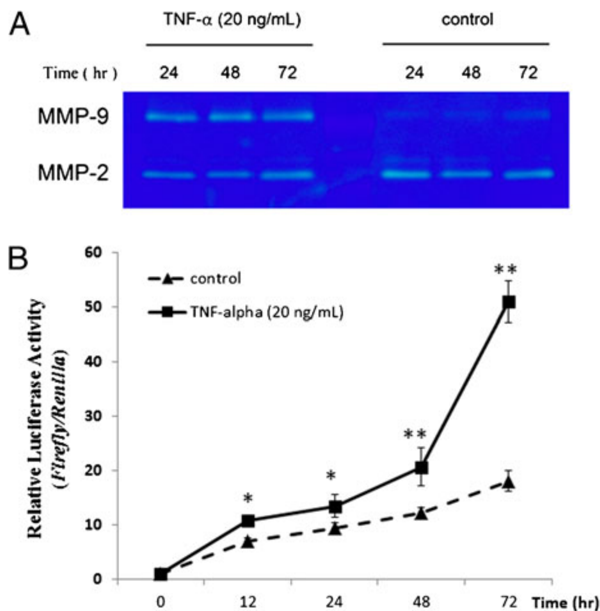
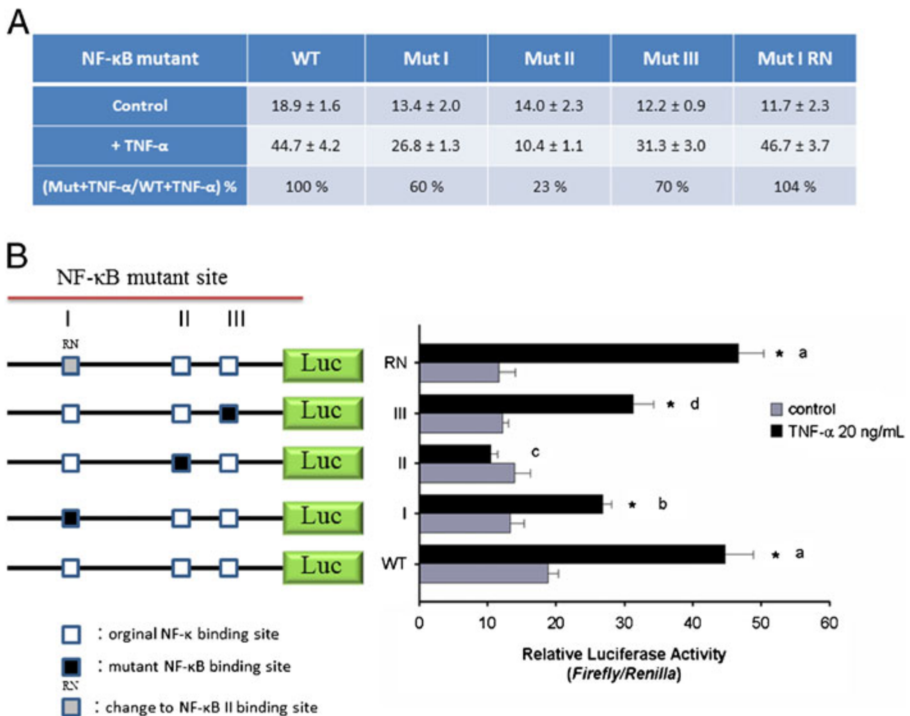


Fig. 3 Induced expression of human *mmp-9* promoter in H9c2 cells. **a** Gelatinolytic activity and MMP-9 expression of H9c2 cells treated with or without TNF-α (20 ng/mL) for 24, 48, and 72 h. The H9c2 cells were grown to 70 % confluence in DMED supplemented with 10 % FBS, and the medium was changed to serum-free medium and stimulated with or without TNF-α (20 ng/mL). Conditioned media was zymographically analyzed for the MMPs activities. **b** The expression of human *mmp-9* promoter transgene in the H9c2 cells after of TNF-α treatments was shown. The H9c2 cells were transiently transfected by phMMP9-2.2 k/luc. After transfection, the cells were stimulated with or without TNF-α (20 ng/mL) as indicated for an additional 0, 12, 24, 48, and 72 h. Values are mean±SD from three independent experiments. The significant differences of gene expression with or without added TNF-α in each group were $^*p<0.05$ and $^{**}p<0.01$



A

NF-κB mutant	WT	Mut I	Mut II	Mut III	Mut I RN
Control	18.9 ± 1.6	13.4 ± 2.0	14.0 ± 2.3	12.2 ± 0.9	11.7 ± 2.3
+ TNF-α	44.7 ± 4.2	26.8 ± 1.3	10.4 ± 1.1	31.3 ± 3.0	46.7 ± 3.7
(Mut+TNF-α/WT+TNF-α) %	100 %	60 %	23 %	70 %	104 %

Fig. 4 Site-directed mutagenesis analysis of NF-κB binding sites required for TNF-α-induced MMP-9 expression. The human 2.2 kb of *mmp-9* promoter construct (*WT*) was mutated at NF-κB I (*I*), NF-κB II (*II*), and NF-κB III (*III*) and replaced NF-κB I with NF-κB II (*RN*). **a** The statistical values presented in **b**. Values are mean±SD from three independent experiments. *Different letters* (*a–d*) indicate the values that were significantly different from all other groups ($p<0.05$). The significant difference of gene expression with or without added TNF-α in each group was $*p<0.05$

can be induced under TNF-α (20 ng/mL) induction, and the induction rates were higher than the NF-κB II mutant clone but lower than the control group (Mut I and Mut III vs. WT in Fig. 4). It showed that the NF-κB II binding site played a more important role than NF-κB I and NF-κB III in *mmp-9* promoter expression. The performance of *mmp-9* promoter activity was affected by NF-κB I mutant construct; the activity decreased to 60 % (Mut I vs. WT in Fig. 4). The NF-κB I (−1418/−1409) confirmed to be a binding site of NF-κB on human *mmp-9* promoter in this study. The *mmp-9* promoter expression could be strengthened, and when NF-κB I was replaced with NF-κB II, the expression activity reached 104 %, similar to normal promoter expression under TNF-α induction (Mut I RN vs. WT in Fig. 4).

Suppression of luciferase activities was shown at multiple site-directed mutageneses of NF-κB binding sites in the *mmp-9* promoter region. The most obvious repression happened in the NF-κB I/II double mutation (Mut I–II); the luciferase activity decreased to 14 % compared with that measured in control, followed by NF-κB II/III double mutation (Mut II–III) and NF-κB I/II/III triple mutation (Mut I–II–III). The luciferase activity decreased to 23 and 25 %, respectively (Fig. 5). The NF-κB I/III double mutation showed the slightest affect (decreased to 67 %) compared with that determined in the control group.

A

NF-κB mutant	WT	Mut I-II	Mut I-III	Mut II-III	Mut I-II-III
control	18.9 ± 1.5	31.0 ± 0.5	16.7 ± 1.7	13.9 ± 1.1	11.4 ± 1.8
+ TNF-α	44.7 ± 4.2	6.4 ± 2.4	30.1 ± 2.4	10.1 ± 1.7	11.2 ± 1.4
(Mut+TNF-α/WT+TNF-α) %	100 %	14 %	67 %	23 %	25 %

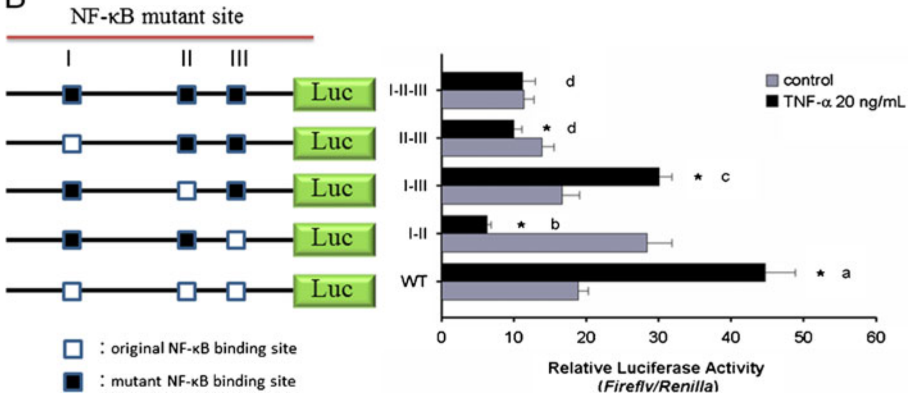


Fig. 5 Multiple site-directed mutagenesis analysis of NF-κB binding sites required for TNF-α-induced MMP-9 expression. The human 2.2 Kb of *mmp-9* promoter construct (*WT*) was mutated at NF-κB I and II (*I–II*), NF-κB I and III (*I–III*), NF-κB II and III (*II–III*), and NF-κB I, II, and III (*I–II–III*). **a** The statistical values presented in **b**. Values are mean±SD from three independent experiments. *Different letters* (*a–d*) indicate the values that were significantly different from all other groups ($p<0.05$). The significant difference of gene expression with or without added TNF-α in each group was *$p<0.05$

Discussion

MMP-9 possesses proteolytic activity against type IV collagen and has been shown in normal physiological processes [31–33]. Human *mmp-9* located at chromosome 20, q11.2–q13.1, includes 13 exons with 7,654 bp genomic size [34]. Transcription of the *mmp-9* yields a 2.5 kb mRNA [19]. The promoter region of *mmp-9* has been covered by many transcription factor binding sites, such as AP-1, AP-2, NF-κB, SP-1, polyoma enhancer activator 3 (PEA3)/Ets, signal transducer and activator of transcription (STAT), GC box, Tcf/Lef (T cell factor/lymphoid enhancer factor), and RAR/RXR (activator acid response element) [18, 19, 35]. Those from *mmp-9* upstream regulatory regions in mammals, such as human, mice, and rats, are highly conserved [36]. Studies found that NF-κB and AP-1 play most important regulatory key role in the human *mmp-9* promoter region when they react with cytokines or growth factors [37–40].

NF-κB is composed of different subunits with homo- or heterodimers. Family members are divided into five different structural proteins: NF-κB1 (p50), NF-κB2 (p52), RelA (p65), RelB, and c-Rel. The amino acid sequences of these five transcriptional factors were composed of more than 45 % similarity, and their three-dimensional structures are similar [41]. The 2.2-kb human *mmp-9* promoter region sequences were analyzed having three candidate NF-κB binding sites, NF-κB I (−1418/−1409), NF-κB II (−626/−617), and NF-κB III (−353/−345), with website bioinformatics tool TFSEARCH and MOTIF Search. The NF-

κB I was identical to be a c-Rel transcription binding site and not yet been confirmed. In the present study, we constructed nine human *mmp-9* promoter expression vectors, including 2.2 kb hMMP9, NF-κB I mutant (Mut I), NF-κB II mutant (Mut II), NF-κB III mutant (Mut III), NF-κB I change to NF-κB II (Mut I RN), NF-κB I/II mutant (Mut I–II), NF-κB I/III mutant (Mut I–III), NF-κB II/III mutant (Mut II–III), and NF-κB I/II/III mutant (Mut I–II–III), by the cloning technique of site-directed mutagenesis. *mmp-9* expression could be induced by TNF-α via a NF-κB signal transduction pathway in mammalian cells [19]. Our results show that *mmp-9* promoter could be activated in different concentrations (ranging from 10 to 100 ng/mL) of TNF-α induction (Fig. 2).

The MMP-9 expression patterns in H9c2 cells showed similarity between endogenous and exogenous human *mmp-9* measured by the construct phMMP9-2.2K/luc transfection. H9c2 cells only expressed MMP-2 protein in normal culture conditions, but when treated by 20 ng/mL TNF-α, MMP-9 protein expression was obvious and constant from 24 to 72 h (Fig. 3a). Luciferase activity analysis showed that phMMP9-2.2K/luc was induced by 20 ng/mL TNF-α induction after transfection into H9c2 cells; the activity rose significantly from 12 to 72 h (Fig. 3b).

Using site-directed mutagenesis techniques, the experiments focus at NF-κB transcription binding sites located in the 2.2-kb human *mmp-9* promoter region and confirmed the important performance of NF-κB transcription factor on *mmp-9* promoter. The experimental results show that when the single mutation of the NF-κB transcription binding site, either NF-κB I, II, or III, the *mmp-9* promoter activity induced by TNF-α was descended to 60, 23, and 70 %, respectively (Fig. 4). In other words, the NF-κB II binding site (−626/−617) within the promoter region of *mmp-9* plays a key role in MMP-9 expression with TNF-α stimulation. Similar results in NF-κB II mutant activity can be found in previous reports [19, 39, 40]. When the NF-κB III transcription binding site was reported by Han et al. [37], the NF-κB III mutant showed a weak binding site even lower than NF-κB I in our result.

NF-κB I is suspected to be one of the NF-κB binding site of c-Rel analyzed by bioinformatics tool that has not yet been confirmed. The experiment was designed with two mutant plasmids to verify this inference. The first type of mutation (Mut I) destroyed the suspected NF-κB binding site through site-directed mutagenesis. The *mmp-9* promoter activity of Mut I decreased to 60 % after TNF-α induction (Fig. 4). The second type of mutation (Mut I RN) replaced the original sequence of the suspected NF-κB binding site in the NF-κB II sequence (Table 1). The *mmp-9* promoter activities of Mut I RN rise up to the same level (104 %) with the original construct (WT, 100 %). The results show that NF-κB I (−1418/−1409) had the capability next to NF-κB II to respond to the induction from TNF-α, induce the *mmp-9* promoter expression, and play a role in the binding ability of NF-κB.

mmp-9 expression maintains a stable and low level of performance under normal physiological conditions, but the gene can be induced with inflammation, trauma repair, ovulation, or uterus resorption after delivery [42, 43]. In addition, the type of transcription factors, numbers, and locations affect the performance of the gene promoter activity. We tried to figure out the *mmp-9* promoter gene performance when mutations occur at multiple NF-κB binding sites. The double mutants at NF-κB I and II (Mut I–II) cause the most serious inhibition of *mmp-9* promoter activity (14 % of activity compared with that of the WT) (Fig. 5). The other mutation combinations, NF-κB II and III (Mut II–III), NF-κB I and III (Mut I–III), and triple mutant at NF-κB I, II and III (Mut I–II–III), were able to reduce TNF-α induced *mmp-9* promoter activation by 23, 67 and 25 % of activity, respectively. Based on results, we verified that the three NF-κB binding sites, NF-κB I (−1418/−1409), NF-κB II (−626/−617), and NF-κB III (−353/−345) play a functional role in TNF-α-induced *mmp-9* transcription. The results also confirmed that the NF-κB II binding sequence should have important physiological

significance. NF-κB II was also found to play a critical role in another inflammatory response element, transforming growth factor beta (TGF-β). The NF-κB II site is indispensable in the suppressive activity of TGF-β in the regulation of *mmp-9* transcription [20].

In conclusion, the promoter expression vector phMMP9-2.2K/Luc of human *mmp-9* was cloned for the study of *mmp-9* promoter expression in vitro. The NF-κB binding sites in the 2.2 kb *mmp-9* promoter region play an important role in associated gene regulation. Compared to the wild-type *mmp-9* promoter, each one of the three NF-κB binding sites of mutant vectors showed loss of gene expression after TNF-α induction. NF-κB II is the key point in the upregulation of *mmp-9* expression, followed by NF-κB I and NF-κB III. We propose that NF-κB I, similar to c-Rel, should be one of the NF-κB families that require further in-depth study.

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