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Inhibitory effect of DA-125, a new anthracyclin analog antitumor agent, on the invasion of human fibrosarcoma cells by down-regulating the matrix metalloproteinases

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Abbreviations:
DMEM, Dulbecco's modified Eagle's medium
ECM, extracellular matrix
EMSA, electrophoretic mobility shift assay
MMPs, matrix metalloproteinases
MT1-MMP, membrane type metalloproteinase
NF-κB, nuclear factor κB
RT-PCR, reverse transcriptase-polymerase chain reaction
TIMP, tissue inhibitor of metalloproteinase

ABSTRACT

Matrix metalloproteinases (MMPs), zinc-dependent proteolytic enzymes, play a pivotal role in tumor metastasis by cleavage of extracellular matrix as well as non-matrix substrates. In this study, we examined the influence of DA-125, a new anthracyclin analog, on the gene expression of MMPs (MMP-2, MMP-9 and MT1-MMP), tissue inhibitor of metalloproteinases (TIMP-1 and TIMP-2) and in vitro invasiveness of human fibrosarcoma cells. Dose-dependent decreases of MMPs and TIMPs mRNA levels were observed in DA-125-treated HT1080 human fibrosarcoma cells detected by reverse transcriptase-polymerase chain reaction. Gelatin zymography analysis also showed a significant down-regulation of MMP-2 and MMP-9 expression in HT1080 cells treated with DA-125 compared to controls. In addition, DA-125 inhibited the invasion, motility and cell migration, and colony formation of tumor cells. These data, therefore, provide direct evidence for the role of DA-125 as a potential cancer chemotherapeutic agent, which can markedly inhibit the invasive capacity of malignant cells. Further, to clarify the transcriptional regulatory pathway, we primarily investigated the role of nuclear factor-κB (NF-κB) in the expression of MMPs by DA-125 in HT1080 cells. Electrophoretic mobility shift assay demonstrated that DA-125 modulates the binding activity of NF-κB. Using the luciferase reporter gene assay, a dose-dependent down-regulation of NF-κB-mediated luciferase expression was also observed. These results suggest that DA-125 down-regulates MMPs expression in HT1080 cells through the NF-κB-mediated pathway.

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1. Introduction

Tumor invasion and metastasis represent a complex process that depends on the cyclic repeat of three fundamental steps: (1) adhesion to the extracellular matrix (ECM) components, (2) local degradation of the ECM and (3) migration through the degraded components [1]. Proteolysis of ECMs, and especially the basement membranes, is considered a key event during this process [1–3]. The matrix metalloproteinases (MMPs) are a multigene family of zinc-dependent endopeptidases that are capable of degrading essentially all of the ECM components and they are thought to play an important role in the matrix degradation during tumor growth, invasion and tumor-induced angiogenesis [4–6]. Among the MMPs, MMP-2 and MMP-9 are thought to play critical roles during tumor invasion and metastasis [7–12].

The MMP activity is tightly regulated at many levels, and this includes transcriptional control, proenzyme activation and inhibition of the activated MMPs by non-specific inhibitors such as α2-macroglobulin [13], or by the more specific endogenous inhibitors, i.e., the tissue inhibitors of metalloproteinase (TIMPs) [14]. TIMPs are thought to bind to the active site and block access to the substrate. An important consequence is that in the circumstances in which there is an approximate molar equivalence of enzyme to the inhibitor, a small change in the concentration of either component can result in a drastic change in the level of enzyme activity [15]. Especially, MMP-2 is secreted from cells as a zymogen (pro-MMP-2) and it is activated post-translationally by a transmembrane MMP designated as membrane type 1-MMP (MT1-MMP) [16–18]. The activation of pro-MMP-2 is regulated by a complex mechanism that involves the formation of a trimolecular complex with MT1-MMP and TIMP-2 [17,19–21]. TIMP-2 bridges the interaction between the MMP-2 zymogen and MT1-MMP via N-terminal binding to the active site of MT1-MMP with the concomitant C-terminal binding to the pro-MMP-2 hemopexin domain [17,19–24].

Coussens et al. [25] have shown that MMP-9 as well as MMP-2 is functionally involved in the progression of invasiveness of cancer cells and in the regulation of oncogenes. Further data have showed that the expression of MMP-9 closely correlates with the metastatic potential of tumor cells [13,26,27]. MMP-9 expression has also been associated with metastasis in a variety of model systems including that of rat sarcomas that were generated by transformation of rat embryo cells with using ras^H and myc [28]. The promotor region of MMP-9 contains three elements that are potential binding sites for the AP-1, SP-1 and NF-κB transcription factors. Among them, it is known that nuclear factor-κB (NF-κB) is involved in inflammation, cell adhesion, cell invasion, metastasis and angiogenesis [29–31]. From these observations, it has been suggested that the suppression of NF-κB activity offers the potential for blocking tumor initiation, promotion and metastasis [32].

DA-125 is a novel anthracycline derivative that has anticancer activity [33,34]. To improve the pharmacological efficacy of anthracycline, a β-alanine moiety and a pyranose ring with fluoride were introduced into the nucleus of the drug (Fig. 1). The anticancer effects of anthracycline are derived from topoisomerase II poisoning and the resultant apoptosis [35].

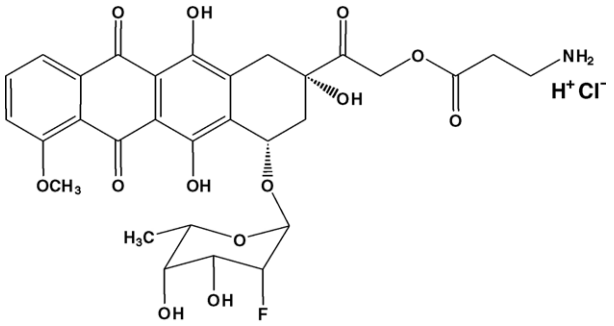

Fig. 1 – Chemical structure of DA-125.

Anthracycline-induced apoptosis may be mediated by the activation of caspase, the loss of mitochondrial membrane potential and the release of apoptogenic factors [36]. However, the studies on the anticancer activity of DA-125 have mainly been focused on the inhibition of topoisomerase II [37,38], whereas the relationship between DA-125 and the metastasis of cancer cells has not yet been clearly established. In this study, we demonstrate that DA-125 decreases the invasiveness of tumor cells in vitro, and this effect is derived from the inhibition of cell–matrix interaction and the suppression of the MMPs. These results suggest that DA-125 can contribute to the reduction of invasion and metastasis in tumors.

This work was undertaken to investigate the influence of DA-125, a new adriamycin analog on the gene expression of MMPs (MMP-2, MMP-9 and MT1-MMP) and TIMPs (TIMP-1 and TIMP-2), and the in vitro invasiveness of human fibrosarcoma cells.

2. Materials and methods

2.1. Materials

All the media for the cell culture were purchased from Gibco BRL (Grand Island, NY). Human type I collagen, bovine serum albumin, gelatin and Bradford reagent were purchased from Sigma (St. Louis, MO). Matrigel was purchased from Becton Dickinson (Bedford, MA). The oligonucleotides were synthesized by DNA International Inc. (Lake Oswego, OR). [γ-^{32}P]-ATP was from Amersham Pharmacia Biotech (Princeton, NJ). Quick spin columns, poly[dI-dC] and PMSF were obtained Boehringer Mannheim Corp. (Indianapolis, IN). T4 polynucleotide kinase was provided from Takara (Shiga, Japan). p(NF-κB)-LUC was from Stratagene (La Jolla, CA, USA). NF-κB oligonucleotide and luciferase assay system were purchased from Promega (Madison, WI). All other chemicals used were of reagent grade.

2.2. Cell culture

HT1080 human fibrosarcoma cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 U/ml penicillin G, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin and 10% heat-inactivated fetal bovine serum. The cultures were maintained at 37 °C in a 5% CO_2/95% air atmosphere.

2.3. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted by the use of TRI reagent (Sigma) and reverse-transcribed at 42 °C for 60 min in 20 μl of 5 mM $MgCl_2$, 10 mM Tris–HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100, 1 mM dNTP, 1 U/μl of recombinant RNasin ribonuclease inhibitor, 15 U/μg of AMV reverse transcriptase and 0.5 μg of oligo$(dT)_{15}$ primer. For the determination of target genes, the following primers were designed using primer3: MMP-2 (forward, +1689 to +1713; reverse, +2055 to +2079), MMP-9 (forward, +1238 to +1260; reverse, +1613 to +1637), TIMP-1 (forward, +233 to +252; reverse, +613 to +632), TIMP-2 (forward, +450 to +473; reverse, +828 to +850) and MT1-MMP (forward, +1320 to +1342; reverse, +1813 to +1836). Sequences of the primer pairs were used to the PCR reaction (Table 1). The PCR mixture consisted of a Taq PCR Master Mix Kit (Qiagen, Valencia, CA), 2 μl of the reverse transcriptase reaction and 20 pmol of primer pairs in a total volume of 50 μl. Thermocycling was performed according to the following profile: 94 °C for 30 s; followed by 94 °C for 30 s, 55 °C for 30 s and 72 °C 30 s, repeated 25 times. Amplification was linear within the range of 20–30 cycles. PCR products were separated by 2% agarose gel electrophoresis, stained with SYBR® Green I (Molecular Probes, Eugene, OR) and visualized using phosphoimaging technology (FLA-2000, Fuji, Stamford, CN).

2.4. Cell invasion and motility assay

5×10^4 cells/chamber were used for each invasion assay. The lower and upper parts of Transwell (Corning Glass) were coated with 10 μl of type I collagen (0.5 mg/ml) and 20 μl of 1:2 mixture of Matrigel:DMEM, respectively. Cells were plated on the Matrigel-coated Transwell. The medium of the lower chambers was also contained 0.1 mg/ml bovine serum albumin. The inserts were incubated for 18 h at 37 °C. The cells that had invaded the lower surface of the membrane were fixed with methanol and stained with hematoxylin and eosin, and photographed.

To determine the effect of the agents on cell motility, cells were seeded into Transwell on membrane filters coated with 10 μl of type I collagen (0.5 mg/ml) which was denatured with 0.1N acetic acid and allowed to stir at room temperature 1–3 h until dissolved, at the bottom of the membrane. Motility in the absence or presence of agents was measured as described in the invasion assay. In addition to this, cell motilities were measured using a wound-healing method and colony dispersion assay. Briefly, for a wound-healing assay, cells were grown almost confluently, and a wound was created with the blunt end of a yellow tip. This was documented through time-lapse photography. For a colony dispersion assay, 1×10^4 cells in 20 μl were seeded in growth medium in the middle of 24-well plate. At 6 h after plating, the medium was removed and the cells were covered with DMEM with 1% fetal bovine serum for 2 h and then exposed to DA-125 for an additional 48–72 h. After washing with phosphate-buffered saline, the cells were fixed with 4% formaldehyde and stained with hematoxylin and eosin. To quantify the expansion of the outgrowth, migratory monolayers were documented through digital photography (Olympus, Japan) with light.

2.5. Preparation of nuclear extracts

Nuclear extracts were prepared by a method of Beg et al. [40]. Briefly, cells were washed in phosphated-buffered saline, pelleted and resuspended in lysis buffer (10 mM Tris–HCl, pH 8.0, 60 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 100 μM PMSF and 0.2% NP-40). After 5 min on ice, the lysates were spun at 2500 rpm in a microcentrifuge at 4 °C for 4 min. The pelleted nuclei were briefly washed in lysis buffer without NP-40. The nuclear pellet was then resuspended in an equal-volume nuclear extract buffer (20 mM Tris–HCl, pH 8.0, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA and 25% glycerol). After a 10-min incubation at 4 °C, the nuclei were briefly vortexed and spun at 14,000 rpm for 5 min. The supernatant was then removed and used as a nuclear extract. Protein concentrations were determined by the Bradford assay [41].

2.6. Electrophoretic mobility shift assay (EMSA)

Following treatment of cells, nuclear extracts were prepared according to the method of Beg et al. [40]. Binding reactions were performed in a 20 μl volume containing 4 μg of nuclear protein extracts, 10 mM Tris–Cl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 0.1 mM dithiothreitol, 10% glycerol and 2 μg of

Table 1 – Sequences of the primer pairs employed in the RT-PCR reactions

Studied gene	Sequences of the primer pairs (5′ → 3′)	Product size (bp)
MMP-2	Forward: AGATCTGCAAACAGGACATTGTATT Reverse: TTCTTCTTCACCTCATTGTATCTCC	400
MMP-9	Forward: CTGGGCTTAGATCATTCCTCAGT Reverse: AGTACTTCCCATCCTTGAACAAATA	400
TIMP-1	Forward: TGGGGACACCAGAAGTCAAC Reverse: TTTTCAGAGCCTTGGAGGAG	400
TIMP-2	Forward: GTCAGTGAGAAGGAAGTGGACTCT Reverse: ATGTTCTTCTCTGTGACCCAGTC	401
MT1-MMP	Forward: GGGCCTGCCTGCGTCCATCAACA Reverse: GCCGCCCTCCTCGTCCACCTCAAT	400
β-Actin [39]	Forward: AGCACAATGAAGATCAAGAT Reverse: TGTAACGCAACTAAGTCATA	188

poly[dI-dC] as a non-specific competitor. After incubation for 25 min room temperature, 40,000 cpm of ^{32}P-labeled specific oligonucleotide probe was then added, followed by further incubation for 25 min at room temperature. NF-κB oligonucleotide was end-labeled with [γ-^{32}P]-ATP (Amersham Pharmacia Biotech) using T4 polynucleotide kinase (Promega). Competition studies were performed by the addition of a molar excess of unlabeled oligonucleotide to the binding reaction. Resultant protein–DNA complexes were resolved on native 5% polyacrylamide gels using 0.25 × TBE buffer (0.05 M Tris–Cl, 45 mM boric acid, 0.5 mM EDTA, pH 8.4) for 2 h. Gels were then dried and subjected to autoradiography.

2.7. Transient transfection and luciferase assay

HT1080 cells were plated in six wells and incubated at 37 °C. At 70–80% confluency, the cells were washed with DMEM and incubated with DMEM without serum and antibiotics for 5 h. Two micrograms of the firefly luciferase reporter construct p(NF-κB)-LUC (Stratagene) was transfected using LipofectAMINE 2000 reagent (Invitrogen). The transfected cells were treated with DA-125. After 24-h incubation, cell lysates were prepared, quantitated by Bradford assay [41]. Determination of firefly luciferase activities was performed using the Luciferase Reporter Assay System (Promega) and was measured using a luminometer (MicroLumat *Plus*, Berthold Technologies, Dortmund, Germany). The results shown from one experiment are representative of at least three independent replicates.

2.8. Statistical analysis

Statistical analyses were performed using the Student's *t*-test and one-way ANOVA. $^{*}P$-value < 0.05 was considered statistically significant. All the statistical analyses were performed using SPSS 10.0 software.

3. Results

3.1. DA-125 decreases the expression of the MMP-2 and MMP-9 genes and the protein in HT1080 cells

To investigate whether DA-125 can inhibit the expression of MMP-2 and MMP-9, HT1080 human fibrosarcoma cells were treated with the compound (2 μM) for various periods of times,

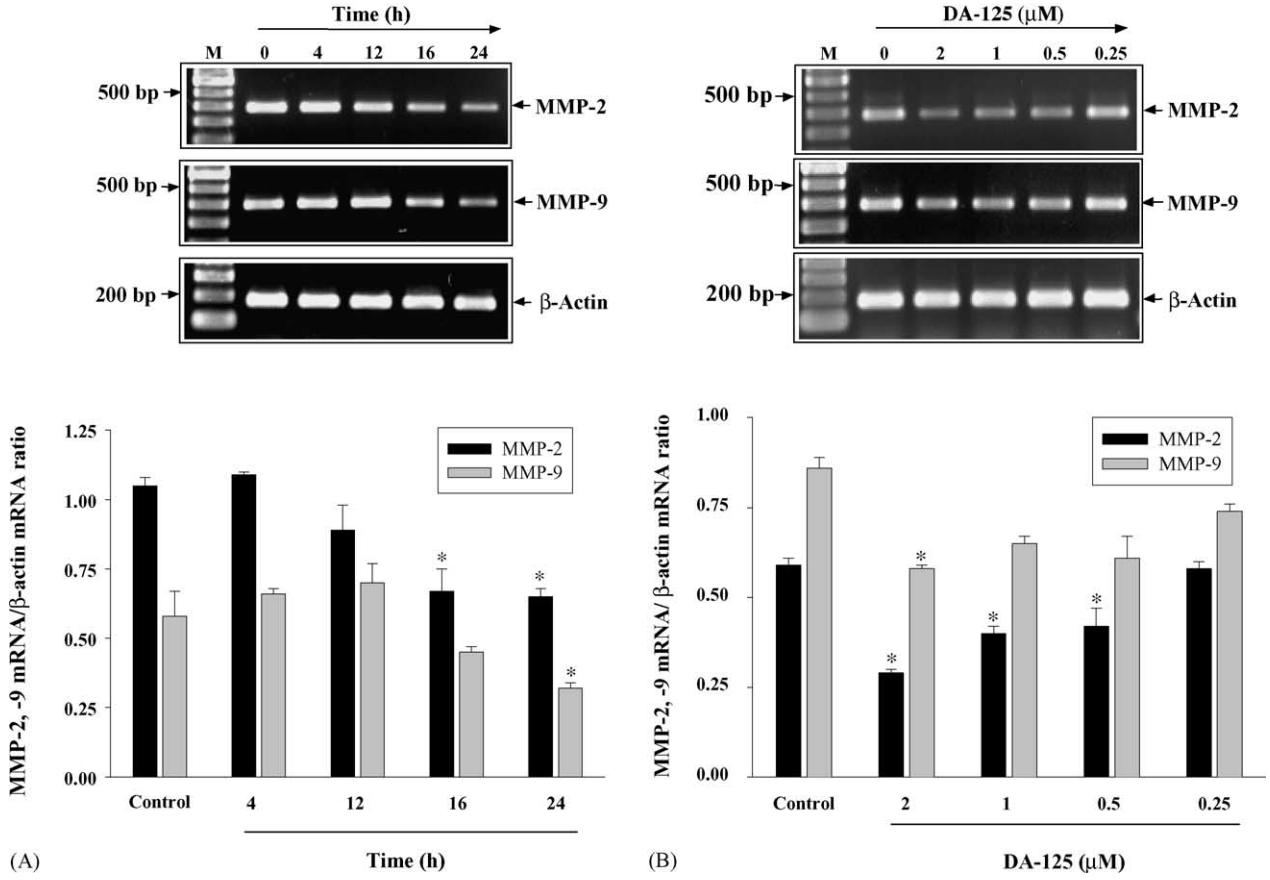

Fig. 2 – Time- and dose-dependent down-regulation of the MMP-2 and MMP-9 mRNA expression in HT1080 cells by DA-125 using RT-PCR analysis. HT1080 cells were exposed to: (A) 2 μM of DA-125 for the indicated period of time or (B) treated with various concentrations of DA-125 for 24 h. PCR products were analyzed by 2% agarose gel electrophoresis and visualized using phosphoimaging (upper panel), followed by densitometric measurements (lower panel). The predicted sizes of RT-PCR products for MMP-2, MMP-9 and β-actin are 400, 401 and 188 bp, respectively. M, molecular weight markers (100-bp DNA ladder). Statistical analyses were performed using the Student's *t*-test and one-way ANOVA. The data shown are the means ± S.D. of four determinations. $^{*}P < 0.05$ was considered statistically significant.

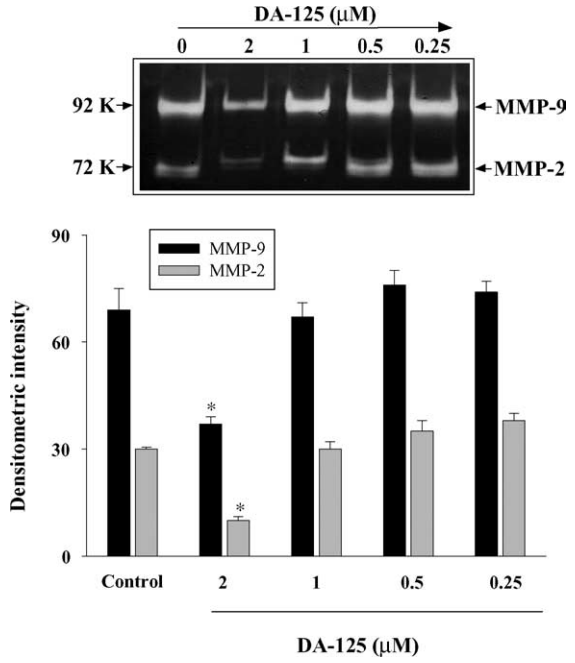

Fig. 3 – Effects of DA-125 on the activities of MMP-2 and MMP-9. HT1080 cells were treated with various concentrations of DA-125. After 48 h, conditioned media were collected and analyzed by gelatin zymography (upper panel), followed by densitometric measurements (lower panel). Statistical analyses were performed using the Student's *t*-test and one-way ANOVA. The data shown are the means ± S.D. of four determinations. $^{*}P < 0.05$ was considered statistically significant.

and the MMP-2 and MMP-9 mRNA levels were determined by semi-quantitative RT-PCR technique. As shown in Fig. 2, mRNA transcripts for MMP-2 and MMP-9 were clearly inhibited in the DA-125-treated cells. Down-regulation of the MMP-2 and MMP-9 expression via treatment with DA-125 was also observed in a time-dependent (Fig. 2A) and dose-dependent manner (Fig. 2B). In addition, gelatin zymography analysis revealed that MMP-2 and MMP-9 proteins were constitutively expressed in untreated HT1080 cells, but the cells treated with DA-125 for 48 h were markedly down-regulated in a dose-dependent manner (Fig. 3).

3.2. *DA-125 down-regulates the expression of the TIMP-1, TIMP-2 and MT1-MMP genes in HT1080 cells*

The TIMPs and MT1-MMP gene expressions were investigated to further explore the modulated activation of the pro-MMPs as mediated by DA-125. As shown in Fig. 4, DA-125 decreased the TIMPs mRNA in a time-dependent (A) and dose-dependent manner (B). The MT1-MMP mRNA levels were also suppressed by the DA-125 treatment (2 μM) in a time-dependent manner (Fig. 5A), dose-dependent down-regulation was observed at 24 h (Fig. 5B), which demonstrates that DA-125 significantly suppresses the expression of the TIMPs and MT1-MMP mRNA levels as well MMP-2 and MMP-9.

3.3. *Effects of DA-125 on invasion and migration activity*

HT1080 cells have an ability to invade through Matrigel. Treatment of DA-125 for 16 h exhibited the significant inhibition of cell invasion in a dose-dependent manner (Fig. 6A). Cell motility through the Transwell (Fig. 6B) and their spreading onto the plasticware (Fig. 6C) were also inhibited by DA-125 treatment. On the colony dispersion assay, the cells were plated at a high density onto the middle of the 24-well plate and they migrated as an outgrowth. As shown in the photos, the HT1080 cells treated by DA-125 were inhibited the migration ability in a dose-dependent manner and the effect was superior to adriamycin (Fig. 6D).

3.4. *NF-κB binding activity*

To determine whether the decrease of MMPs gene expression via DA-125 is associated with inhibition of NF-κB's binding activity, HT1080 tumor cells were treated with 2 μM DA-125 for 2 and 24 h, and then the nuclear extracts were prepared and analyzed by the electrophoretic mobility shift assay with using ^{32}P end-labeled NF-κB oligonucleotides. As shown in Fig. 7A, the incubation with DA-125 for 24 h down-regulated the NF-κB binding activity (lanes 3–5). This result was well correlated with the suppressive effects on the MMPs' mRNA gene expression in the DA-125-treated HT1080 tumor cells. The binding activity was completely inhibited by an unlabeled competitor DNA that contained the consensus NF-κB sequence (lane 6). However, when treated with DA-125 for 2 h, a remarkable increase of NF-κB binding activity was observed in a dose-dependent manner (0.25–2 μM) (Fig. 7B). To further clarify the induction of NF-κB binding activity via DA-125 for a short-time incubation, the cells were pre-treated for 30 min with the NF-κB inhibitors PDTC or curcumin before a co-exposure to DA-125 (2 μM) for 2 h. Indeed, the pre-treatment with PDTC (Fig. 7C) or curcumin (Fig. 7D) resulted in the inhibition of NF-κB activation when the cells were exposed to DA-125.

3.5. *DA-125-mediated down-regulation of MMPs gene expression is associated with NF-κB-mediated transcription*

To further determine that the NF-κB binding site plays the critical role in the DA-125-mediated suppression of the MMPs' gene expression the HT1080 cells were transfected with the NF-κB-regulated luciferase reporter construct, p(NF-κB)-LUC. As shown in Fig. 8, exposure of transfected cells to 2 or 1 μM DA-125 markedly inhibited the NF-κB-mediated transcription, as was measured by luciferase activity.

4. Discussion

Adriamycin is an anthracycline antibiotic with antitumor activity against a broad spectrum of human neoplasms, and it is one of the most commonly employed anticancer drugs in clinical use [42,43], but its use is often limited by the severe cardiotoxicity and other undesirable side effects [44,45]. DA-

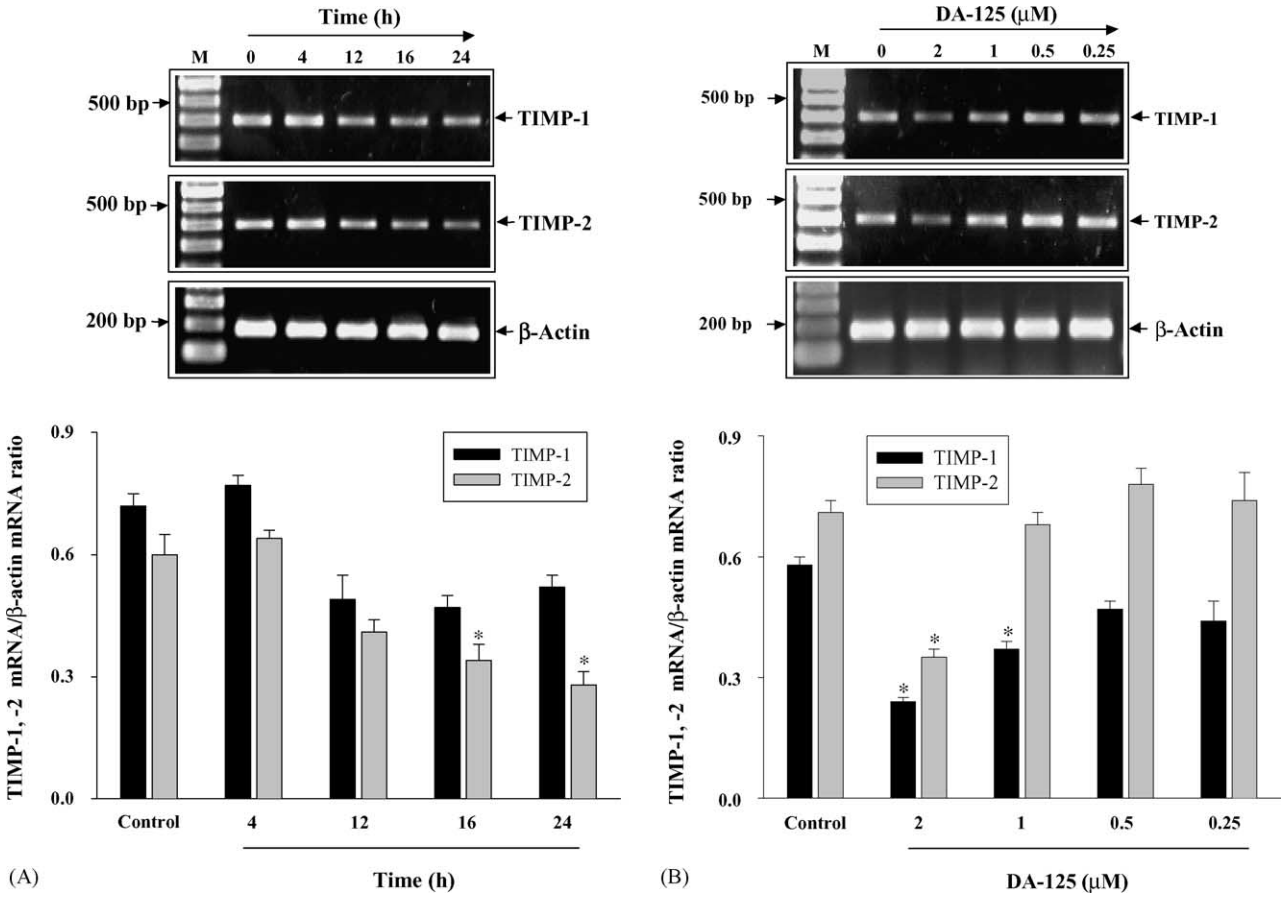

Fig. 4 – Time- and dose-dependent down-regulation of the TIMP-1 and TIMP-2 mRNA expression in HT1080 cells by DA-125 using RT-PCR analysis. HT1080 cells were exposed to: (A) 2 μM of DA-125 for the indicated period of time or (B) treated with various concentrations of DA-125 for 24 h. PCR products were analyzed by 2% agarose gel electrophoresis and visualized using phosphoimaging (upper panel), followed by densitometric measurements (lower panel). The predicted sizes of RT-PCR products for TIMP-1, TIMP-2 and β-actin are 400, 401 and 188 bp, respectively. M, molecular weight markers (100-bp DNA ladder). Statistical analyses were performed using the Student's *t*-test and one-way ANOVA. The data shown are the means ± S.D. of four determinations. $^{*}P < 0.05$ was considered statistically significant.

125 (Fig. 1), a new adriamycin analog, was recently developed by the Research Laboratory of the Dong-A Pharmaceutical Company in Korea with the aim of achieving enhanced anticancer activity with lower cardiotoxicity than adriamycin. DA-125 has exhibited the potential antitumor activity in animal models and its anticancer activity is undergoing clinical trials in Korea [34,46]. One possible mechanism of the antitumor activity of DA-125 has been suggested by the topoisomerase II poison mechanism and the DNA intercalator mechanism [37,38]. Further studies on this drug's mechanisms of action are still needed. In this study, we have explored whether DA-125 modulates cancer invasion and metastasis in cultured HT1080 cells with using the representative biomarkers of the MMP expressions. As a result, DA-125 significantly suppressed the gene expression of the MMPs (MMP-2, MMP-9 and MT1-MMP) and the TIMPs (TIMP-1 and TIMP-2) in HT1080 human fibrosarcoma cells: further, the non-toxic ranges of DA-125 markedly decreased the MMPs activity, cell migration, motility and invasiveness (Figs. 3 and 6).

MMPs mediate invasion and metastasis through the degradation of the extracellular matrix and the basement membrane, and this allows tumor cells to invade surrounding tissues and enter the blood stream to travel to distant sites [1,2,47]. Especially, MMP-2 and MMP-9 are considered to be particularly good targets for anticancer drugs because both enzymes degrade gelatins, which are major component of the basement membrane, and the expression of both these enzymes correlates with an aggressive, advanced, invasive or metastatic tumor phenotype [48–52]. Furthermore, knockout mice for either gelatinase have a reduced tumor burden and decreased metastasis, as well as reduced tumor angiogenesis without developmental abnormalities [12,53]. On this line, DA-125-mediated suppression of MMP-2 and MMP-9, and the MT1-MMP gene expression (Figs. 2 and 5), suggests that DA-125 might inhibit cancer cell invasion (Fig. 3).

TIMPs play an important role in regulating the activity of the secreted metalloproteases. In addition to their ability to bind at the active site, TIMP-1 and TIMP-2 can form complexes

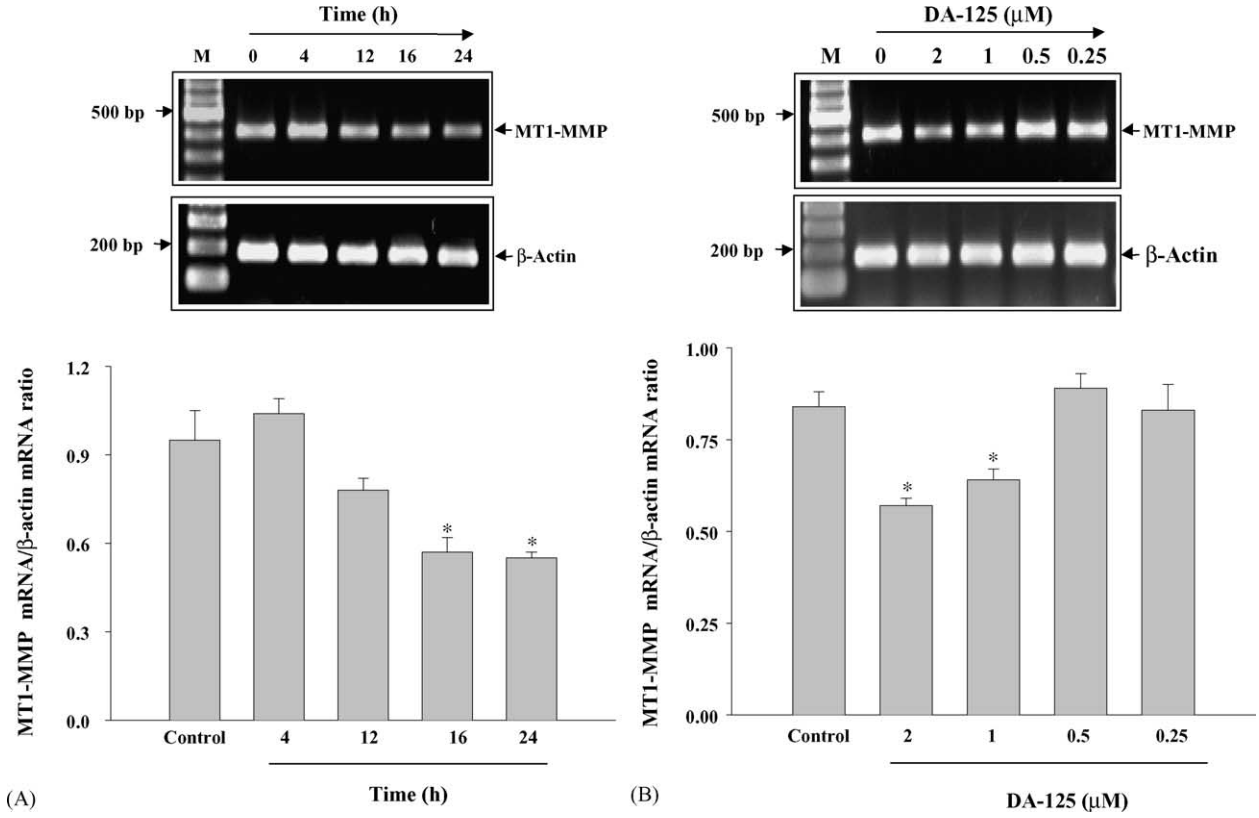

Fig. 5 – Analysis of the MT1-MMP mRNA. HT1080 cells were exposed to: (A) 2 μM of DA-125 for the indicated period of time or (B) treated with various concentrations of DA-125 for 24 h. PCR products were analyzed by 2% agarose gel electrophoresis and visualized using phosphoimaging (upper panel), followed by densitometric measurements (lower panel). The predicted sizes of RT-PCR products for MT1-MMP and β-actin are 400 and 188 bp, respectively. M, molecular weight markers (100-bp DNA ladder). Statistical analyses were performed using the Student's *t*-test and one-way ANOVA. The data shown are the means ± S.D. of four determinations. $^{*}P < 0.05$ was considered statistically significant.

with specific metalloproteinases. TIMP-1 is a 28-kDa *N*-glycosylated protein and it forms a 1:1 complex with the 92-kDa procollagenase [54]. TIMP-2 is a 21-kDa non-glycosylated protein and it selectively forms a complex with the 72-kDa procollagenase. In the present study, DA-125 exerts its action through the regulation of TIMP-1 and TIMP-2. Interestingly, DA-125 suppressed the TIMP-1 and TIMP-2 mRNA levels (Fig. 4). Although TIMP-1 and TIMP-2 are considered to be inhibitors of MMP-9 and MMP-2, respectively, the expressions of these inhibitors are differentially regulated in in vivo as well as in a cell culture system [55,56]. DA-125 was found to be a suppressor of the MMP-2, MMP-9, MT1-MMP, TIMP-1 and TIMP-2 mRNA levels. These findings seem to be quite different compared to the previous reports: genistein decreases the MMP-2 and MMP-9 mRNA levels, whereas it increases TIMP-1 mRNA levels in MDA-MB-231 and MCF-7 cells [57], and ursolic acid (UA) decreases MMP-9 mRNA levels but MMP-2 and membrane type MMP mRNA levels were constantly expressed, and TIMP-1and TIMP-2 mRNA levels were also not changed after 3 and 6 days of treatment with UA in HT1080 cells [58]. 1α,25-Dihydroxyvitamin D_3 and its analogs also down-regulates MMP-9 and uPA, whereas it up-regulate TIMP-1 and uPAI-1 levels in MDA-MB-231 cells [59]. Silibinin is a flavonoid antioxidant, and it markedly decreased the MMP-2 and uPA levels: it increased the TIMP-2 protein level without affecting the mRNA level, and it did not affect the TIMP-1 protein or mRNA levels [60]. Collectively, the DA-125 down-regulated MMP-2 and MMP-9 gene expressions are at least in part, mediated at the level of transcription regardless of the TIMP-1 and TIMP-2 expressions (Figs. 2 and 4). In addition, zymography analysis showed that the down-regulation of the MMP-2 and MMP-9 genes by DA-125 is correlated with a significant and dose-dependent down-regulated of MMP-2 and MMP-9 protein in HT1080 cells (Fig. 3). To further clarify the transcriptional regulatory pathway, we envisaged the role of NF-κB in the expression of MMPs via DA-125 in the HT1080 cells.

NF-κB regulates a variety of genes whose products are involved in many diverse biological processes, including cell growth, differentiation, apoptosis, cell invasion and metastasis [29–31,61–63]. Many studies have revealed that blocking NF-κB activity suppresses the tumor growth and metastasis of human cancer cells including prostate and melanoma cells due to the inhibition of angiogenesis and cell invasion [47]. Further, the expression of MMP-9 and uPA require NF-κB [64,65] as well as AP-1. For example, selenite has been shown

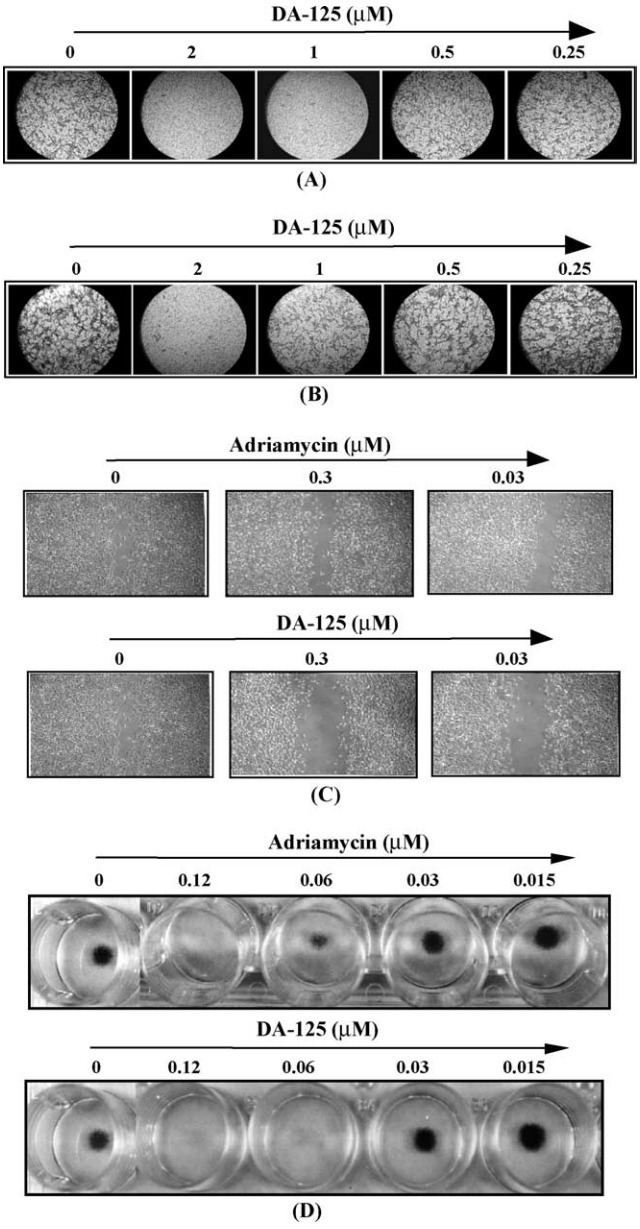

Fig. 6 – Effect of DA-125 on invasion, motility, wound healing and colony dispersion assay. (A) HT1080 cells were treated with 0, 0.25, 0.5, 1 or 2 μM DA-125 for 18 h using Matrigel-coated Transwell. (B) The same method used in (A) excepting Matrigel-coated on upper parts of Transwell. (C) Confluent cultures of HT1080 cells were wounded with a tip. The cells were incubated with increasing concentrations of DA-125 for 16 h, fixed and stained. (D) HT1080 cell migration was determined after 3-day incubation by various concentration of DA-125 shown in a colony dispersion assay. Data shown are representatives of three independent experiments.

to inhibit the binding of NF-κB to DNA [66,67] by oxidizing the critical cysteine I residues in their DNA-binding domains like AP-1. Based on this, we examined whether DA-125 might affect the NF-κB activity. The treatment of DA-125 for 24 h inhibited the NF-κB activity in HT1080 cells (Fig. 7A), but the mechanism of the suppressive effect on NF-κB expression remains to be investigated. These results are comparable with the MMP gene expression and they are also in agreement with earlier reports on the inhibition of NF-κB by selenite in HT1080 cells [32,66]. Therefore, the inhibitory effect of DA-125 on NF-κB activity might be able to explain the suppression of the MMP-2 and MMP-9 genes. In contrast, we found that a short-time incubation of DA-125 for 2 h significantly increased the NF-κB binding activity in a dose-dependent manner (Fig. 7B). This result is consistent with the recent report by Ho et al. [68] in which nuclear lysates obtained from treatment by 5 μM adriamycin for periods ranging from 1 to 6 h in MDA-MB-231 and HEK293 cell lines evoked a large increase in the NF-κB binding activity. It is also assumed that the increase of NF-κB binding activity might be correlated with oxidative stress or apoptosis [38]. To support the possible role of oxidative cellular stress in the DA-125-induced activation of NF-κB, the binding activity was determined with using treatment of PDTC or curcumin. PDTC is a the radical-scavenging thiol compound, and it is widely used as an inhibitor of NF-κB activation [69]: curcumin is a diferuloylmethane, and it has been known to specifically inhibit the activation of NF-κB by preventing the degradation of IκB, a NF-κB inhibitory subunit, and by blocking the translocation of NF-κB into the nuclear compartment [70]. As a result, the pre-treatment with PDTC or curcumin attenuated the enhanced NF-κB binding activity by DA-125 (Fig. 7C and D), suggesting that the DA-125-induced NF-κB binding activity might be correlated with the oxidative stress caused by the short period of incubation. However, a longer exposure with DA-125 seems to down-regulate the NF-κB binding activity in the cells. In addition, to further confirm the involvement of NF-κB in the transcriptional regulation of the MMPs genes we investigated the role of NF-κB in the DA-125-mediated inhibition of MMPs in HT1080 cells by using a transient transfection assay that contains a NF-κB reporter vector linked to a luciferase reporter gene. As shown in Fig. 8, DA-125 was found to inhibit luciferase activity in a dose-dependent manner. These results clearly indicate that the down-regulation of the MMPs gene expression by DA-125 is associated with transcriptional inhibition of the NF-κB. It has been suggested that the suppression of NF-κB activity might potentially block tumor initiation, promotion and metastasis [32]. Therefore, the DA-125-mediated suppression of NF-κB activity might be a new finding with potential antimetastatic activity, and so further studies on the antimetastatic properties of DA-125 are needed to gain a complete understanding.

In conclusion, we report here that DA-125 down-regulates the expression of invasion-associated proteases. The inhibition was also related, at least in part, to the suppression of the transcriptional level and the regulation of such transcription factors as NF-κB; however, its mechanism of action still remains elusive. In addition, DA-125 inhibits degradation and cellular invasion of the extracellular matrix and the basement membrane. This study shows a possible activity of the anti-invasive potential that goes beyond the antitumor activity mediated by DA-125.

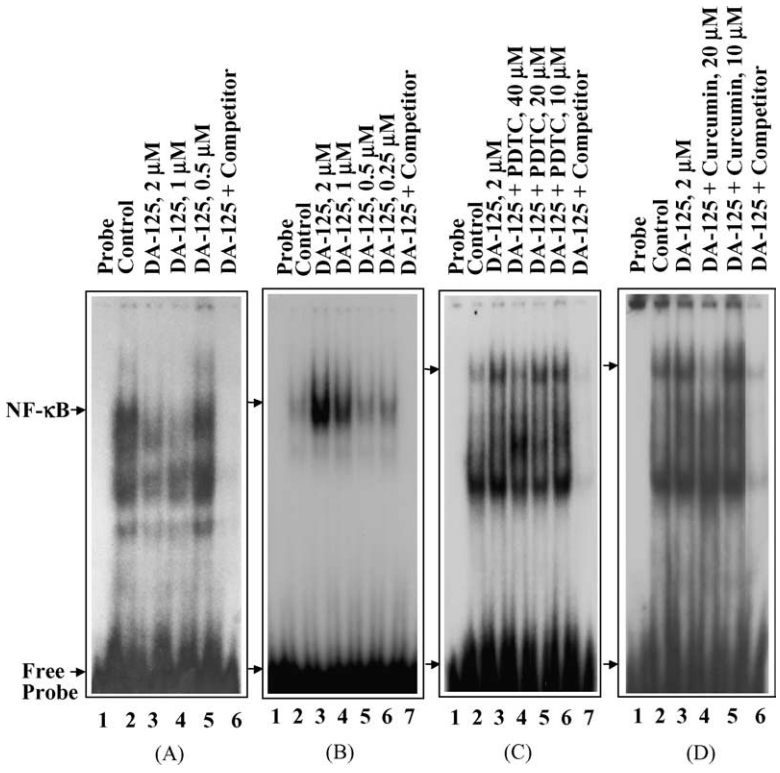

Fig. 7 – Time-dependent regulation of DA-125-induced NF-κB DNA-binding activity in HT1080 cells as measured by EMSA. Lane 1: probe alone. Cells were either untreated (lane 2) or treated for 24 h (A) or 2 h (B) with decreasing doses of DA-125 (A: lanes 3–5; B: lanes 3–6). Competition study was performed by the addition of excess unlabeled oligonucleotide (A: lane 6; B: lane 7) using nuclear extracts from cells treated with 2 μM DA-125. (C and D) Pre-treatment with PDTC or curcumin blocks DA-125-induced NF-κB DNA-binding activity in HT1080 cells as measured by EMSA. Cells were pre-treated for 30 min with indicated concentrations of PDTC (C), curcumin (D), before a 2 h treatment with 2 μM of DA-125 (C: lanes 4–6; D: lanes 4 and 5). Lane 1, probe alone; lane 2, untreated; lane 3, treatment with 2 μM DA-125; lane 7 (C); lane 6 (D), competition study performed by the addition of excess unlabeled oligonucleotide using nuclear extract from cells treated with 2 μM DA-125. Data shown are representatives of three independent experiments.

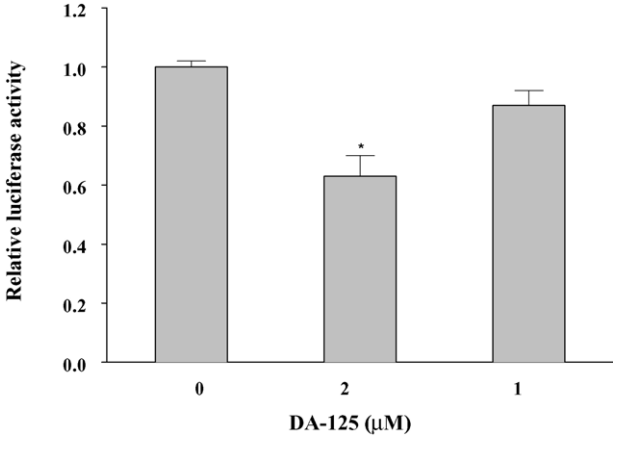

Fig. 8 – Effects of DA-125 on the activities of NF-κB. To elucidate the effects of DA-125 on NF-κB activity, a reporter vector that has NF-κB binding sites was transfected, and various concentrations of DA-125 were treated and then incubated for 24 h. The cells were then lysed, and luciferase activity was measured. Statistical analyses were performed using the Student's t-test and one-way ANOVA. The data shown are the means ± S.D. of four determinations. *$P < 0.05$ was considered statistically significant.

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