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# The role of annexin A1 in expression of matrix metalloproteinase-9 and invasion of breast cancer cells

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ABSTRACT

Matrix metalloproteinase-9 (MMP-9) plays an important role in the invasion and metastasis of cancer cells. However, the regulatory mechanism of MMP-9 expression and its biological effects on breast cancer development remain obscure. In the current study, we examined the potential role of annexin A1 (ANXA1) in regulating migration and invasion in breast cancer cell lines. Both ANXA1 mRNA and protein are expressed in the highly invasive, hormone-insensitive human breast cancer cell lines MDA-MB-231 and SKBr3, but not in the hormone-responsive cell lines MCF-7 and T47D. Downregulation of ANXA1 expression with specific small interfering RNAs (ANXA1 siRNA) in MDA-MB-231 cells resulted in decreased cancer cell migration and invasion. Ablation of ANXA1 expression decreases the expression of MMP-9 at both the mRNA and protein levels and also reduces the proteolytic activity of MMP-9 in MDA-MB-231 cells. Moreover, silencing *ANXA1* also decreases the transcriptional activity of MMP-9 by the suppression of nuclear factor kappa-B (NF-κB) activity. Collectively, these results indicate that ANXA1 functions as a positive regulator of MMP-9 expression and invasion of breast cancer cells through specific activation of the NF-κB signaling pathway.



## 1. Introduction

One of the basic properties of cancer cells is their ability to invade surrounding tissues and metastasize to other organs [1]. Tumor cell invasion requires cell migration and degradation of extracellular matrix (ECM) proteins [2]. Invasive cancer cells utilize matrix metalloproteinases (MMPs) to degrade the ECM and basement membrane during metastasis [3]. The metalloproteinases MMP-2 and MMP-9, both of which perform critical roles in the degradation of type IV collagen, are highly expressed in various malignant tumors and closely related to invasion, metastasis, and the epithelial–mesenchymal transition (EMT) in cancer cells [4,5]. While MMP-2 is constitutively overexpressed in highly metastatic tumors, MMP-9 is regulated by various biochemical stimulators, including growth factors, cytokines, and phorbol 12-myristate 13-acetate (PMA), via activation of various intracellular signaling pathways [6]. Induction of MMP-9 is particularly important to determine the invasiveness of human cancers, including breast cancer [7]. Blockade of MMP-9-mediated invasion suppresses the metastasis of breast cancer cells into other organs [8]. The activity of MMP-9 in various tumor cells is tightly regulated, primarily at the transcription level [9]. The human MMP-9 promoter contains cis-acting regulatory elements and transcription factor-binding sites, including sites for AP-1 (−533 bp, −79 bp) and NF-κB (−600 bp), which participate in the regulation of MMP-9 gene transcription [10]. Nuclear factor kappa-B (NF-κB) and activator protein-1 (AP-1) are well-known transcription factors that can be induced by multiple stimuli and have critical roles in the activation of genes encoding MMP-9 [11]. The transcription factors regulate the expression of a number of proteins involved in metastasis, tumorigenesis, and inflammation [12].

Annexin A1 (ANXA1), also known as lipocortin, is a member of the annexin superfamily of calcium- and phospholipid-binding proteins [13]. ANXA1 is an endogenous mediator of the anti-inflammatory effects of glucocorticoids [14]. A recent report indicated that ANXA1 enhances activation of NF-κB through its interaction with the IκB kinase (IKK) complex, and subsequently induces C-X-C chemokine receptor type 4 (CXCR4)-mediated migration of breast cancer cell lines in response to C-X-C chemokine ligand 12 (CXCL12) [15]. As a potential marker for tumor progression, ANXA1 expression levels are upregulated in pancreatic, hepatic, stomach cancers, and breast cancers [16]. By contrast, reduced ANXA1 expression levels have been observed in prostate, head and neck, and esophageal cancers [17]. In addition, loss of ANXA1 expression is correlated with the development and progression of breast cancer [18]. The amplification of ANXA1 levels has been reported to occur in several multidrug-resistant (MDR) tumor cells with or

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without P-gp1 and MRP1 expression [19,20]. When comparing MDA-MB-231 ($ANXA1^+$) and MCF-7 ($ANXA1^-$) cells, MDA-MB-231 cells have greater resistance to adriamycin, melphalan, and etoposide than MCF-7 cells do [20]. Recent studies also suggest the existence of potential correlations between the level of ANXA1 expression and EMT in breast cancer [21]. However, the mechanism by which ANXA1 functions as a tumor promoter in breast cancer cells remains unclear.

In this study, we investigated the effects of ANXA1 on MMP-9 expression in breast cancer cells and explored the underlying upstream signaling mechanism. We found that ANXA1 significantly enhances MMP-9 gene expression by increasing NF-κB activity and consequently, induces invasion and migration of human breast cancer cells.

## 2. Materials and methods

### 2.1. Cells and reagents

The human breast cancer cell lines drug sensitive-MCF-7 [20], MDA-MB 231, T47D, and SKBr3 were maintained in RPMI 1640 supplemented with 10% heat-inactivated FBS. Lipofectamine 2000 reagent was purchased from Invitrogen (Carlsbad, CA). Anti-ANXA1, anti-tubulin, and anti-p65 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-MMP-9 antibody was from Cell signaling (Beverly, MA). Phorbol 12-myristate 13-acetate (PMA) was purchased from Calbiochem (San Diego, CA). All the chemicals not included above were from Sigma.

### 2.2. Western blot analysis

Cell lysates were separated on 10% SDS–polyacrylamide gels and transferred to nitrocellulose membranes. The blots were incubated with anti-ANXA1 and anti-MMP-9 antibodies and developed with the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech., Piscataway, NJ). The same blot was stripped and reprobed with anti-tubulin antibody for use as an internal control.

### 2.3. In vitro invasion assay

The 8-μm pore size polycarbonate nucleopore filter inserts in a 24-well transwell chamber were coated with 30 μg/well Matrigel (Sigma). Transfected-MDA-MB-231 cells were seeded into the upper part of the Matrigel-coated filter, and serum-free RPMI was added to the lower part. After 36 h, the cells that had migrated through the Matrigel and the 8-μm pore-size membranes were fixed, stained, and counted under a light microscope.

### 2.4. Wound healing assay

Transfected-MDA-MB-231 cells were incubated until 90–100% confluency. After cells were scratched by a P-10 pipette tip, cells were incubated for various time periods and the pictures were taken. The pictures were taken at 0 and 24 h. Phase contrast images were taken by Nikon microscopy system (Nikon Instrument). The wound-healing gap distance was measured using the computer program Image J. Results were expressed as the mean ± SE. $p \leqslant 0.05$ was considered significant (Student's $t$ test).

### 2.5. RNA interference of ANXA1

Scrambled control and human ANXA1 specific siRNA were purchased from Bioneer (Daejeon, Korea). For RNA interference experiments, cells were plated at a density of $5 \times 10^5$ cells/well in a 6-well plate. After 24 h, cells were transfected with siRNA using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions.

### 2.6. RNA extraction and semi-quantitative RT-PCR

Total RNA was extracted from cells with Trizol (Invitrogen) according to the manufacturer's protocol. Approximately 1 μg of total RNA was used to prepare cDNA using the Superscript First Strand cDNA synthesis Kit (Bioneer, Daejeon, South Korea). The following primers were used in this study: 5′-AGCGTCAACAGATCAAAG CAGCAT-3′ and 5′-AGACCCTGTTAATGTCTCTGATTT-3′ for ANXA1; 5′-TCCCTGGAGACCTGAGAACC-3′ and 5′-CGGCAAGTCTT CCGAGTAGTT-3′ for MMP-9; 5′-CCATCACCATCTTCCAGGAG-3′ and 5′- CCTGCTTCACCACGTTCTTG-3′ for GAPDH. PCR was performed with Platinum Taq polymerase (Invitrogen) under the following conditions: 30 cycles of 96 °C for 40 s, 55 °C (ANXA1 and MMP-9) or 60 °C (GAPDH) for 40 s, and 72 °C for 1 min followed by 10 min at 72 °C. All the PCR reactions were repeated at least three times. GAPDH was amplified as an internal control. The intensity of each band amplified by RT-PCR was analyzed using MultiImageTM Light Cabinet (version 5.5, Alpha Innotech Corp., San Leandro, CA.

### 2.7. Gelatin zymography

The presence of MMP-9 in the supernatants of ANXA1 siRNA transfected-MDA-MB-231 cells was analyzed with gelatin zymograms. Briefly, cells were incubated in serum-free RPMI and the supernatants were collected after incubation for 24 h, clarified by centrifugation, normalized to the total protein concentration of the cell lysate, mixed with non-reducing Laemmli sample buffer, and separated by electrophoresis in 10% SDS–PAGE containing 1 mg/ml gelatin (DIFCO). Subsequent steps were performed as described [22] and MMP-9 activities were visible as clear bands on a blue background where the gelatin substrate had been hydrolyzed by enzyme activity.

### 2.8. Elisa

The supernatants were collected for measuring secreted-MMP-9 protein. The total and active MMP-9 protein was assayed according to SensoLyte PlusTM 520 MMP-9 assay system (AnaSpec, San Jose, CA). MMP-9 activity unit was expressed as a change in fluorescence intensity at excitation of 490 nm/emission of 520 nm.

### 2.9. Transient transfection and luciferase reporter assay

Transcriptional activities of MMP-9, NF-κB and AP-1 were measured by the luciferase reporter assay using the pMMP-9-Luc, pNF-κB-Luc and pAP-1-Luc reporter plasmids. MDA-MB-231 cells were seeded into 6-well plates. Cells at 70–80% confluency were co-transfected with 0.2 μg of MMP-9 or NF-κB reporter constructs and 0.2 μg of pSV-β-galatosidase for 24 h. The luciferase and β-galactosidase activities were assayed according to the manufacture's protocol (Promega), using a Luminometer 20/20n (Turner BioSystems, Sunnyvale, CA).

### 2.10. Electrophoretic mobility shift assay (EMSA)

Double-stranded oligonucleotides containing the NF-κB (5′-AGTTGAGGGGACTTTCCCAGGC-3′) or consensus sequences were 5′-end-labeled with $\gamma$-$^{32}$P ATP using T4 polynucleotide kinase. Unincorporated nucleotide was removed by passage over a Bio-Gel P-6 spin column (Bio-Rad, Inc., Hercules, CA). Nuclear extract was incubated with radiolabeled probe for 20 min, and protein-DNA complexes were separated from free probes by

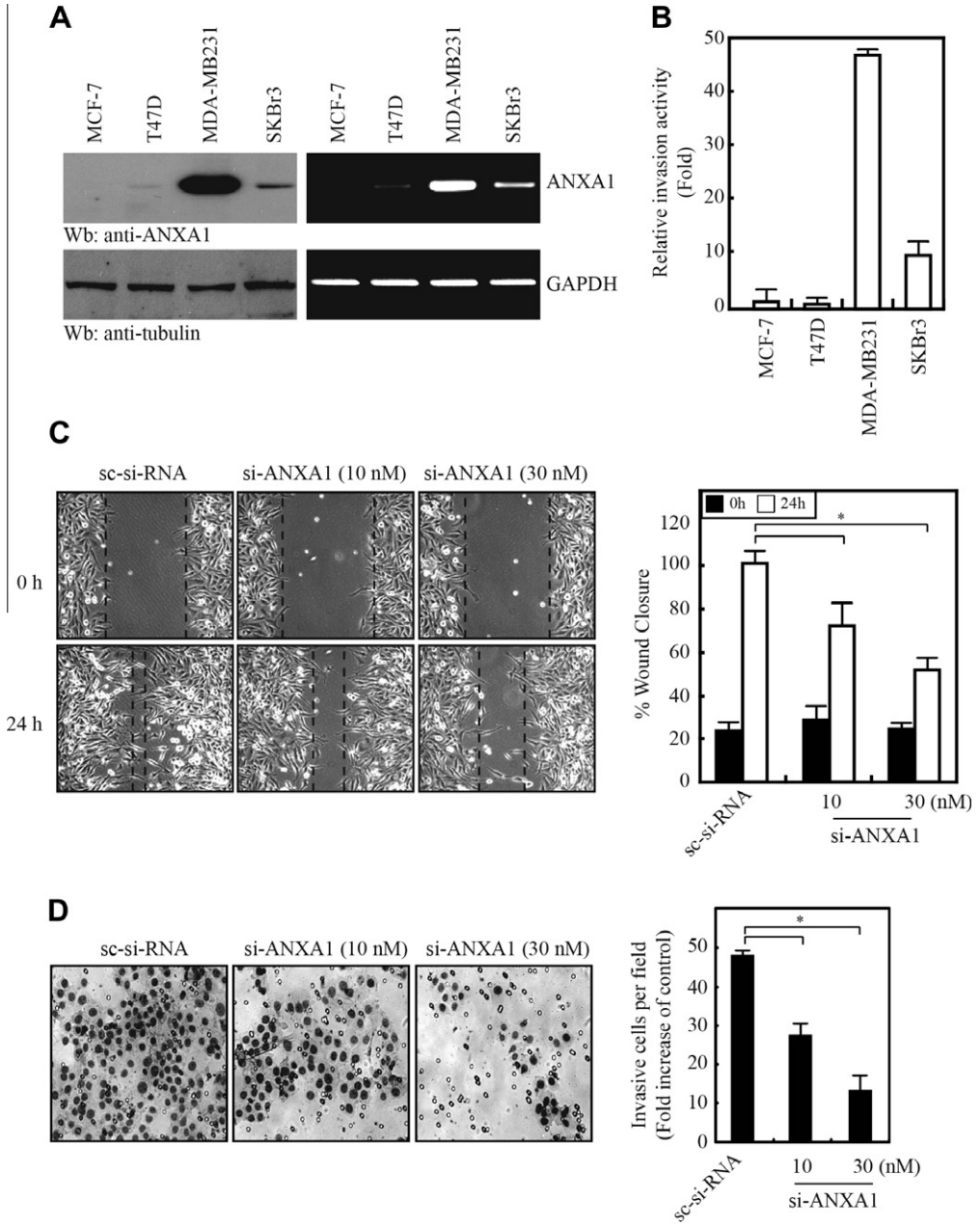


**Fig. 1.** ANXA1 siRNA inhibits invasion and migration of MDA-MB-231 cells. (A) Expression of ANXA1 protein and mRNA in cultured breast cancer cell lines (MCF-7, T47D, MDA-MB-231, and SKBr3) were assessed by western blotting and semiquantitative RT-PCR. Expression of Protein and mRNA levels were normalized with α-tubulin or GAPDH. (B) MCF-7, T47D, MDA-MB-231, and SKBr3 cells were analyzed for quantification of cell migration using transwells as described in Materials and Methods. Data are expressed as the mean ± SD and one way analysis of variance (ANOVA) was performed to determine statistical significance (*$p < 0.05$). (C) MDA-MB-231 cells were transfected with the ANXA1 siRNA. Representative images of wound healing were taken at the time of the scratch and 24 h of the wound scratch. The level of cell migration into the wound scratch was quantified as the percentage of wound healing. Data are presented as the mean ± SD. (D) MDA-MB-231 cells were transfected with the indicated amounts of ANXA1 siRNA, and after 36, Matrigel invasion under normal growth conditions was measured. Data are presented as the fold change of invasion relative to control inserts (*$p < 0.05$).

electrophoresis on a 4% native polyacrylamide gel in 0.5 × Tris–HCl (pH 7.5), 1 mM $MgCl_2$, 50 mM NaCl, 0.5 mM EDTA, 4% glycerol, 0.5 mM DTT, and 50 mg/ml of poly (dI-dC). Dried gels were visualized by autoradiography.

### 2.11. Chromatin immunoprecipitation assay

For ChIP experiments, approximately $4 \times 10^7$ cells were used per each sample. Cells were washed with PBS and treated with 1% formaldehyde in medium for 10 min at 25 °C followed by addition of glycine to a final concentration of 0.125 M for 5 min. Cells were then scraped into PBS and centrifuged at 1000*g* for 5 min at 4 °C. ChIP assays were performed by co-precipitating the DNA–protein complexes with anti-p65 antibody or control-IgG. The promoter region −690 to −506 and −485 to −301 of MMP-9 were amplified from the prepared DNA samples using the oligomers.

### 2.12. Statistics analysis

Data are presented as the mean ± S.D. Statistical evaluation was carried out by the Student's *t*-test. Data were considered statistically significant when $p < 0.05$. All statistical analysis was

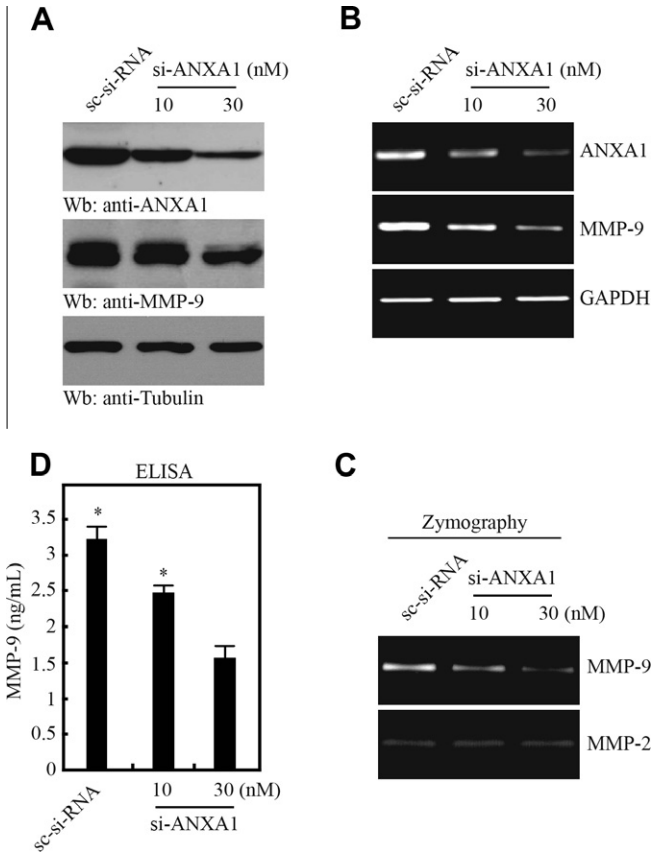


**Fig. 2.** ANXA1 siRNA suppresses expression of MMP-9 in MDA-MB-231 cells. (A and B) MDA-MB-231 cells were transfected with the indicated amounts of ANXA1 siRNA. After 24 h, cells were assessed by western blotting and semiquantitative RT-PCR. Western blotting and RT-PCR analysis showing downregulation of MMP-9 in ANXA1-silenced MDA-MB-231 cells by 80% respectively, as compared to scramble siRNA. Tubulin or GAPDH is used as a loading control. (C and D) MDA-MB-231 cells were transfected with the indicated amounts of ANXA1 siRNA. The MMP-9 protein levels in the supernatants were determined using an ELISA (C), and the proteolytic activity of MMP-9 was determined by gelatin zymographic analysis (D). Results are shown as the mean ± SD of three experiments performed in triplicate.

performed by the computer program Prism (GraphPad Software, La Jolla, CA).

## 3. Results

### 3.1. ANXA1 regulates invasion and migration of breast carcinoma cells

To investigate the potential role of the ANXA1 in breast cancer cell migration and invasion, we first examined both ANXA1 expression and cell invasion in four breast cancer cell lines. Western blotting and RT-PCR analysis showed that ANXA1 expression was high in the MDA-MB-231 cell line, but low in the SKBr3 and T47D cell lines, and non-existent in the MCF-7 cell line (Fig. 1A). These results agree with a previous study reporting that ANXA1 was not expressed in drug-sensitive MCF-7 cells, but highly expressed in MDA-MB-231 cells [19,20]. Transwell migration assays demonstrated that cell lines with high ANXA1 expression levels were invasive in nature, whereas lines with poor or no ANXA1 expression were poorly migratory in nature (Fig. 1B). These results display an excellent correlation between ANXA1 expression and cell migration (Fig. 1A and B). We then investigated the importance of ANXA1 expression in cell migration and invasion; ANXA1 levels were depleted by performing a siRNA knockdown in MDA-MB-231 cells. An ANXA1 knockdown performed in MDA-MB-231 cells resulted in a markedly decreased ability of cellular migration and invasion compared with the scrambled control siRNA-transfected cells, as examined by wound healing and a transwell matrix penetration assay (Fig. 1C and D). These results strongly suggest a role for ANXA1 in modulation of the invasiveness of breast cancer cells.

### 3.2. si-ANXA1 suppresses the expression and proteolytic activity of MMP-9

MMP-9 is upregulated in metastatic cancer cells and has been implicated in a wide range of pathological conditions, including inflammation and tissue repair [4]. To examine whether ANXA1 is involved in MMP-9 gene expression, we performed an ANXA1 siRNA knockdown in MDA-MB-231 cells, which exhibit higher expression levels of ANXA1 than other breast cancer cell lines. The mRNA and protein expression levels of MMP-9 were decreased in a dose-dependent manner in cells transfected with ANXA1 siRNA compared with cells transfected with a scramble control siRNA, i.e., a siRNA with a scrambled sequence (Fig. 2A and B). We then examined whether ANXA1 induces the expression of MMP-9 protein using an ELISA assay. Consistent with the RT-PCR and western blotting results, ANXA1 siRNA-transfected cells showed lower MMP-9 protein production compared with cells transfected with a scramble control siRNA (Fig. 2C). We also examined the effect of ANXA1 on the proteolytic activity of MMP-9 using zymographic analysis. ANXA1 siRNA reduced the proteolytic activity of MMP-9 in MDA-MB-231 cells (Fig. 2D). These results indicate that ANXA1 is involved in the regulation of endogenous MMP-9 gene transcription and subsequently, in the proteolytic activity of MMP-9.

### 3.3. si-ANXA1 inhibits the transcriptional activity of MMP-9 via suppression of NF-κB activity

To understand the transcriptional activity of *MMP-9* gene promoter induced by ANXA1, we isolated the 5′-regulatory region of the human *MMP-9* gene located 925 bp upstream of the transcriptional start sited and subcloned the insert into the pGL4-luc luciferase reporter vector, yielding the pMMP-9 plasmids (−925/+13). We co-transfected the MMP-9 promoter–luciferase construct into MDA-MB-231 cells with ANXA1 siRNA or scramble control siRNA. As shown in Fig. 3A, the promoter activity of MMP-9 in cells transfected with ANXA1 siRNA was downregulated by 2.2-fold compared with scramble control siRNA-transfected MDA-MB-231 cells. The presence of ANXA1 siRNA decreased promoter activity of MMP-9 in a dose-dependent manner (Fig. 3A), indicating that ANXA1 regulates MMP-9 expression at the transcriptional level. The promoter of human MMP-9 contains cis-acting regulatory elements that bind to transcription factors such as NF-κB or AP-1 [10]. The transcription factor binding sites in the MMP-9 promoter include binding sites for NF-κB (−600 bp), SP-1 (−569 bp) and AP-1 (−533 bp and −79 bp) [10]. To determine which of the transcription factors participate in regulation of the MMP-9 promoter by ANXA1, we generated serial fragments of the MMP-9 promoter containing the various transcription factor–binding sites. Although ANXA1 siRNA reduced promoter activity of MMP-9 (−925/+13) by 2.2-fold, the promoter activities of NF-κB deletion mutants (−580/+13 and −450/+13) displayed no difference in their response to ANXA1 siRNA. These results indicate that the NF-κB binding site (−600 bp) in the MMP-9 promoter is involved in the modulator effect of ANXA1 on transcriptional activity of MMP-9. Furthermore, we generated the promoter with a mutation in the NF-κB binding site (MMP-9 mNF-κB). The results showed that ANXA1 siRNA did not affect the promoter activity of MMP-9 mNF-κB (Fig. 3C). These results indicated that the binding of NF-κB to the MMP-9 promoter

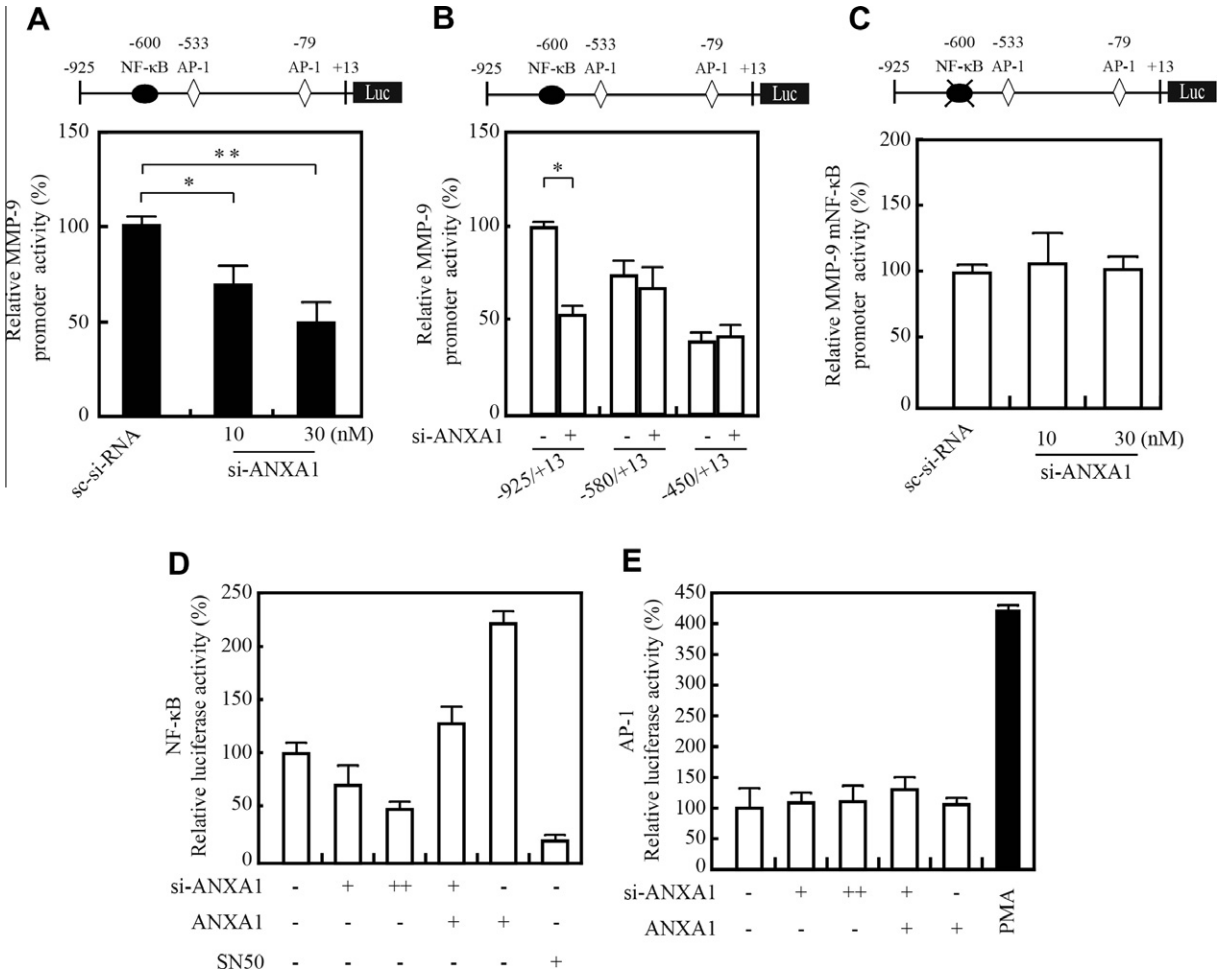


**Fig. 3.** ANXA1 siRNA inhibits the transcription of MMP-9 promoter constructs via suppression of NF-κB activity. (A) MDA-MB 231 cells were co-transfected with the indicated amounts of ANXA1 siRNA and pMMP-9-luciferase (−925/+13). The luciferase activity was determined after 24 h of transfection. The luciferase activity was normalized to β-galactosidase activity, and the experiments were performed in triplicate. Data are expressed as the mean ± SD and are presented as the relative luciferase activity. (B and C) ANXA1 siRNA was co-transfected with the deletion mutants pMMP-9-Luc (B) and NF-KB binding site mutant (mNF-KB) pMMP-9-Luc (C) into MDA-MB-231 cells and then, the luciferase activities were determined. The luciferase activity was normalized to β-galactosidase activity, and the experiments were performed in triplicate. Data are expressed as the mean ± SD and are presented as the relative luciferase activity. (D and E) MDA-MB-231 cells were transfected with reporter plasmids containing tandem NF-κB or AP-1 binding sites. The luciferase activity driven by the tandem NF-κB promoter was significantly decreased by ANXA1 siRNA, but rescue of ANXA1 expression in ANXA1-silenced cells or only ANXA1 expression cells resulted in up-regulation of NF-κB activity. SN50 was used as a positive control for NF-κB activity (D). The luciferase activity driven by the tandem AP-1 promoter was not significantly changed by ANXA1 siRNA or ANXA1. PMA was used as a positive control for AP-1 activity (E).

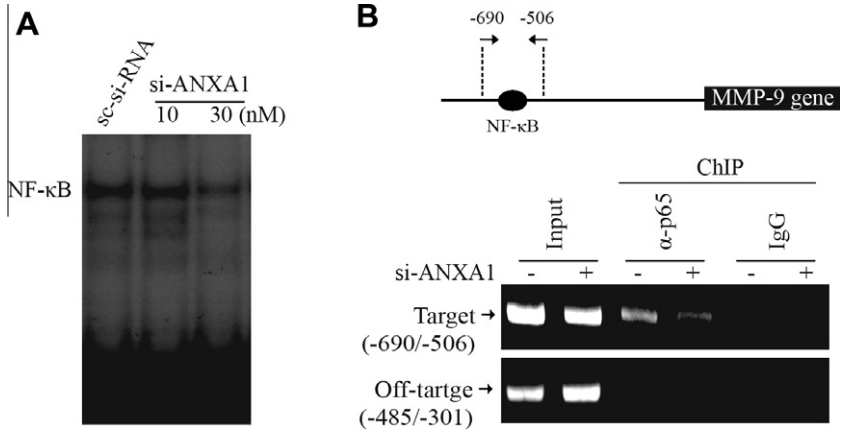


**Fig. 4.** ANXA1 siRNA inhibits the basal NF-κB activity in MDA-MB-231 cells. (A) MDA-MB-231 cells were transfected with the indicated amount of ANXA1 siRNA for 24 h. Then the nuclear extract was assayed by EMSA. (B) MDA-MB-231 cells were transfected with the scramble siRNA and ANXA1 siRNA, cross-linked, lysed, and immunoprecipitated with anti-p65 antibody and rabbit IgG as a negative control. The precipitated DNA was subjected to PCR with primers specific for the off-target region (−485/−301) or the target region (−690/−506). One aliquot of input DNA was used as a positive control.

contributes to the inhibitory effect of ANXA1 siRNA on MMP-9 transcription activity. For further investigation, luciferase reporter vectors containing tandem repeats of the NF-κB– or AP-1–binding sites were used. As shown in Fig. 3D, the luciferase activity in cells transfected with the NF-κB reporter was decreased in a dose-dependent manner by ANXA1 siRNA. However, reconstitution of ANXA1 rescued NF-κB reporter activity compared with ANXA1 siRNA. As a positive control, SN50, a specific inhibitor of NF-KB, completely abolished MMP-9 promoter activity. However, no significant changes were observed in cells transfected with the AP-1 reporter (Fig. 3E). These results indicate that NF-κB transcription factor and its binding sites on the MMP-9 promoter region contribute to the inhibitory effect of ANXA1 siRNA on MMP-9 transcription.

### *3.4. si-ANXA1 inhibits the DNA binding activity of NF-κB in the MMP-9 promoter*

To determine the effect of ANXA1 on the DNA binding activities of NF-κB, we performed electrophoretic mobility shift assays and chromatin immunoprecipitation (ChIP) assays. As shown in Fig 4A, ANXA1 siRNA reduced the DNA-binding activities of NF-κB in a dose-dependent manner, whereas the DNA-binding activity of AP-1 was unaffected by ANXA1 siRNA (data not shown). The ChIP assay revealed that in scramble control siRNA-transfected cells, NF-κB had already been recruited to the promoters of MMP-9, and the recruitment was reduced by ANXA1 siRNA (Fig. 4B). Therefore, ANXA1 siRNA antagonizes the NF-κB stimulatory effect on MMP-9 expression, resulting in blockage of breast cancer cell migration induced by NF-κB.

## 4. Discussion

MMPs belong to a family of zinc-dependent endopeptidases that are involved in degradation and remodeling of ECM proteins associated with tumorigenic processes [3]. These MMPs promote tumor invasion and metastasis, as well as regulate host defense mechanism and normal cell function. Therefore, MMP inhibitors are expected to be useful chemotherapeutic agents in the treatment of malignant cancer, osteoarthritis, and rheumatoid arthritis [23]. Recent studies have shown that MMP-9 is a critical determinant in the invasive ability of human breast cancer [10,24], and inhibition of MMP-9-mediated invasion suppresses metastasis of breast cancer cells [4]. Therefore, identification of proteins that regulate MMP-9 expression could be effective in the treatment of breast cancer. The current study was designed to estimate the invasive potential of ANXA1 and to explore the molecular mechanism underlying its function. We first evaluated the effect of ANXA1 on promoting migration and invasion in MDA-MB-231 cells. We demonstrated that (1) ANXA1 siRNA inhibits invasion and migration, (2) ANXA1 regulates MMP-9 expression and activity, and (3) ANX-1 siRNA inhibits the activation of NF-κB in MDA-MB-231 cells.

While MMP2 is usually expressed constitutively, the synthesis and secretion of MMP-9 is stimulated by a variety of stimuli during various pathological processes such as tumor invasion, atherosclerosis, inflammation, and rheumatoid arthritis [4,9]. Several reports have shown that NF-κB and AP-1 induce invasion and migration of breast cancer cells by MMP-9 secretion [11]. The MDA-MB-231 cells, which contain endogenous ANXA1, are usually highly invasive. Our results show that siRNA knock-down of ANXA1 decreases the gene expression and secretion of MMP-9 in MDA-MB-231 cells. Transcription of the MMP-9 gene is regulated by the upstream promoter sequences, including the binding sites for AP-1, NF-κB, and SP-1 [6]. The AP-1 and NF-κB elements of the MMP-9 promoter are involved in the induction of MMP-9 [11]. To gain a comprehensive understanding of the ANXA1-related signaling cascade underlying MMP-9 expression in MDA-MB-231 cells, we examined MMP-9 promoter activity by using deleted and mutated reporter plasmids. We found that the NF-κB binding site is necessary for the inhibition of MMP-9 expression by ANXA1 siRNA-transfected MDA-MB-231 cells. Furthermore, results from a luciferase assay showed that ANXA1 siRNA suppresses tandem NF-κB promoter activity, but not AP-1 promoter activity. Our results are supported by a recent report that ANXA1 is a constitutive activator of NF-κB, in particular with an upstream signaling pathway, such as the IKK complex [15]. The binding of ANXA1 to IKKγ or NEMO has been documented by gel-filtration analysis and immunoprecipitation. This report suggests that ANXA1 enhances the activation of NF-κB. Our results indicate that ANXA1 regulates the NF-κB activity that is involved in MMP-9 expression during invasion.

In summary, ANXA1 enhances breast cancer invasion, at least in part, through the activation of NF-κB and expression of the MMP-9 gene. Given that ANXA1 is involved in human breast cancer progression, it is an attractive therapeutic target.

## Acknowledgments

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## References

[1] E.C. Woodhouse, R.F. Chuaqui, L.A. Liotta, General mechanisms of metastasis, Cancer 80 (1997) 1529–1537.
[2] E.I. Deryugina, G.X. Luo, R.A. Reisfeld, M.A. Bourdon, A. Strongin, Tumor cell invasion through matrigel is regulated by activated matrix metalloproteinase-2, Anticancer Res. 17 (1997) 3201–3210.
[3] H. Sato, T. Takino, Y. Okada, J. Cao, A. Shinagawa, E. Yamamoto, M. Seiki, A matrix metalloproteinase expressed on the surface of invasive tumour cells, Nature 370 (1994) 61–65.
[4] Y. Itoh, H. Nagase, Matrix metalloproteinases in cancer, Essays Biochem. 38 (2002) 21–36.
[5] L.S. Orlichenko, D.C. Radisky, Matrix metalloproteinases stimulate epithelial-mesenchymal transition during tumor development, Clin. Exp. Metastasis 25 (2008) 593–600.
[6] A. Hozumi, Y. Nishimura, T. Nishiuma, Y. Kotani, M. Yokoyama, Induction of MMP-9 in normal human bronchial epithelial cells by TNF-alpha via NF-kappa B-mediated pathway, Am. J. Physiol. Lung Cell Mol. Physiol. 281 (2001) L1444–1452.
[7] J. Yao, S. Xiong, K. Klos, N. Nguyen, R. Grijalva, P. Li, D. Yu, Multiple signaling pathways involved in activation of matrix metalloproteinase-9 (MMP-9) by heregulin-beta1 in human breast cancer cells, Oncogene 20 (2001) 8066–8074.
[8] R.L. Thangapazham, N. Passi, R.K. Maheshwari, Green tea polyphenol and epigallocatechin gallate induce apoptosis and inhibit invasion in human breast cancer cells, Cancer Biol. Ther. 6 (2007) 1938–1943.
[9] N. Johansson, M. Ahonen, V.M. Kahari, Matrix metalloproteinases in tumor invasion, Cell Mol. Life Sci. 57 (2000) 5–15.
[10] H. Sato, M. Seiki, Regulatory mechanism of 92 kDa type IV collagenase gene expression which is associated with invasiveness of tumor cells, Oncogene 8 (1993) 395–405.
[11] S.K. Park, Y.S. Hwang, K.K. Park, H.J. Park, J.Y. Seo, W.Y. Chung, Kalopanaxsaponin A inhibits PMA-induced invasion by reducing matrix metalloproteinase-9 via PI3K/Akt- and PKCdelta-mediated signaling in MCF-7 human breast cancer cells, Carcinogenesis 30 (2009) 1225–1233.
[12] X. Dolcet, D. Llobet, J. Pallares, X. Matias-Guiu, NF-kB in development and progression of human cancer, Virchows Arch. 446 (2005) 475–482.
[13] R.J. Flower, N.J. Rothwell, Lipocortin-1: cellular mechanisms and clinical relevance, Trends Pharmacol. Sci. 15 (1994) 71–76.
[14] G. Cirino, R.J. Flower, J.L. Browning, L.K. Sinclair, R.B. Pepinsky, Recombinant human lipocortin 1 inhibits thromboxane release from guinea-pig isolated perfused lung, Nature 328 (1987) 270–272.
[15] P. Bist, S.C. Leow, Q.H. Phua, S. Shu, Q. Zhuang, W.T. Loh, T.H. Nguyen, J.B. Zhou, S.C. Hooi, L.H. Lim, Annexin-1 interacts with NEMO and RIP1 to constitutively activate IKK complex and NF-kappaB: implication in breast cancer metastasis, Oncogene 30 (2011) 3174–3185.

[16] S.H. Ahn, H. Sawada, J.Y. Ro, G.L. Nicolson, Differential expression of annexin I in human mammary ductal epithelial cells in normal and benign and malignant breast tissues, Clin. Exp. Metastasis 15 (1997) 151–156.
[17] D. Shen, F. Nooraie, Y. Elshimali, V. Lonsberry, J. He, S. Bose, D. Chia, D. Seligson, H.R. Chang, L. Goodglick, Decreased expression of annexin A1 is correlated with breast cancer development and progression as determined by a tissue microarray analysis, Hum. Pathol. 37 (2006) 1583–1591.
[18] D. Shen, H.R. Chang, Z. Chen, J. He, V. Lonsberry, Y. Elshimali, D. Chia, D. Seligson, L. Goodglick, S.F. Nelson, J.A. Gornbein, Loss of annexin A1 expression in human breast cancer detected by multiple high-throughput analyses, Biochem. Biophys. Res. Commun. 326 (2005) 218–227.
[19] Y. Wang, X.Q. Pan, F. Lheureux, E. Georges, Overexpression of a 40-kDa protein in human multidrug resistant cells, Biochem. Biophys. Res. Commun. 236 (1997) 483–488.
[20] Y. Wang, L. Serfass, M.O. Roy, J. Wong, A.M. Bonneau, E. Georges, Annexin-I expression modulates drug resistance in tumor cells, Biochem. Biophys. Res. Commun. 314 (2004) 565–570.
[21] S. Maschler, C.A. Gebeshuber, E.M. Wiedemann, M. Alacakaptan, M. Schreiber, I. Custic, H. Beug, Annexin A1 attenuates EMT and metastatic potential in breast cancer, EMBO Mol. Med. 2 (2010) 401–414.
[22] Y. Kim, H. Kang, S.W. Jang, J. Ko, Celastrol inhibits breast cancer cell invasion via suppression of NF-kB-mediated matrix metalloproteinase-9 expression, Cell Physiol. Biochem. 28 (2011) 175–184.
[23] N.G. Lia, Z.H. Shib, Y.P. Tang, J.A. Duan, Selective matrix metalloproteinase inhibitors for cancer, Curr. Med. Chem. 16 (2009) 3805–3827.
[24] M.J. Duffy, T.M. Maguire, A. Hill, E. McDermott, N. O'Higgins, Metalloproteinases: role in breast carcinogenesis, invasion and metastasis, Breast Cancer Res. 2 (2000) 252–257.