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Metallothionein-2A overexpression increases the expression of matrix metalloproteinase-9 and invasion of breast cancer cells

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ABSTRACT

The overexpression of metallothionein-2A (MT-2A) is frequently observed in invasive human breast tumors and has been linked with more aggressive breast cancers. MT-2A overexpression led to the induction of MDA-MB-231 breast cancer cell migratory and invasive abilities. The reduction of MT-2A expression through small interfering RNA (siRNA) targeting MT-2A in invasive MDA-MB-231 cells completely inhibited both cell invasion and migration. In addition, the expression of matrix metalloproteinase-9 (MMP-9) and the transcriptional activity of AP-1 and NF-κB were upregulated by MT-2A overexpression. Collectively, our results provide the first demonstration that MT-2A promotes breast cancer cell invasion by upregulating MMP-9 via AP-1 and NF-κB activation. Furthermore, we found that MT-2A silencing can inhibit breast cancer invasiveness.
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1. Introduction

The rapid increase in the incidence rate of breast cancer has led to the search for and identification of biomarkers that can predict risk for and future behavior of this malignancy and can aid in its management. In this regard, several proteins, including estrogen receptors bcl-2 and metallothionein (MT), have been identified as potential biomarkers in breast cancer [1].

MTs are a group of low-molecular-weight (6–7 kDa) intracellular metal-binding proteins that are expressed in many tissues [2]. MT-1 and MT-2 are ubiquitously expressed, whereas MT-3 and MT-4 are expressed predominantly in the central nervous system and in the squamous epithelia, respectively [3]. MT plays an important role in apoptosis and proliferation, which can influence the carcinogenic process. MT-1 and MT-2 isoforms are expressed coordinately in most organs, but the precise role of these MT isoforms has not been well elucidated. The MT-2A mRNA transcript, which has been reported to be the highest among all the functional isoforms of MT detected in breast tissues, positively correlates with cell proliferation and histological grade [4]. Histological grade 3 tumors were observed to have a higher expression of MT-2A mRNA than grade 1 and 2 tumors. Recently, it has been demonstrated that the downregulation of MT-2A arrests growth in the MCF-7 human breast cancer cell lines, suggesting the close involvement of the MT-2A isoform in the proliferative activity of breast cancer cells [5,6]. Although the growth-promoting mechanism of MT-2A has been well characterized, the mechanism by which MT-2A enhances cell invasion and tumor metastasis is still unknown.

Metastasis is a complex, multi-step process that involves the separation of cells from the primary tumor, intravasation, extravasation, and the establishment of tumor cells at a secondary site [7]. The invasive ability of tumor cells is important in all of these steps. Invasion requires the degradation of the basement membrane to permit the egress and eventual ingress of malignant cells from the primary tumor. Several invasion-related proteases, including matrix metalloproteinase's (MMPs), cysteine proteinases, and serine proteases, are responsible for this process [8–10]. In particular, MMP-2 and MMP-9, the two predominately expressed MMPs in cancer cells that are capable of degrading type IV collagen, are thought to be directly involved in tumor cell growth and progression during metastasis [11,12]. Increased MMP activity is observed in human breast, prostate, lung, and ovarian tumors [13,14].

Here we report that MT-2A induces MMP-9 upregulation, invasion and migration of breast cancer cells. We further show that the activation of both NF-kB and AP-1 signaling pathways are required for the MT-2A-induced MMP-9 upregulation and for the

Abbreviations: MMPs, matrix metalloproteinase's; MT, metallothionein; siRNA, small interfering RNA; TIMPs, tissue inhibitors of metalloproteinase's

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subsequent induction of the invasive and migratory abilities of breast cancer cells.

2. Materials and methods

2.1. Establishment of MT-2A-overexpressing stable transfectants

MT-2A was amplified by RT-PCR using the primers 5′-GAATT-CATGGATCCCAACTGCT-3′ and 5′-GGATCCTCTGGCGCAGCAGCTG-3′. The MT-2A expression vector was constructed by inserting the fragment of MT-2A into pMEX-HA (Dualsystems Biotech AG, Zurich, Switzerland) and designated pMEX-MT-2A-HA. The human breast cancer cell line MDA-MB231 (American Type Culture Collection, Manassas, VA) was grown in DMEM supplemented with 2 mM glutamine and 10% FBS (Gibco-BRL-Life Technologies (Grand Island, NY), at 37 °C in an atmosphere containing 5% CO_2. The MT-2A expression vector was transfected into MDA-MB231 cells and subjected to antibiotic selection for 4 weeks. The stable transfectants with the highest MT-2A expression were used for the investigation.

2.2. Transient transfection and luciferase assays

The MMP-9 promoter, MMP-9 mAP-1, and MMP-9 mNF-kB constructs cloned into the pGL3-Basic luciferase vector (Promega) were kind gifts from Dr. Wolfgang Eberhardt. MDA-MB231 cells were transiently co-transfected with pCMV-β-gal and pGL3-MMP-9-Luc or pGL3-MMP-9 mAP-1-Luc or pGL3-MMP-9 mNF-kB-Luc, using the Lipofectamine 2000 reagent (Invitrogen, San Diego, CA, USA) according to the manufacturer's protocol. Cells were then lysed, and luciferase activity was measured using a luminometer (Luminoscan Ascent; Thermo Electron Co.). Luciferase activity was normalized to β-galactosidase activity in cell lysates and was expressed as an average of three independent experiments.

2.3. RNA preparation, semi-quantitative RT-PCT and real-time PCR

Total RNA was isolated from cells using an RNA isolation kit (Takara Shuzo, Kyoto, Japan) according to the manufacturer's protocol. The quality of the RNA was confirmed by a ratio of >1.8 for the optical densities at 260 and 280 nm. The PCR amplification protocol was 30 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min. Amplified products were resolved by 1.5% agarose gel electrophoresis. PCR product formation was continuously monitored during the PCR using the Sequence Detection System software, version 1.7 (Applied Biosystems, Foster City, CA, USA). Accumulated PCR products were detected directly by monitoring the increase in the reporter dye (SYBR) signal. The following primers were used in this study: 5′-TGACAGCGACAAGAAGTG-3′ and 5′-CAGTGAA-GCGGTACATAGG-3′ for MMP-9; 5′-CAATTCCGACCTCGTCATCA-3′ and 5′-TCAGAGCCTTGGAGGAGCT-3′ for TIMP1; 5′-CCGGCTCCTGC-AAATGCAAAGAGTG-3′ and 5′-CGCTCCCAGATGTAAAGAACGCGAC-3′ for MT-2A; and 5′-GATGATATCGCCGCGCTCGTCGTCGAC-3′ and 5′-AGCCAGGTCCAGACGCAGGATGGCATG-3′ for β-actin. The quantity of each transcript was calculated as described in the instrument manual and normalized to the levels of β-actin.

2.4. Western blot analysis

Cells were washed with phosphate-buffered saline (PBS) and were lysed on ice for 30 min in 100 μl of lysis buffer (120 mM NaCl, 40 mM Tris, pH 8, 0.1% Nonidet P-40). Lysates were centrifuged at 12 000 rpm for 30 min, and protein concentrations were determined using a BCA protein assay kit. Lysates (40 μg) were boiled for 5 min and were electrophoresed in 10% SDS–polyacrylamide gels. The separated proteins were transferred onto nitrocellulose membranes and were probed with antibodies against HA-Tag (detection of MT-2A-HA), MMP-9 (Cell Signaling Technology, Beverly, MA) or β-actin (Santa Cruz, CA, USA), followed by anti-mouse or anti-rabbit HRP conjugated secondary antibodies. The protein bands were detected using an enhanced chemiluminescence Western blotting detection kit.

2.5. Cell migration and invasion assay

Cell migration was assessed with a Transwell insert system (Millipore Corp., Bedford, MA, USA) using polycarbonate membranes of 8 μm pore size. Invasion assays were performed using Transwell inserts precoated with Matrigel. MDA-MB231 cells were incubated in DMEM with 10% FBS and were collected by trypsinization. Cells (1×10^5 cells/mL) in serum-free medium were added to the inner cup of a 48-well Transwell chamber that had been coated with 50 μl of Matrigel (1:10 dilution in serum-free medium; BD Biosciences, Franklin Lakes, NJ, USA). Medium supplemented with 10% serum or the indicated compounds were added to the outer cup. After 24 h, the cells that had migrated through the Matrigel and the 8-μm pore-size membrane were fixed, stained, and counted under a light microscope. Each experiment was performed in triplicate.

2.6. Gelatin zymography

The enzymatic activities of MMP-2 and MMP-9 were determined by gelatin zymography. Briefly, cells were seeded to confluence for 24 h and were maintained in serum-free medium. Conditioned media was collected 24 h after stimulation, mixed with non-reducing sample buffer, and subjected to electrophoresis in 10% polyacrylamide gels containing 0.1% (w/v) gelatin. The gel was washed in washing buffer containing 2.5% Triton X-100 and 50 mM Tris–HCl, pH 7.5, and was incubated at 37 °C for 24 h in 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 5 mM $CaCl_2$, 1 mM $ZnCl_2$, and 40 mmol/l NaN_3. The gel was stained with 0.25% (w/v) Coomassie brilliant blue in 45% (v/v) methanol and 1% (v/v) acetic acid. Gelatinolytic activity was normalized against the protein content of the cultured cells.

2.7. RNA interference

Small interfering RNA (siRNA) was synthesized by Genolution (Seoul, South Korea) with the following sequences: MT-2A, 5′-CCG GCT CCT GCA AAT GCA AAG AGT G-3′. Cells, grown to 50% confluence were transfected using oligofectamine transfection reagent (Invitrogen, Carlsbad, CA, USA) with MT-2A or scrambled siRNA according to the manufacturer's instructions.

2.8. Chromatin immunoprecipitation assay (CHIP assays)

Cells were cross-linked with formaldehyde, and the sonicated chromatin–DNA complexes were precipitated with anti-p65 and anti-*c*-Jun antibody. ChIP was carried out with Chromatin Immunoprecipitation Assay Kit (Upstate Biotechnology Inc., Lake Placid, NY). Then, the procedure followed manufacturer's protocol. PCR analysis was carried out using 1 μl of purified DNA, and Platinum *Taq* DNA polymerase (Invitrogen). After 40 amplification cycles, PCR products were analyzed on 2% agarose gel with ethidium bromide.

2.9. Statistical analyses

All experiments were performed in triplicate. Differences between the two groups were evaluated using the Student's t test. A one-way analysis of variance (ANOVA) was used to determine the significance of differences between treatment groups. The Newman-Keuls test was used for multi-group comparisons. Statistical significance was accepted for P-values < 0.01.

3. Results

3.1. Overexpression of MT-2A enhances migration and invasion of MDA-MB231 cells

Cancer cell tissue invasion is important during metastasis. It has previously been demonstrated that MDA-MB231 cells are able to migrate through a Matrigel matrix [15]. As shown in Fig. 1A, we transfected MDA-MB231 breast cancer cells with control (pMEX-HA, Con) or MT-2A expression vectors (pMEX-MT-2A-HA) and selected a stable cell line expressing a high level of the MT-2A. In vitro transwell migration assays demonstrated that MT-2A enhanced the migration of MDA-MB231 cells (Fig. 1B). In addition, the transwell invasion assay indicated that MT-2A-overexpressing cells exhibited a higher invasive ability than MDA-MB231 cells alone (Fig. 1C). Also, MT-2A overexpression led to the induction of MCF-7 breast cancer cell migratory and invasive abilities (Supplementary data). These data suggest that MT-2A enhances the metastatic potential of cancer cells.

3.2. Upregulation of MMP-9 by MT-2A

MMP-9 is highly expressed in human cancers, and a direct relationship between cancer progression and gelatinase expression and activity has been established in many studies [18]. Real-time PCR analysis was performed to determine the effects of MT-2A on MMP-9 mRNA expression levels. As shown in Fig. 2A and B, when the expression of MMP-9 in MT-2A-overexpressing cells was investigated, we observed an increase in MMP-9 mRNA levels. As the activity of MMP-9 is tightly regulated by the tissue inhibitors of metalloproteinase's (TIMPs) [16], we sought to investigate the levels of TIMP mRNA following MT-2A overexpression; however, no differences were observed in MT-2A-overexpressing cells (Fig. 2A). We further confirmed the upregulation of MMP-9 as gelatin zymography assays demonstrated that the levels of active MMP-9 increased in the culture medium of MT-2A-overexpressing cells (Fig. 2C). In addition, immunoblotting confirmed the upregulation of MMP-9 in the conditioned medium (Fig. 2C). These data suggest that MT-2A increases the expression and enzymatic activity of MMP-9 with no changes in TIMP-1 activity.

3.3. Silencing of MT-2A inhibits migration and invasion of MDA-MB231 cells

As tumor invasion and metastasis are often associated with the enhanced expression of MT-2A, we next sought to knock down MT-2A in the MT-2A-overexpressing cells (Fig. 3A). We examined the gene expression levels of MT-2A at 24 h post-transfection of

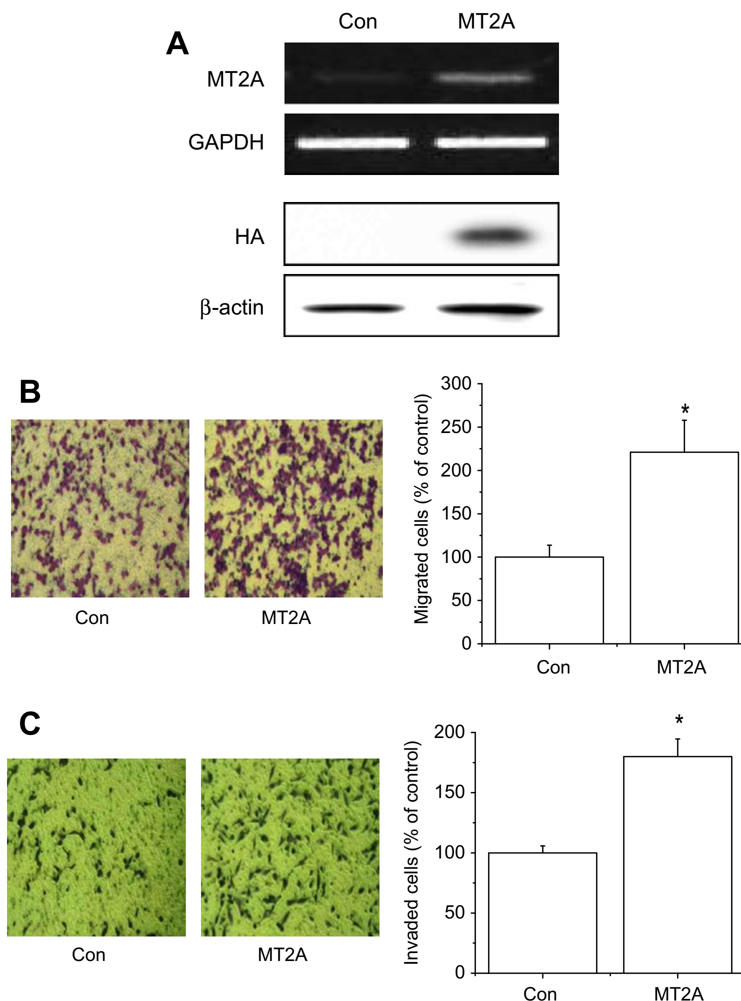

Fig. 1. Increase of migration and invasion by MT-2A. MDA-MB-231 cells were transfected with control or MT-2A expression vectors to establish stable transfectants. (A) The expression of MT-2A was determined by western blot and semi-quantitative RT-PCR analysis. (B and C) Cells were subjected to the in vitro invasion assay (B) and Transwell migration assay (C). After 24 h, the cells on the bottom side of the filter were counted. Migrating cells were imaged under a phase-contrast microscope. Each bar represents the mean ± S.D. calculated from three independent experiments. *Significantly different from control ($P < 0.01$).

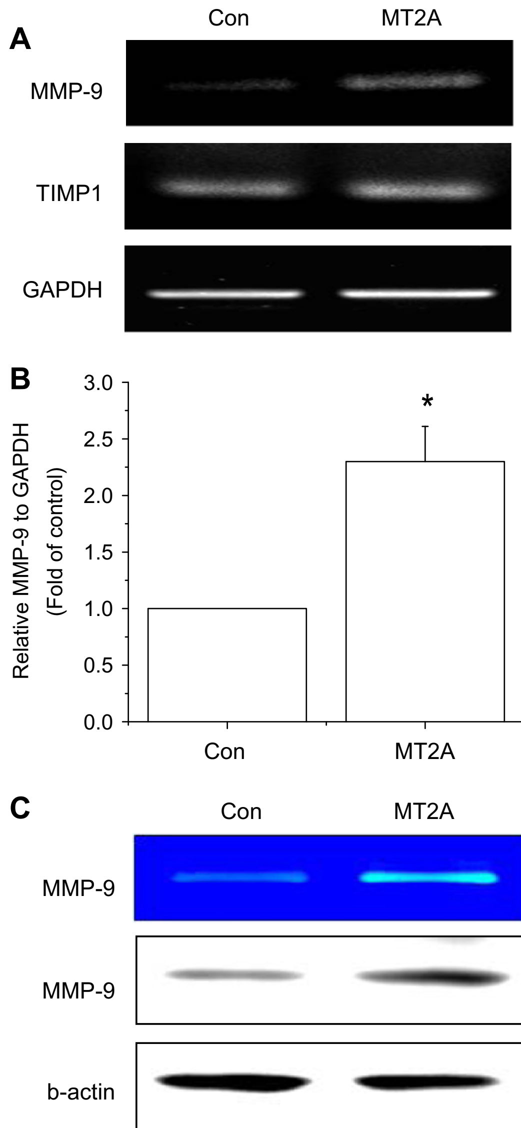

Fig. 2. MT-2A increases the expression and secretion of MMP-9. (A and B) Total RNAs were isolated from control- and MT-2A-overexpressing cells and the expression of MMP-9 and TIMP-1 were measured by semi-quantitative RT-PCR and real-time PCR. Bars show the mean ± S.D. of three independent experiments, performed in triplicate. $^{*}P < 0.01$, significantly different from control. (C) Conditioned media was collected and analyzed for the expression of and MMP-9 by western blot analysis. Zymography assays were performed, and the gelatinolytic activity of active MMP-9 (92 kDa) was visualized as clear bands against a blue background of stained gelatin.

MT-2A-siRNA in MDA-MB231 cells and observed a 70% reduction in MT-2A expression (Fig. 3B). The MT-2A knockdown was confirmed; Western blot analysis revealed that the expression and activity of MMP-9 in MDA-MB231 cells was effectively decreased following MT-2A silencing (Fig. 3C). We further observed that the reduction of MT-2A expression correlated with a significant reduction in MDA-MB231 cell invasion and migration (Fig. 3D and E), demonstrating that MT-2A plays a critical role in the induction of these characteristics in MDA-MB231 cells.

3.4. AP-1 and NF-κB are crucial for the MT-2A-induced MMP-9 upregulation, invasion and migration of MDA-MB231 cells

The expression of MMP-9 is regulated at the transcriptional level through the binding of AP-1 and NF-κB at specific sequences in the MMP-9 gene promoter [17,18]. To test whether these transcription factors regulate MMP-9 expression in MT-2A-overexpressing cells, cells were transiently transfected with a reporter gene that included the wild-type MMP-9 promoter, a promoter with mutations in the NF-κB site, or a promoter with mutations in AP-1 sites. As shown in Fig. 4A, MT-2A-overexpressing cells increased wild-type MMP-9 promoter activity compared to the control cells. However, the transcriptional activity of the MMP-9 AP-1 and NF-κB mutant reporters were unaffected in MT-2A-overexpressing cells, suggesting that the target of MT-2A was the AP-1 and NF-κB transcription factors (Fig. 4A). To confirm these findings, cells were transiently transfected with reporter vectors that included tandem repeats of the AP-1 and NF-κB binding sites. We observed that MT-2A-overexpressing cells increased the levels NF-κB and AP-1 transcriptional activity, confirming MT-2A activation of these signaling pathways (Fig. 4B). We subsequently performed ChIP assays using antibodies against NF-κB and AP-1, which further confirmed the direct binding of endogenous NF-κB and AP-1 to the MMP-9 promoter (Fig. 4C). To address the functional roles of NF-κB and AP-1 in MMP-9 expression and invasion/migration of MT-2A-overexpressing cells, we used the pharmacological inhibitors curcumin (a specific inhibitor of AP-1 activation), and Bay11-7802 (an inhibitor of NF-κB). Fig. 4D demonstrates that treatment with 10 μM curcumin or Bay11-7802 significantly inhibited MT-2A-induced MMP-9 expression as observed by gelatin zymogram assays. Moreover, the invasion and migration of MT-2A-overexpressing cells was significantly inhibited by 10 μM of curcumin or Bay11-7802 (Fig. 4E). These data demonstrate that the MT-2A-induced MMP-9 upregulation, and subsequent invasive and migratory ability of MDA-MB231 cells, requires the activation of NF-κB and AP-1 signaling pathways.

4. Discussion

Because metastasis is the principal cause of death from breast cancer, strategies for the development of therapeutic interventions to regulate breast cancer metastasis are of great importance. MTs have been implicated in breast cancer progression as oncogenic proteins, promoting cell invasion in several types of cancers [19]. Although the downregulation of MT-2A by siRNA in breast cancer cells results in the induction of growth arrest and apoptosis, the exact mechanism by which MT-2A affects cancer cell invasion in breast cancer has not been well elucidated [6]. In the current study, we attempted to elucidate the mechanisms of MT-2A mediated induction of breast cancer invasion. In MT knockout mice, impaired angiogenesis and wound healing have been reported in a model of cortical freeze injury [20]. The MT-2A data suggests a possible relationship between the invasiveness of breast cancer cells and the expression of MT-2A. Furthermore, there is evidence that MT-2A regulates the genes involved in angiogenesis and metastasis. Using siRNA mediated knock-down of MT-2A, the present study reveals MT-2A as an important target in the invasion and migration of MDA-MB-231 cells consistent with the reported major regulatory role of MT in cancer metastasis.

The originally described tumor-associated role of MMP-9 is in the degradation of the basement membrane. Indeed, the enhanced invasive activity of cancer cells is observed when MMP-9 is ectopically expressed. Pathological investigations also demonstrate that the expression of MMP-9 is strongly associated with tumor metastasis and is frequently negatively correlated with patient's survival. Clinical studies have demonstrated a correlation between MT-2A expression and MMP-9 in breast cancer [20]. It is possible that the poor prognosis of breast cancer is associated with MT-2A overexpression and may be mediated by MMP-9. In the present study,

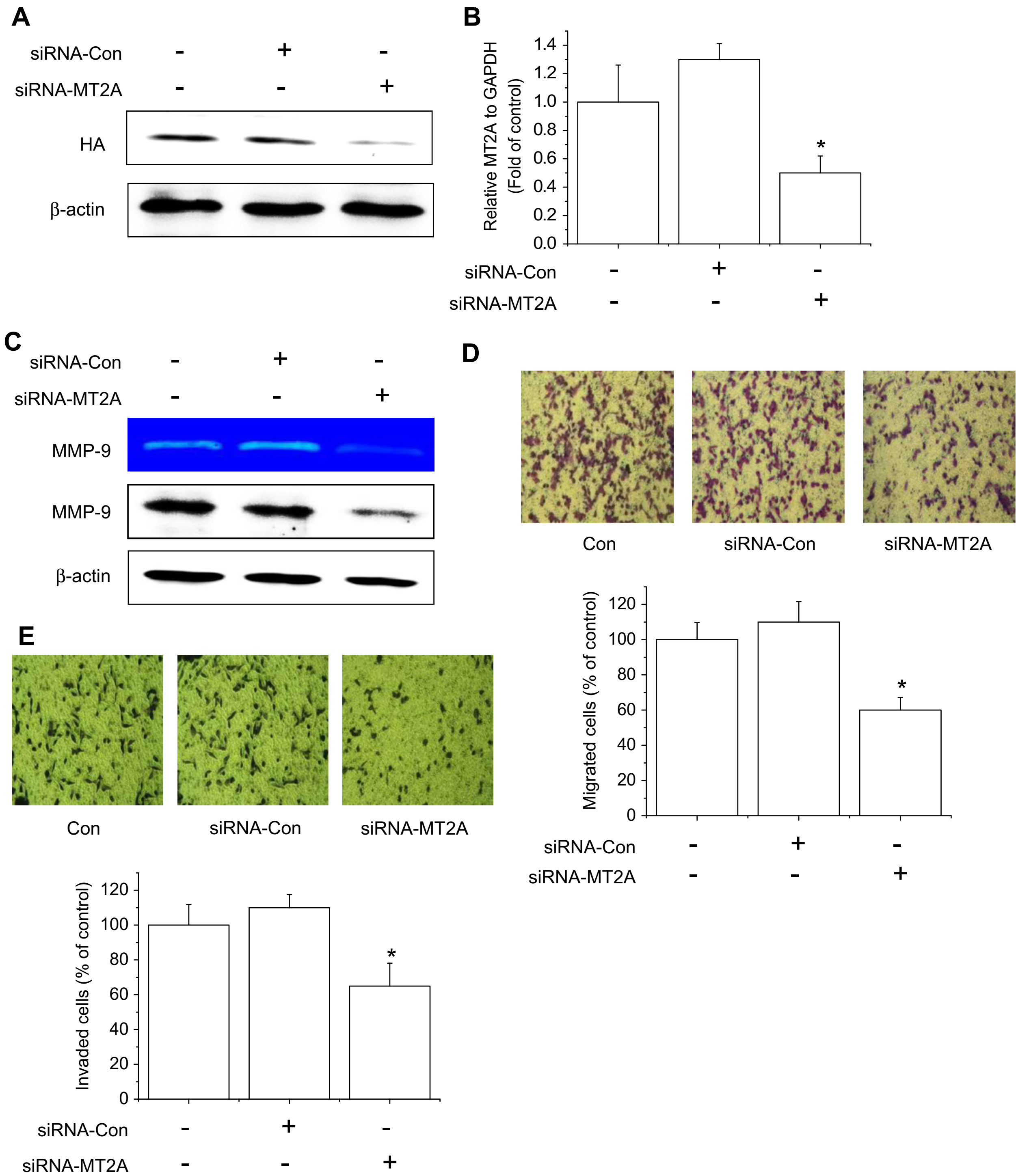

Fig. 3. Inhibition of MMP-9 expression and cell invasion by MT-2A siRNA. MDA-MB-231 cells or MT-2A-overexpressing cells were transfected with control (Con) or MT-2A siRNA vectors for 24 h. (A) Cellular proteins were also extracted for the determination of MT-2A protein levels by western blot analysis in MT-2A-overexpressing cells. (B) Total RNA was harvested for the study of MMP-9 expression by real-time PCR in MDA-MB-231 cells. (C) Conditioned media was collected, and MMP-9 activity in MDA-MB-231 cells was determined by gelatin zymography and by western blot analysis. (D and E) Cells transfected with siRNA for MT-2A were subjected to transwell migration assay (D) or in vitro invasion assays (E) in MDA-MB-231 cells. After 24 h, the cells on the bottom side of the filter were counted. Migrating cells were imaged under a phase-contrast microscope. Each bar represents the mean ± S.D. calculated from three independent experiments. *Significantly different from control ($P < 0.01$).

we demonstrate that overexpression of MT-2A increases MMP-9 expression to promote migration and invasion of breast cancer cells. We sought to further investigate if direct gene transcription is a potential mechanism for the MT-2A-induced MMP-9 upregulation of MDA-MB231 through luciferase reporter assays. The promoter region of MMP-9 has binding sites for AP-1 and NF-κB, and the important roles of AP-1 and NF-κB have been reported in MMP-9 gene activation [21,22]. In the present study, mutational analyses of the promoter region revealed that the major target of MT-2A was AP-1 and NF κB, a finding that was further confirmed through the use of reporter plasmids containing synthetic elements specific for various transcription factors. In addition, a direct

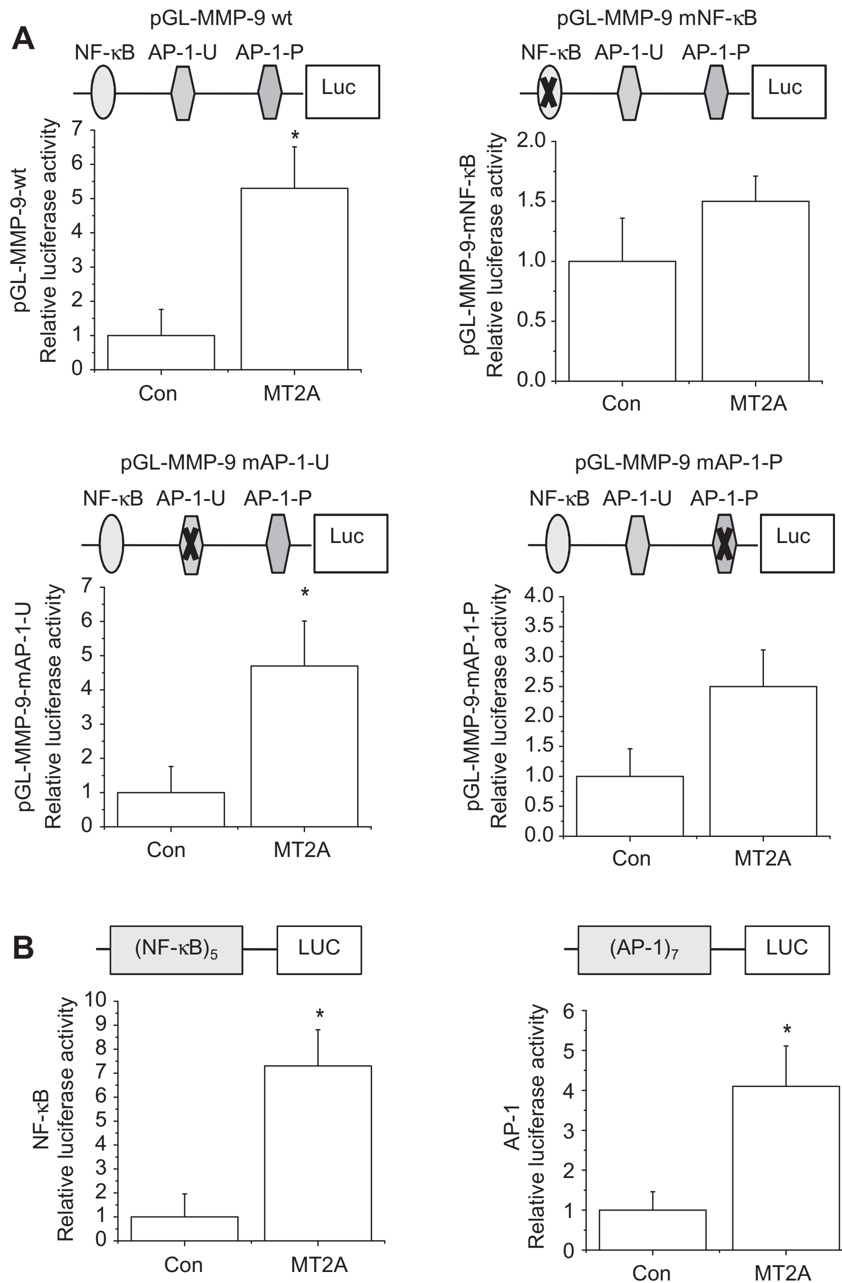

Fig. 4. NF-κB and AP-1 activity is required for the MT-2A-induced MMP-9 upregulation, invasion and migration. (A and B) Mutations were introduced in the NF-κB or AP-1 binding sites of pGL-MMP-9WT, and MDA-MB-231 cells or MT-2A-overexpressing cells were transfected with (A) pGL-MMP-9WT, pGL-MMP-9mNF-κB, or pGL-MMP-9mAP reporter plasmids, or (B) with reporter plasmids containing tandem NF-κB or AP-1 binding sites. Cells were then harvested and assayed for luciferase activity. Each bar represents the mean ± S.D. calculated from three independent experiments. *Significantly different from control ($P < 0.01$). (C) Chromatin immunoprecipitation using the anti-NF-κB and anti-AP-1 antibodies was performed on chromatin extracted from MDA-MB-231 cells or MT-2A-overexpressing cells, and the specific MMP-9 promoter regions were amplified by PCR. (D) MT-2A-overexpressing cells were treated with 10 μM of curcumin or Bay11-7802 for 24 h. Conditioned media was collected, and the gelatinolytic activity of secreted MMP-9 was determined by gelatin zymogram assay. Relative band intensities were determined by the quantitation of each band. (E) MDA-MB-231 cells or MT-2A-overexpressing cells were pretreated with 10 μM of curcumin or Bay11-7802 and were subjected to in vitro migration assays (upper panel) and invasion assays (lower panel) for 24 h. Each bar represents the mean ± S.D. calculated from three independent experiments. *Significantly different from control ($P < 0.01$). **Significantly different from MT-2A-overexpressing control ($P < 0.01$).

association between AP-1/NF-κB and the MMP-9 promoter by chromatin immunoprecipitation was observed. We further confirmed this finding by demonstrating that MT-2A-induced invasion and migration were significantly inhibited in the presence of curcumin or Bay11-7802, specific inhibitors of the AP-1 or NF-κB pathways, respectively.

Taken together, our results provide the first evidence that the upregulation of MMP-9 is one of the mechanisms by which MT-2A promotes cell invasion. Our results also raise the possibility that the inhibition of MT-2A may suppress tumor invasion. We also provide evidence for the importance of the AP-1 and NF-κB pathways in MMP-9 upregulation and invasion of MDA-MB231 cells by MT-2A. Given that MT-2A is one of the most important proteins in human breast cancer and thus is an attractive therapeutic target, our findings may provide a molecular basis for the promoting role of MT-2A in breast cancer progression.

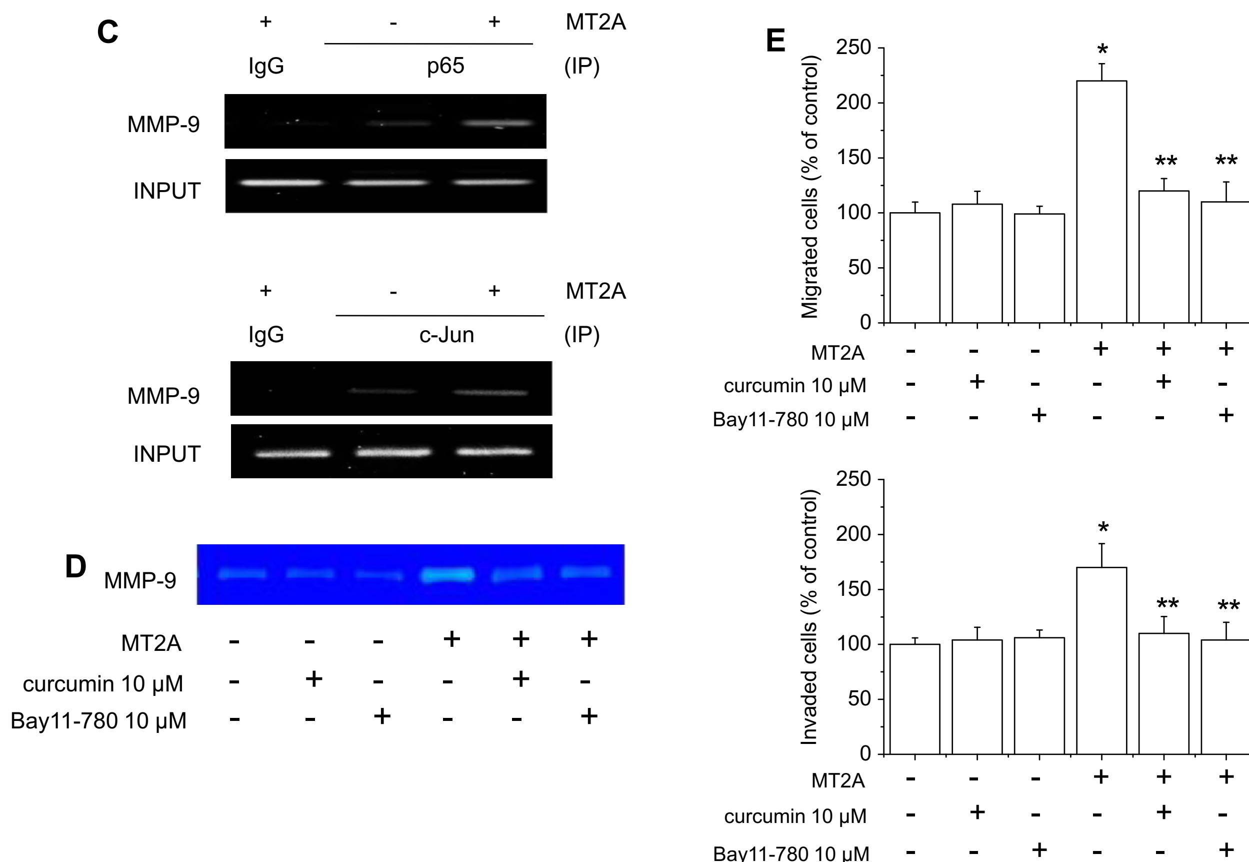

Fig. 4 (*continued*)

Conflict of interest statement

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2010.12.030.

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