Inhibition of endothelial cell migration through the down-regulation of MMP-9 by A-kinase anchoring protein 12

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Abstract. Matrix metalloproteinases (MMPs) play an important role in the degradation of extracellular matrix (ECM) molecules. ECM degradation is associated with tumor metastasis and angiogenesis. Therefore, the regulation of MMPs is of potential benefit in the treatment of various diseases, including cancer. A-kinase anchoring protein 12 (AKAP12) has been identified as a potential tumor suppressor. However, the function of AKAP12 as a tumor suppressor is not well understood. Herein, to determine the relationship between AKAP12 and MMP-9 in cancer, we first investigated the expression of MMP-9 under normoxic and hypoxic conditions in human fibrosarcoma cells. The expression of MMP-9 was not detected under normoxic conditions.; however, it was markedly increased under hypoxia in HT1080 cells. The effect of AKAP12 on the expression of MMP-9 was subsequently investigated. Hypoxia-induced MMP-9 mRNA expression was significantly reduced by overexpression of AKAP12, as was MMP-9 protein expression. In addition, when the AKAP12 transfectant-conditioned media (CM) were transferred into human endothelial cells, cell migration was significantly inhibited compared to the control group. Notably, the inhibition of AKAP12 expression by siRNA targeting AKAP12 resulted in an increase in the expression of active MMP-2 under normoxia, as well as of MMP-9. Endothelial cell migration was also strongly increased by treatment with CM of siRNA against AKAP12, as compared to the control group. Taken together, the results indicate that AKAP12 is involved in the regulation of endothelial cell migration through the inhibitory regulation of MMP-9 expression in tumor cells.

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Introduction

Matrix metalloproteinases (MMPs) are regarded as the main critical proteins that assist in the migration of tumor cells during metastasis. Tumor-induced angiogenesis is required for tumor growth, invasion and metastasis. MMPs modulate the interaction between the extracellular matrix (ECM) and the cellular surface, and various alterations may affect cellular behavior such as adhesion and migration. Degradation of the ECM plays a critical role in tumor metastasis. MMP-9 in particular is involved in many stages of tumor growth, including angiogenesis, reduces the immune response to cancer and promotes cancer cell invasion (1-3). *In vivo* studies showed a reduction in the ability of tumors to metastasize and reduced angiogenesis in MMP-9 null mice (4,5). In general, MMP-9 expression is quite low, but is highly increased by several cytokines and growth factors, such as interleukin-1, tumor necrosis factor-α and transforming growth factor-β (6). The regulation of MMP-9 is complex and its mechanims are controversial as multiple pathways are involved. Therefore, studies on the mechanisms involved in the regulation of MMP-9 expression are important for understanding the processes behind cancer progression.

A-kinase anchoring protein 12 (AKAP12) was originally identified as a tumor suppressor. Its expression was found to be down-regulated in *src*- and *ras*-transformed fibroblast cells (7). Previously, we demonstrated that AKAP12 regulates angiogenesis through the inhibition of vascular endothelial growth factor (VEGF) expression (8). Furthermore, the suppression of VEGF was associated with the ability of AKAP12 to inhibit lung metastasis formation (9). Moreover, AKAP12 was found to be highly expressed in untransformed cell lines and undifferentiated prostate cancer; however, its expression was significantly down-regulated in human prostate, breast and gastric cancers (10). Re-expression of AKAP12

suppresses tumor cell viability and *src*-induced oncogenic properties, such as metastatic growth (11), suggesting that AKAP12 plays a crucial role as a tumor suppressor. As mentioned above, although the AKAP12 gene has been isolated as a tumor suppressor gene, molecular signaling by AKAP12 in tumor cells is not yet well determined. In the present study, we investigated the effect of AKAP12 on MMP-9 expression in HT1080 fibrosarcoma cells and endothelial cell migration.

Materials and methods

Cell culture and hypoxic conditions. HT1080 fibrosarcoma cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin (Invitrogen, Grand Island, NY, USA), and maintained in an incubator in a humidified atmosphere of 95% O_2 and 5% CO_2 at 37˚C. For the hypoxia experiments, HT1080 cells were incubated for 24 h in a hypoxic chamber (Forma Scientific, San Bruno, CA, USA) that maintained the cells under low-oxygen tension (5% CO_2 with 1% O_2, balanced with N_2). Human umbilical vein endothelial cells (HUVECs) were grown on gelatin-coated plates in M199 medium supplemented with 20% FBS, 2 ng/ml basic FGF (Millipore, Bedford, MA, USA) and 10 U/ml heparin (Sigma, St. Louis, MO, USA).

Transient transfection and preparation of the conditioned media. Full-length AKAP12 cDNA was subcloned into pcDNA3. Transient transfections were performed using Lipofectamine Plus reagent (Invitrogen). All transfections were performed according to the manufacturer's instructions. HT1080 cells were transiently transfected with 5 μg AKAP12 expression vector after seeding on 100-mm culture dishes. To prepare the conditioned medai (CM) for the treatment of HUVECs, media from the transfected HT1080 cells were exchanged with M199 medium containing 1% FBS for 24 h, collected and filtered through a 0.22-μm pore size membrane (Millipore, San Francisco, CA, USA), and then concentrated four times using centrifugal filters (Millipore).

RNA preparation and RT-PCT. Total RNA was isolated from the indicated cells using TRIzol reagent (Invitrogen). RT-PCR analysis was performed as described previously (12). PCR products were analyzed on 1.5% agarose gels, and the gels were digitally imaged. The following mouse-specific primer pairs were used: *Akap12*, 5'-GCCAGTCCTGACACTTG-3' and 5'-TGAGCCCATGCCTCCAGAA-3', or 5'-AGGGCACC TCCGGTTCTC-3' and 5'-GGTTCGCTTTCTTTGGATGC-3'; *MMP-9*, 5'-TTGAGTCCGGCAGACAATCC-3' and 5'-CTTA TCCACGCGAATGACG-3'; β-actin, 5'-TCTACAATGAGC TGCGTGTG-3' and 5'-AATGTCACGCACGATTTCCC-3'.

Migration assay. HUVECs were plated on 60-mm culture dishes upon reaching 95% confluence, wounded with a razor blade 2 mm in width, and marked at the injury line. After wounding, the cultures were washed in serum-free medium and further incubated in M199 media with 1% serum and 1 mM thymidine. HUVECs were allowed to migrate for 12 h and were then rinsed with serum-free medium, followed by fixing with absolute methanol and staining by Giemsa.

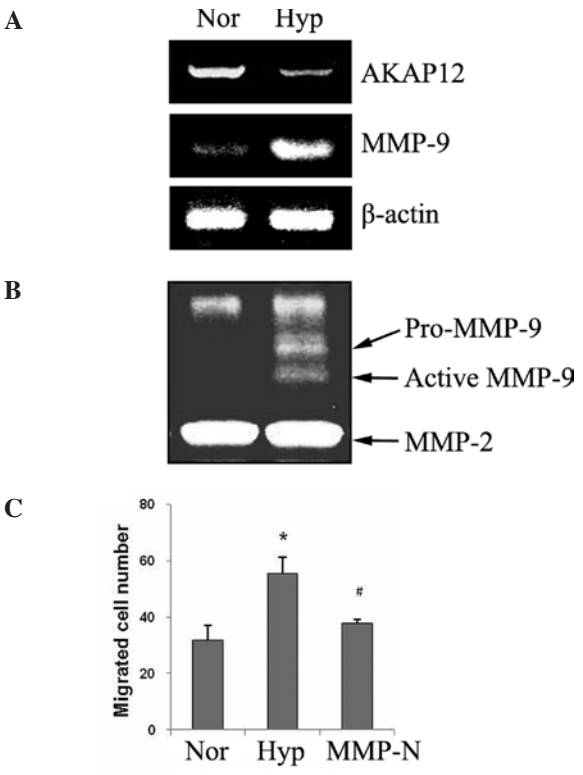

Figure 1. Hypoxia induced the expression of MMP-9 in migrating HT1080 fibrosarcoma and endothelial cells. (A) Human fibrosarcoma cells were exposed to normoxia (Nor) or hypoxia (Hyp) for 24 h. MMP-9 in total RNA was analyzed by RT-PCR under the indicated levels of oxygen tension. (B) Cell lysates were isolated to investigate the level of MMP-9 protein after exposure to hypoxia, and 30 μg of cell lysates were loaded for zymography analysis. (C) For the endothelial cell migration assay, CM prepared from normoxia- or hypoxia-treated human fibrosarcoma cells were transferred into human umbilical vein endothelial cells (HUVECs), which were subsequently treated with MMP-9-neutralized hypoxia-CM (MMP-N). A quantitative graph from triplicate experiments is shown. Control IgG-neutralized normoxia-CM (Nor) or IgG-neutralized hypoxia-CM (Hyp) was used as the control. *$p<0.01$ compared to HUVECs cultured with normoxia-CM; #$p<0.01$ compared to HUVECs cultured with hypoxia-only CM. Independent experiments were repeated three times.

Migration was quantitated by counting the number of cells that moved beyond the reference line (8,12).

MMP zymography. Standard gel zymography was used to measure the levels of MMP-2 and MMP-9 (13). Total protein (30 μg) was loaded and separated by a 10% Tris-glycine gel with 0.1% gelatin as substrate, washed with renaturing buffer (Invitrogen) for 90 min, and further incubated with developing buffer (Invitrogen) at 37˚C for 24 h. Finally, the gels were stained with 0.5% Coomassie blue R-250 for 1 h and then destained appropriately.

RNA interference. HT1080 fibrosarcoma cells were grown to 80% confluence, and small interfering RNAs (siRNAs) (50 μM) were transfected into the cells using Lipofectamine Plus or Lipofectamine 2000 reagent (Invitrogen). All transfections were performed according to the manufacturer's instructions. siRNAs and the control nonsilencing RNAs were designed by Dharmacon (Lafayette, CO, USA). The AKAP12 target sequence used was: 5'-AGACGGATGTAG TGTTGAA-3'.

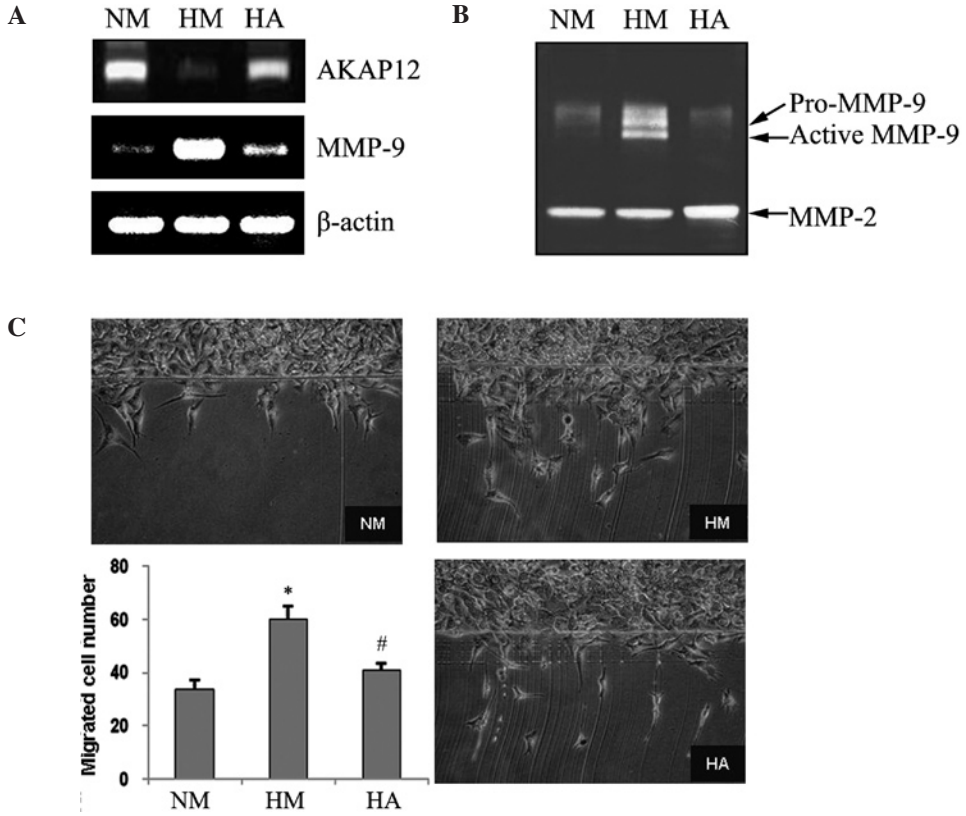

Figure 2. Inhibitory effect of AKAP12 on MMP-9 expression. Total RNA were isolated for RT-PCR (A) and harvested cell lysates for zymograpy assay (B) after transfection of the AKAP12 gene. Human fibrosarcoma cells were transfected with the AKAP12 vector, incubated for 12 h, and then exposed to 24 h of normoxia or hypoxia. NM, empty mock vector-transfected cells under normoxia; HM, empty mock vector-transfected cells under hypoxia; HA, AKAP12 vector-transfected cells under hypoxia. (C) CM from the AKAP12 transfectant were prepared under normoxia or hypoxia conditions for the endothelial cell migration assay, then transferred to human umbilical vein endothelial cells (HUVECs). A quantitative graph from triplicate experiments is shown. $^{*}p<0.01$ compared to HUVECs cultured with normoxia-mock vector transfectant CM; $^{\#}p<0.01$ compared to HUVECs cultured with hypoxia-mock vector transfectant CM. Independent experiments were repeated three times. NM, HUVECs with CM of the mock vector-transfected cells under normoxia; HM, HUVECs with CM of the mock vector-transfected cells under hypoxia; HA, HUVECs with CM of the AKAP12 vector-transfected cells under hypoxia.

Statistical analysis. Data are expressed as the mean ± SD. Statistical analysis was performed using ANOVA and an unpaired Student's t-test. A p-value of ≤0.05 was considered statistically significant. Statistical calculations were performed using SPSS software for Windows (version 10.0; SPSS, Chicago, IL, USA).

Results

Effect of hypoxia on MMP-9 and endothelial cell migration. To investigate the expression of MMP-9, RT-PCR and zymography analysis were performed. MMP-9 expression was very weak under normoxic conditions (Fig. 1A and B). However, when HT1080 cells were exposed to hypoxic conditions (24 h), MMP-9 expression was greatly increased at the mRNA and protein levels. Additionally, MMP-2 expression was not affected by hypoxia as indicated by the zymography analysis (Fig. 1B). At the beginning of the experiment, there was no increase in MMP-9 expression after hypoxia treatment using culture media not including the serum. However, after adding 2-5% of serum, hypoxia induced increased expression of MMP-9. Therefore, all the hypoxia-treatment experiments were performed using media with 2% serum. The expression of AKAP12 was strongly decreased by hypoxia treatment (Fig. 1A). To investigate the effect on angiogenesis, CM was collected under normoxic and hypoxic conditions from the HT1080 cells, then transferred to the HUVECs. Upon treatment with the hypoxic CM, endothelial cell migration was increased compared to that in the normoxic CM. However, the increased migration was reduced by neutralizing MMP-9 from the hypoxic CM (Fig. 1C). These data indicate that hypoxia-induced MMP-9 is involved in endothelial cell migration.

Overexpression of AKAP12 reduced MMP-9 expression. To verify the effect of AKAP12 on the expression of MMP-9, the AKAP12 gene was transfected into HT1080 cells. As expected, hypoxia-induced MMP-9 expression was markedly decreased by overexpression of AKAP12 at the RNA and protein levels (Fig. 2A and B). When the hypoxic CM was transferred into the endothelial cells, migration was increased compared to the control normoxic group (Fig. 2C). However, the hypoxic CM of the AKAP12 transfectant did not induce endothelial cell migration compared to the hypoxia-only CM.

Effect of AKAP12-siRNA on MMP-9 expression. To identify the inhibitory signaling pathway by AKAP12, the effect of AKAP12-siRNA on MMP-9 expression was investigated. Inhibition of AKAP12 expression by siRNA-targeting AKAP12 resulted in an increase in the expression of active MMP-9 under normoxic conditions (Fig. 3A). In addition,

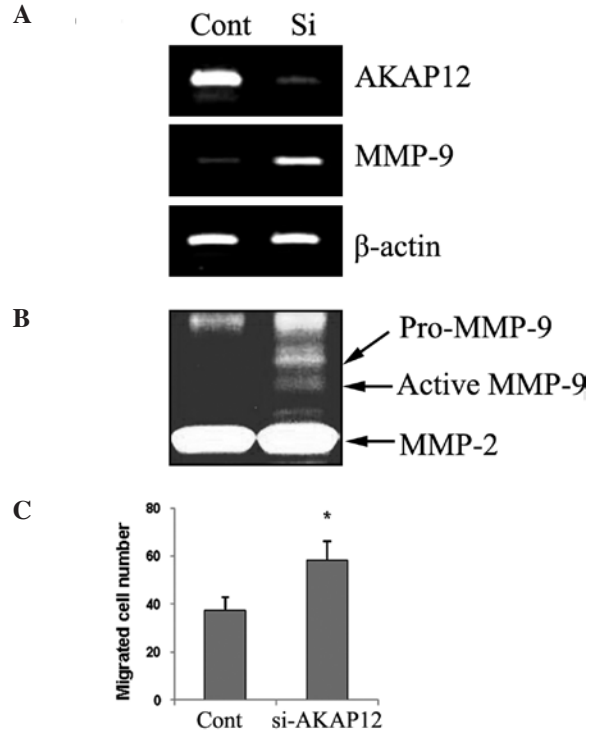

Figure 3. Knockdown of AKAP12 gene expression increased MMP-9 expression under normoxic conditions. HT1080 cells were transfected with siRNA targeting AKAP12 and incubated for 48 h under normoxic conditions, then harvested for RT-PCR (A) and zymography analysis (B). Cont, scrambled siRNA transfectant; si, siRNA targeting AKAP12 transfectant. (C) Prepared CM from the transfectants of AKAP12-siRNA or scrambled siRNA were transferred to the human umbilical vein endothelial cells (HUVECs) for the migration assay. A quantitative graph from triplicate experiments is shown. $^{*}p<0.05$ compared to HUVECs cultured with scrambled siRNA transfectant CM. Cont, scrambled siRNA transfectant CM-treated endothelial cells; si-AKAP12, siRNA targeting AKAP12 transfectant CM-treated endothelial cells.

zymography analysis performed to identify the protein expression level of MMP-9 indicated that MMP-9 expression was significantly induced by treatment of AKAP12-siRNA (Fig. 3B). To verify the paracrine effect on endothelial cell migration after silencing of AKAP12 using siRNA, the CM of the AKAP12-siRNA group and control group were transferred to the human endothelial cells. Notably, even under normoxic conditions, endothelial cell migration was significantly increased by inhibition of AKAP12 expression (Fig. 3C).

Discussion

MMPs are key players in the degradation of the ECM and basement membranes. ECM degradation is an essential process in tumor invasion and metastasis. Moreover, hypoxia is one of the main factors involved in tumor angiogenesis. Many growth factors are induced by hypoxia. In particular, MMP-9 is a promising therapeutic target for many solid tumors. However, MMP-9 and MMP-2 expression patterns under conditions of hypoxic remain controversial (14,15). MMP-9 is an endopeptidase that facilitates tumor invasion and angiogenesis through the breakdown of the ECM. MMP-9 mRNA levels are regulated by a number of transcription factors, including nuclear factor (NF)-B and activator protein-1 (AP-1), which are induced by the p42/p44 MAPK and PI-3K pathways in response to cytokines and growth factors (16,17). HIF-1 has not been implicated in the regulation of MMP-9, although some *in vitro* studies have found this enzyme to be induced by hypoxia (18-20). The induction of MMP-9 may be due to NF-B induced by hypoxia (21,22). The correlation between HIF-1 and MMP-9 raises the possibility that HIF-1 may have a regulatory role in the expression of MMP-9. Alternatively, it may indicate that both factors are induced by stimuli such as hypoxia or EGFR (23). Further investigation is required to elucidate whether or not HIF-1 is involved in the regulation of MMP-9.

Herein, we investigated the expression of MMP-9 under normoxic and hypoxic conditions using HT1080 human fibrosarcoma cells. RT-PCT was performed to investigate the RNA expression level of MMP-9. MMP-9 expression was found to be very weak under normoxic conditions. In addition, no MMP-9 (pro- and active MMP-9) expression was detected at the protein level under normoxic conditions (Fig. 1B). However, MMP-9 expression was markedly increased by hypoxia in the HT1080 cells at the mRNA level (Fig. 1A). Also, as indicated by the zymography assay, an increase in the active MMP-9 form was noted under hypoxic conditions (Fig. 1B). MMP-2 expression was not altered in the HT1080 fibrosarcoma cells by hypoxia treatment. In previous reports, it was determined that MMP-2 activation was substantially attenuated in myofibroblasts by hypoxia treatment. The migration of the myofibroblasts was independent of MMP-2 expression (24). In a previous study, hypoxia was found to down-regulate the secretion of MMP-2 and MMP-9 in porcine pulmonary artery endothelial and smooth muscle cells (15). However, human breast tumors demonstrated a strong relationship between hypoxic microenvironments and MMP-2 activation (25). In the case of hepatic stellate cells, an increase in MMP-2 expression was found to be most notable after a 6-h treatment with hypoxia (26). Based on these previous reports, we determined that the effect of hypoxia on the expression of MMP-2 is variable in different cell types and under different conditions.

Transcriptional factor AP-1 is one of the key factors functioning as an inducer of MMP-9 (17). In our previous study, we found that AKAP12 down-regulates AP-1 transcriptional activity. Also, AKAP12 was reported to be involved in the inhibition of angiogenesis (8). Based on these findings, we investigated the effect of AKAP12 on the expression of MMP-9. As expected, hypoxia-induced MMP-9 mRNA expression was significantly reduced by the overexpression of AKAP12 (Fig. 2A). In addition, MMP-9 protein expression was strongly decreased by AKAP12 (Fig. 2B). However, MMP-2 expression was not affected by hypoxia (24 h) (Fig. 1B). These data indicate the possibility that AKAP12 has an inhibitory function on MMP-9, but not on MMP-2, expression, through the regulation of the AP-1 transcriptional factor. The zymography analysis carried out after transfection of siRNA against AKAP12 indicated that MMP-9 expression was markedly increased. However, there was no significant change in MMP-2 expression in the AKAP12 knock-down group compared to the control (Fig. 3B). In addition, to investigate the effect of AKAP12-transfected tumor cells on endothelial cell migration, the AKAP12 transfectant-CM was

transferred into endothelial cells. In the AKAP12 transfected group, endothelial cell migration was significantly inhibited compared to the control group (Fig. 2C). These data suggest that MMP-9 is one of the most important inducers of endothelial cell migration during tumor angiogenesis. Taken together, the results suggest that AKAP12 plays a key role in the inhibition of tumor growth and angiogenesis via the regulation of MMP-9 expression.

In this study, we found that AKAP12 is involved in the regulation of endothelial cell migration by controlling the expression of MMP-9. Therefore, the regulation of AKAP12 is crucial for the reduction of tumor angiogenesis.

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