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Astrocyte Elevated Gene-1 Upregulates Matrix Metalloproteinase-9 and Induces Human Glioma Invasion

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Abstract

The poor prognosis of malignant gliomas is largely attributed to their highly invasive nature. The molecular mechanism underlying the invasiveness of glioma cells, however, remains to be elucidated. The present study found that astrocyte elevated gene-1 (AEG-1) was upregulated in human glioma cell lines and glioma tissues compared with normal astrocytes and brain tissues. AEG-1 was found to be upregulated in 265 of 296 (89.5%) glioma sections, and the AEG-1 expression level significantly correlated with clinicopathologic stages of gliomas. Ectopic expression or short hairpin RNA silencing of AEG-1 significantly enhanced or inhibited, respectively, the invasive ability of glioma cells. At the molecular level, we showed that upregulated AEG-1 in glioma cells interacted with matrix metalloproteinase-9 (MMP-9) promoter and transactivated MMP-9 expression, whereas knockdown of AEG-1 expression reduced the level of MMP-9. Two regions in MMP-9 promoter were found to be involved in the interaction with AEG-1. Suppression of endogenous MMP-9 abrogated the effects of AEG-1 on invasiveness. Consistent with these observations, immunostaining analysis revealed a significant correlation between the expressions of AEG-1 and MMP-9 in a cohort of clinical glioma samples. Moreover, intracranial xenografts of glioma cells engineered to express AEG-1 were highly invasive compared with the parental cells and expressed high level of MMP-9. Collectively, these findings provide evidence that AEG-1 contributes to glioma progression by enhancing MMP-9 transcription and, hence, tumor cell invasiveness, and underscore the importance of AEG-1 in glioma development and progression. *Cancer Res; 70(9); 3750–9. ©2010 AACR.*

Introduction

Gliomas represent the most common and aggressive type of tumors in the central nervous system (CNS). In spite of the enormous improvements made in neurosurgery, chemotherapy, and radiotherapy, the prognosis of malignant gliomas has remained poor over the last decades in the United States and Europe (1). The cumulative 1-year survival rate is <30%, and the median survival time of the grade 4 glioma, glioblastoma multiforme (GBM), is only 15 months (2, 3). Such suboptimal efficacy in the management of glioma is largely attributable to the highly invasive nature of glioma cells capable of diffusely infiltrating and widely migrating in the surrounding brain tissue, leading to restricted and incomplete surgical resection and, thus, high recurrence rates (1, 4). Biologically, the invasion process of glioma cells into the neighboring areas involves cell adhesion and proteolytic degradation of the extracellular matrix (ECM; ref. 5). Previously, mounting evidence has shown that matrix metalloproteinase (MMP) family members, including MMP-2 and MMP-9, are tightly involved in augmenting the invasive capability of gliomas and correlated with the degree of histologic malignancy as well as the prognosis of gliomas (6–10). Hence, a better understanding of the molecular mechanism mediating the regulation of MMP expression in gliomas is key to development of efficacious therapeutic strategy that abolishes the infiltration and invasion of glioma cells.

Astrocyte elevated gene-1 (AEG-1) was initially identified as a novel protein induced by HIV-1 or tumor necrosis factor-α in primary human fetal astrocytes (11–14). Numerous recent studies have shown that AEG-1 is upregulated in various human cancer types, including melanoma, breast cancer, GBM, esophageal squamous cell carcinoma, neuroblastoma, and prostate cancer (14–24). Meanwhile, AEG-1 has been reported to play important roles in multiple biological processes during cancer development and progression, including malignant transformation, apoptosis regulation, angiogenesis, invasion, and metastasis of tumor cells via

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activation of various oncogenic signaling pathways (25–29). The expression of AEG-1 could be induced by Ha-ras through the activation of the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway due to binding of c-Myc to key E-box elements in the AEG-1 promoter region (26). Furthermore, AEG-1 synergizes with Ha-ras to increase the colony-forming ability of nontumorigenic immortalized melanocytes and augment invasion of transformed cells, thereby acting as a positive autofeedback activator (28). Ectopic expression of AEG-1 could inhibit serum starvation–induced apoptosis by provoking constitutive activation of PI3K/Akt signaling, which further induces expression of MDM2, rescinding the function of p53 and repressing the expression of Bad and p21. In contrast, silencing AEG-1 expression could stimulate apoptosis due to enhanced FOXO3a activity mediated by reduction of Akt activity (15, 28). We have previously found that AEG-1 promotes proliferation in breast cancer via suppressing FOXO1 (29). In addition, the molecular mechanism by which AEG-1 contributes to oncogenesis is also associated with the activation of NF-κB pathway (15, 24, 27). It has been reported that upregulation of AEG-1 could induce the cytoplasm/nucleus translocation of NF-κB and enhance its DNA-binding activity (27), and inhibition of NF-κB attenuates AEG-1–induced enhancement of colony formation in soft agar and increases invasion of HeLa cells in Matrigel (24). Moreover, AEG-1 has also been found to be crucial for the progression of hepatocellular carcinoma, possibly mediated by Wnt/β-catenin signaling through extracellular signal-regulated kinase p42/44 activation and upregulation of lymphoid-enhancing factor 1/T-cell factor 1 (21). Recently, Hu et al. (22) have shown that ALDH3A1 and MET contribute to chemoresistance of breast cancer induced by AEG-1. Taken together, all available evidence suggests that AEG-1 might function as a potential oncogene contributing to the development and progression of human cancers. Nevertheless, whether AEG-1 plays a role in tumor invasion still remains to be investigated.

In the current study, we report that AEG-1 could promote the invasiveness of glioma and transcriptionally upregulate MMP-9 expression through directly binding to the MMP-9 promoter. We also show that the expression of AEG-1 is associated with histologic staging and expression of MMP-9 in gliomas.

Materials and Methods

Cell lines. Primary normal human astrocytes (NHA) were purchased from Sciencell Research Laboratories and cultured under the condition as the manufacturer suggested. Glioma cell lines, including LN443, LN444, LN464, U118G, T98G, U251MG, U87MG, D247MG, LN340, A172, LN319, LN382T, and SNB19, were kindly provided by Dr. Shi-Yuan Cheng (University of Pittsburgh, Pittsburgh, PA) and grown in DMEM supplemented with 10% fetal bovine serum (HyClone). All above-mentioned cells have been characterized as $GFAP^+$ cells with immunofluorescence using antibody against GFAP.

Patient information and tissue specimens. A total of 296 paraffin-embedded glioma specimens were collected for this study, which had been histopathologically and clinically diagnosed at the Sun Yat-sen University–Affiliated First Hospital from 2000 to 2005. For the use of these clinical materials for research purposes, prior patient's consents and approval from the Institutional Research Ethics Committee were obtained. Clinical information of the samples is described in detail in Supplementary Table S1. Normal brain tissues were obtained by donation from individuals who died in traffic accident and confirmed to be free of any prior pathologically detectable conditions.

Chromatin immunoprecipitation. Chromatin immunoprecipitation (ChIP) was done using the Chromatin Immunoprecipitation kit (Upstate) according to the manufacturer's instruction. Briefly, 2×10^6 indicated cells in a 100-mm culture dish were treated with 1% formaldehyde to cross-link proteins to DNA. The cell lysates were sonicated to shear DNA to sizes of 300 to 1,000 bp. Equal aliquots of chromatin supernatants, into which 1 μg of anti–AEG-1 antibody (Invitrogen) or anti-IgG as negative control was added, were incubated overnight at 4°C with rocking. After reverse cross-link of protein/DNA complexes to free DNA, PCR was done using specific primers of MMP-9 promoter as follows: primer 1, GCCATGTCTGCTGTTTTCTAGAGG (forward) and CACACTCCAGGCTCTGTCCTCTTT (reverse; product, 207 bp); primer 2, AAAGAGGACAGAGCCTGGAGTGTG (forward) and GGGAACTGTATGAAAGGGAGGGAG (reverse; product, 225 bp); primer 3, CTCAGGGAGTCTTCCATCACTTTC (forward) and AGCATGAGAAAGGGCTTACACCAC (reverse; product, 250 bp); primer 4, TGGTGTAAGCCCTTTCTCATGCTG (forward) and CAGCTGCTGTTGTGGGGGCTTTAA (reverse; product, 161 bp).

Luciferase assay. Cells (3.5×10^4) were seeded in triplicates in 48-well plates and allowed to settle for 24 hours. Luciferase reporter plasmids (100 ng) containing different fragments of MMP-9 promoter, or the control luciferase plasmid, plus 1 ng of pRL-TK *Renilla* plasmid (Promega) were transfected into glioma cells using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's recommendation. Luciferase and *Renilla* signals were measured 48 hours after transfection using the Dual-Luciferase Reporter Assay kit (Promega) according to a protocol provided by the manufacturer. Three independent experiments were done and the data are presented as the mean ± SD.

Intracranial brain tumor xenografts, immunohistochemistry, and H&E staining. U87MG (5×10^5) or U87MG-AEG-1–expressing cells (5×10^5) were stereotactically implanted into individual nude mouse brains with five mice per group. The glioma-bearing mice were scarified after 3 weeks of implantation and the whole brains were removed, and 4-μm sections were cut and subjected to immunohistochemistry and H&E staining. After deparaffinization, immunohistochemistry was conducted using an anti–AEG-1 antibody (Invitrogen) and an anti–MMP-9 antibody (Cell Signaling). For H&E staining, deparaffinized tumor sections were stained with Mayer's hematoxylin solution. The images were captured using the AxioVision Rel.4.6 computerized image analysis system (Carl Zeiss).

Statistical analysis. All statistical analyses were carried out using the SPSS 10.0 statistical software package. The

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χ^2 test was used to analyze the relationship between AEG-1 expression and the clinicopathologic characteristics. Bivariate correlations between study variables were calculated by Spearman's rank correlation coefficients. $P < 0.05$ in all cases was considered statistically significant.

Results

Upregulation of AEG-1 in glioma cell lines and primary glioma. Western blotting and real-time reverse transcription-PCR (RT-PCR) analyses revealed that the expression of AEG-1, at both protein and mRNA levels, was markedly higher in all 13 glioma cell lines in comparison with that in NHA (Fig. 1A; Supplementary Fig. S1A). Furthermore, comparative analysis on paired glioma tumor and adjacent nontumor tissues (ANT), with each pair obtained from the same patient, showed that the mRNA and protein levels of AEG-1 were also higher in all eight glioma samples compared with each corresponding ANT tissue (Fig. 1B; Supplementary Fig. S1B), clearly showing notable upregulation of AEG-1 in both glioma cell lines and clinical primary glioma tissues.

Increased expression of AEG-1 correlates with progression of gliomas. To further investigate whether AEG-1 protein is overexpressed in clinical samples of glioma, we examined 296 paraffin-embedded, archived glioma tissues, including 39 cases of grade 1 (13.2%), 121 cases of grade 2 (40.9%), 88 cases of grade 3 (29.7%), and 48 cases of grade 4 gliomas (16.2%) by immunohistochemistry. As presented in Fig. 1C and Supplementary Table S1, positive AEG-1 staining was shown in 265 of 296 (89.5%) cases, among which 143 (48.3%) were identified as low-level AEG-1 expression and 153 cases (51.7%) as high-level AEG-1 expression. Quantitative analysis indicated that the average mean optical densities of AEG-1 staining intensity in histologic grade 1 to 4 primary tumors were statistically significantly higher than those in normal brain tissues ($P < 0.001$; Fig. 1C; Supplementary Fig. S1C). Further analysis showed that AEG-1 expression strongly correlated with the age of patients ($P < 0.001$) and the clinicopathologic grades ($P < 0.001$; Supplementary Table S2), which was confirmed by a Spearman correlation analysis (Supplementary Table S3). Taken together, our results suggested that the expression of AEG-1 significantly correlated with clinicopathologic grades of gliomas.

***Modulation of the invasive ability of glioma cells by AEG-1* in vitro.** To investigate the biological significance of upregulated AEG-1 in the development and progression of gliomas, the gain or loss of function of AEG-1 in glioma cell models with ectopic expression of AEG-1 cDNA (Fig. 2A, left) or RNA interference (RNAi)–mediated AEG-1 knockdown (Fig. 2A, right), respectively, was tested. The

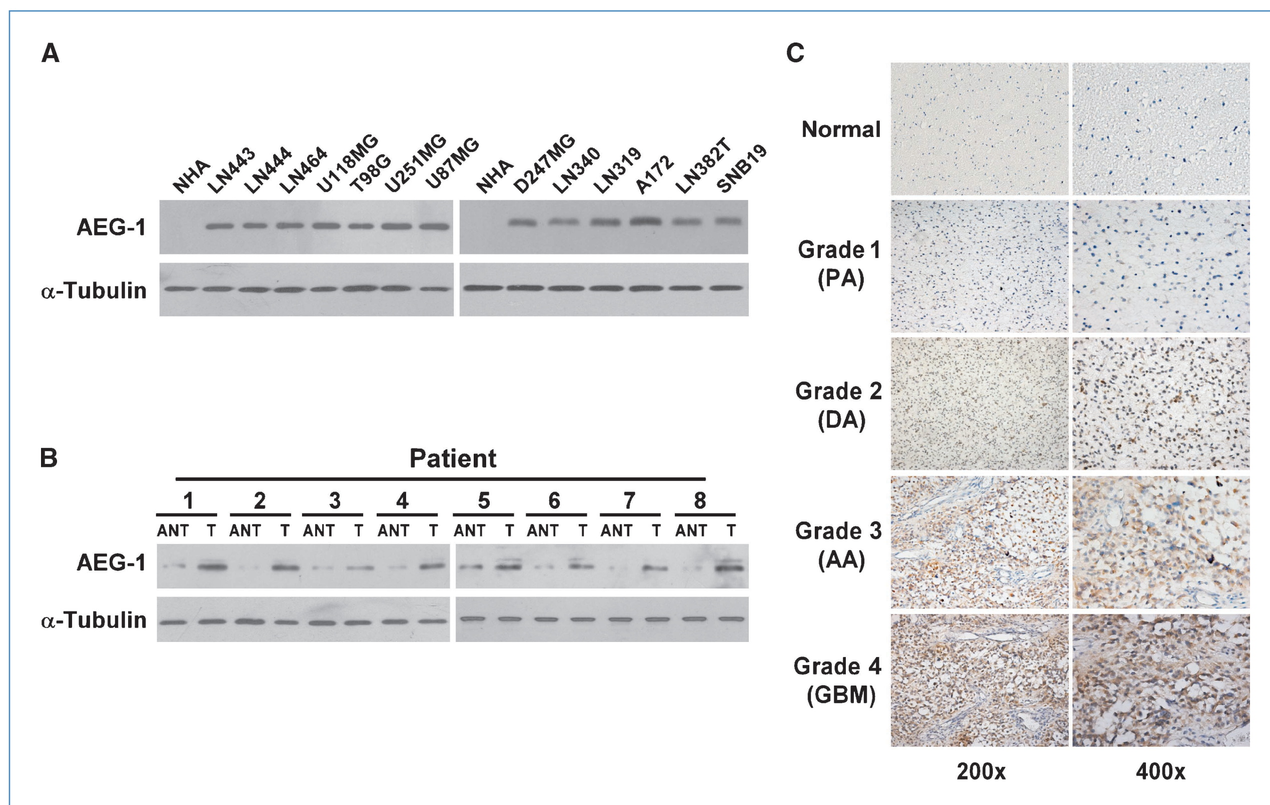

Figure 1. AEG-1 expression in glioma cell lines and primary gliomas. A, expression of AEG-1 protein in NHA and cultured glioma cell lines (LN443, LN444, LN464, U118G, T98G, U251MG, U87MG, D247MG, LN340, LN319, A172, LN382T, and SNB19). B, comparative quantification of AEG-1 protein in paired primary glioma tissues (T) and ANTs, with each pair obtained from the same patient. Protein expression levels were normalized with α-tubulin. C, AEG-1 protein is upregulated in glioma sections (WHO grade 1–4) compared with normal brain tissue as examined by immunohistochemical staining.

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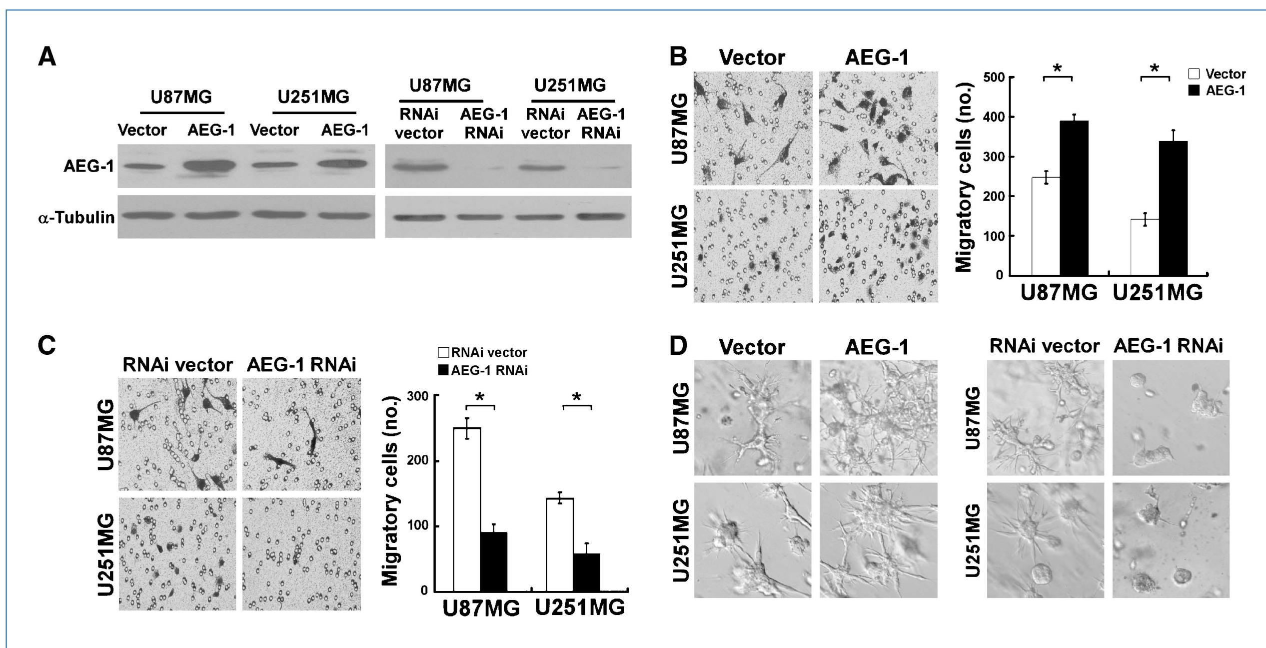

Figure 2. AEG-1 modulates the invasive ability of glioma cells *in vitro*. A, ectopic expression of AEG-1 (left) and knockdown of AEG-1 (right) in glioma cell lines U87MG and U251MG were analyzed by immunoblotting using an anti–AEG-1 antibody. Protein expression levels were normalized with α-tubulin. B and C, representative pictures (left) and quantification (right) of penetrated cells were analyzed using the Transwell matrix penetration assay. The quantification of penetrated cells was represented as the mean of three different experiments. D, representative micrographs of indicated cultured cells after 10-d culture in three-dimensional spheroid invasion assays. *, $P < 0.05$.

AEG-1–overexpressing U87MG and U251MG glioma cells exhibited markedly increased ability of invasion compared with the vector control–transduced cells, as examined by Transwell matrix penetration assay (Fig. 2B). In contrast, silencing endogenous AEG-1 expression dramatically reduced the invasive ability of glioma cells (Fig. 2C). Furthermore, three-dimensional spheroid invasion assay, which has been considered to be better mimicry of *in vivo* tumor invasion, revealed that both AEG-1–transduced glioma cell lines, after being cultured in Matrigel for 10 days, displayed morphologies typical of highly aggressive invasiveness, presenting more outward projections from nearly all individual cells, as opposed to the vector-transduced control cells (Fig. 2D, left). Conversely, the AEG-1 RNAi–transduced glioma cells presented immotile and spheroid morphologies (Fig. 2D, right). These data strongly suggest a role of AEG-1 in the modulation of the invasiveness of glioma cells.

AEG-1 promotes invasive ability of glioma cells through activation of MMP-9 expression. Because the invasion ability of glioma cells is biologically and clinically linked to expression and activation of MMP-9 (6–10), we were prompted to examine whether the invasive phenotype enhanced by AEG-1 was associated with a change in MMP-9 expression. Real-time RT-PCR analysis was done to determine the expression levels of MMP-9 in glioma cells expressing ectopic AEG-1 and RNAi knocked down for AEG-1 expression, and as shown in Fig. 3A, in both U87MG and U251MG glioma cells, ectopically overexpressing AEG-1 (left) increased the expression of MMP-9 mRNA, and inversely, knockdown AEG-1 drastically repressed MMP-9 mRNA expression (right). Consistent with the real-time RT-PCR data, ELISA assay and gelatin zymography assay of MMP-9 showed that the AEG-1–transduced glioma cells displayed higher MMP-9 production and proteolytic activity than the vector control cells. By contrast, the AEG-1 RNAi–transduced glioma cells exhibited lowered MMP-9 activities compared with the vector control–infected cells (Fig. 3B and C).

To further show a functional link between AEG-1 and MMP-9 expression, we sought to test whether in clinical glioma tissues the upregulated expression of AEG-1 was associated with increase in the MMP-9 level. The correlation between the expression levels of AEG-1 and MMP-9 was examined in 296 paraffin-embedded glioma clinical specimens. As shown in Fig. 3D, glioma samples with high-level AEG-1 expression exhibited strong MMP-9 staining signals, whereas MMP-9 expression in specimens with low AEG-1 levels was low or absent. Spearman correlation analysis showed a strong correlation between AEG-1 and MMP-9 expression in the tested tissue samples ($r = 0.748$; $P < 0.001$; Supplementary Table S4), suggesting that upregulation of AEG-1 was clinically relevant to increased expressions of MMP-9 in human gliomas.

To determine whether MMP-9 is a key mediator for the increased invasiveness of glioma cells induced by AEG-1, we examined the effect of silencing MMP-9 on the AEG-1–mediated invasiveness. Functional assays (i.e., Transwell matrix penetration assay and three-dimensional invasion assay) revealed that knockdown of MMP-9 in the AEG-1–overexpressing U87MG and U251MG glioma cells reversed

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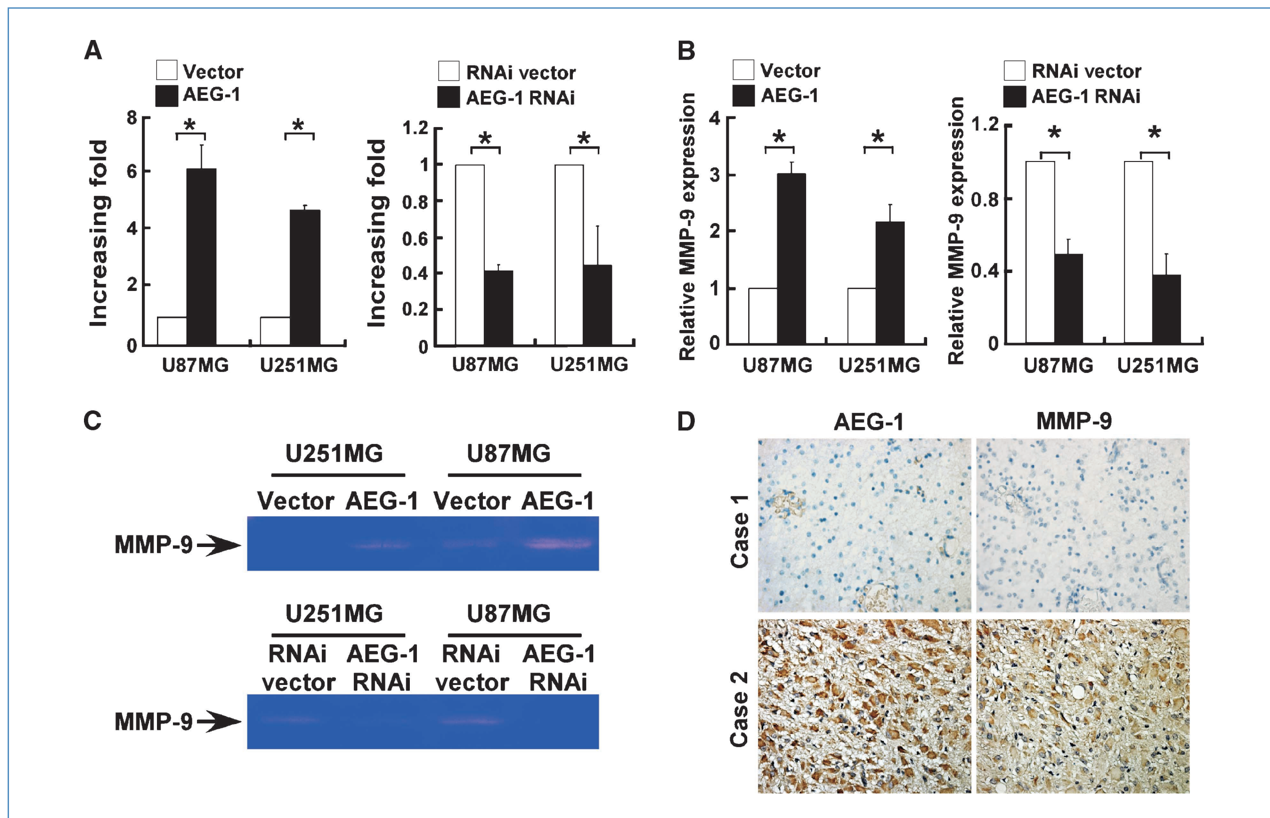

Figure 3. AEG-1 promotes invasive ability of glioma cells through activation of MMP-9 expression. A, quantification of changes of MMP-9 mRNA levels in AEG-1–transduced and AEG-1 RNAi–transduced cells. mRNA expression levels are presented as increasing fold compared with the vector control cells and were normalized with *GAPDH*. B, MMP-9 protein levels in the supernatants of indicated cells were assessed using ELISA. C, gelatinase activity of MMP-9 in indicated cells was assessed using gelatin zymography assays. D, immunohistochemical staining of AEG-1 and MMP-9 in glioma specimens. *, $P < 0.05$.

the invasive ability of both glioma cell lines (Fig. 4), indicating that MMP-9, at least partially, plays important roles in the invasiveness of glioma cells induced by AEG-1.

AEG-1 regulates MMP-9 promoter activity in glioma cells. To understand the mechanism via which AEG-1 upregulates MMP-9 expression, luciferase-based test was done to determine whether AEG-1 regulates MMP-9 promoter activity. We cotransfected the MMP-9 promoter-luciferase construct pGL3 into U87MG and U251MG together with pcDNA3.1-AEG-1 or the control vector, or AEG-1–small interfering

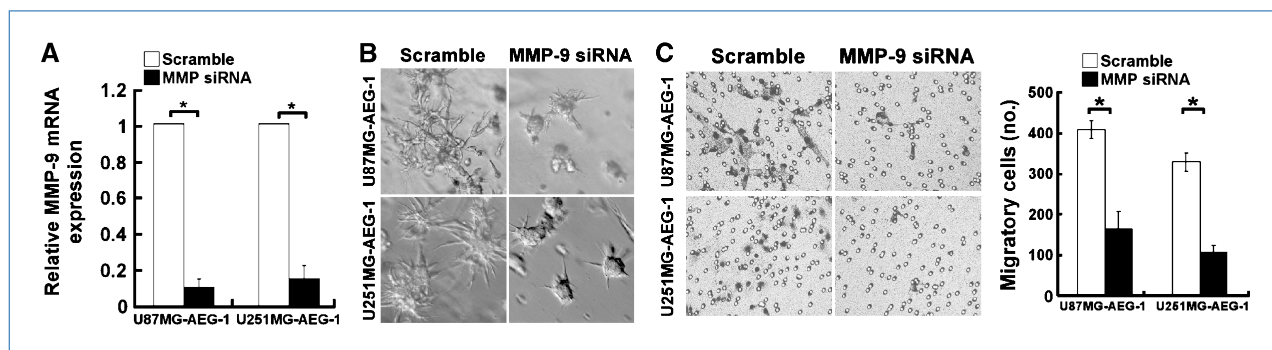

Figure 4. Knockdown of MMP-9 inhibited the invasive properties of glioma cells induced by AEG-1. A, knockdown of MMP-9 mRNA in indicated cells confirmed by real-time RT-PCR. mRNA expression levels were normalized with *GAPDH*. B, knockdown of MMP-9 inhibited the invasive properties of glioma cells induced by AEG-1. Representative micrographs of indicated cultured cells after 10-d culture in three-dimensional spheroid invasion assay. C, representative pictures of penetrated cells (left) and quantification of indicated cells (right) analyzed using the Transwell matrix penetration assay. Quantification of penetrated cells was represented as the mean of three different experiments. *, $P < 0.05$.

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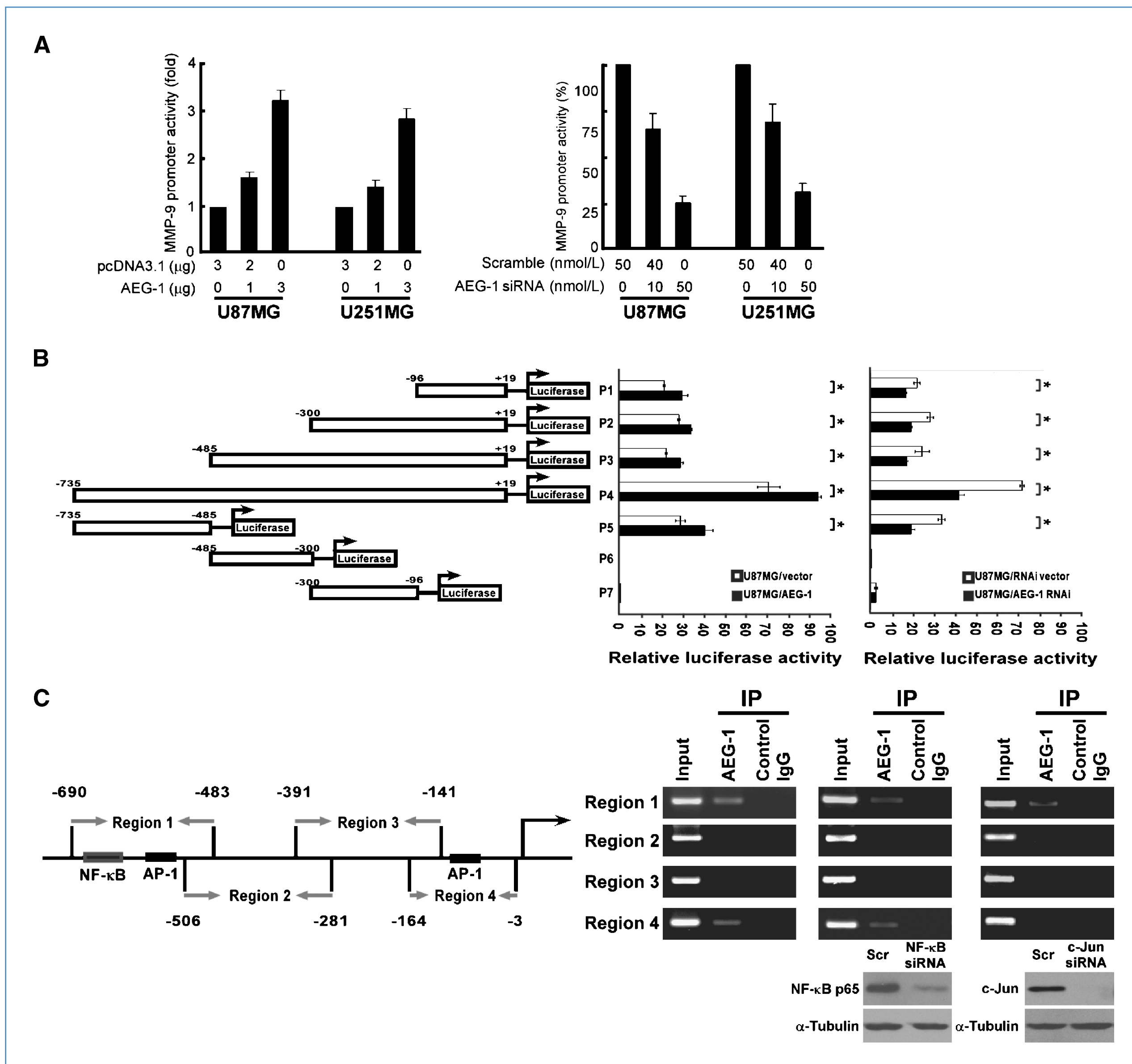

Figure 5. AEG-1 transcriptionally regulates the expression of MMP-9 through association with MMP-9 promoter. A, transactivation of the MMP-9 promoter by AEG-1 (left) and repression of the MMP-9 promoter by AEG-1 siRNA (right) in U87MG and U251MG glioma cells, as shown in luciferase activity assays. B, left, schematic illustration of cloned fragments of the human MMP-9 promoter. The promoter region was cloned as seven fragments (P1 to P7). Right, transactivating activity of AEG-1 on serial MMP-9 promoter fragments as indicated in U87MG cells. The luciferase activities of the promoter constructs were measured after normalization to *Renilla* luciferase activity. Columns, mean of three independent experiments; bars, SD. C, regions of MMP-9 promoter physically associated with AEG-1 were analyzed using ChIP assay. Left, schematic illustration of PCR-amplified fragments of MMP-9 promoter; right, ChIP assays were done with U87MG cells using AEG-1 antibody to screen AEG-1–bound MMP-9 promoter regions for PCR amplification in U87MG, U87-NF-κB siRNA, and U87-AP-1/c-Jun siRNA cells. IgG was used as a negative control. *, $P < 0.05$.

RNA (siRNA) or scramble control. As shown in Fig. 5A (left), cotransfection with pcDNA3.1-AEG-1 activated the luciferase activity driven by the MMP-9 promoter in a dose-dependent manner in both glioma cell lines. Conversely, a consistent and dose-dependent reduction of luciferase activity of MMP-9 promoter on AEG-1 siRNA transfection was shown in both U87MG and U251MG glioma cell lines (Fig. 5A, right). Furthermore, when the luciferase activities driven by serial fragments cloned from the MMP-9 promoter region, including those covering nucleotides −96 to +19 (P1), −300 to +19 (P2), −485 to +19 (P3), −735 to +19 (P4), or −735 to −485 (P5; nucleotide numbering illustrated in Fig. 5B), were tested, the result showed that the luciferase activity could be significantly increased by ectopic overexpression of AEG-1 or decreased by AEG-1 knockdown (Fig. 5B; Supplementary Fig. S2) compared with the vector control cells, whereas

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the luciferase activity in cells transfected with MMP-9 promoter fragments representative of nucleotides −485 to −300 (P6) and −300 to −96 (P7) displayed no difference in their response to AEG-1 overexpression, knockdown, or their controls (Fig. 5B; Supplementary Fig. S2). These results indicate that AEG-1 expression was involved in MMP-9 promoter activity and that the transaction might be through two regions in the MMP-9 promoter [i.e., the P1 region (nucleotides −96 to +19) and the P5 region (nucleotides −735 to −485)].

Consistent with the results obtained from the luciferase activity assay, ChIP assay using U87MG cells revealed that AEG-1 was able to bind region 1 (nucleotides −690 to −483) and region 4 (nucleotides −164 to −3) as defined by the ChIP PCR primers within the MMP-9 promoter area (Fig. 5C), suggesting a physical interaction between AEG-1 and MMP-9 promoter. Because AEG-1 protein itself does not contain any DNA-binding domain, we hypothesized that the association of AEG-1 with MMP-9 promoter elements might be cooperating with other transcription factor(s). The MMP-9 promoter region was examined for transcriptional binding site using prediction tools, which identified a potential NF-κB–binding site and an activator protein (AP)-1–binding element (ABE) between nucleotide positions −690 and −483 bp, as well as an ABE between nucleotides −164 and −3 bp, of the MMP-9 promoter, as indicated in Fig. 5C (left).

To determine whether AEG-1 binds to MMP-9 promoter required for NF-κB or AP-1, ChIP assay was done. Our results showed that the binding efficiency of AEG-1 with MMP-9 promoter region 1 could be reduced by silencing NF-κB p65 and that the binding efficiencies of AEG-1 with MMP-9 promoter regions 1 and 4 could be reduced by silencing AP-1/c-Jun using RNAi (Fig. 5C, right), which indicated that binding of AEG-1 to MMP-9 promoter elements might involve cooperation with NF-κB p65 and/or AP-1. Moreover, real-time PCR assay revealed that the expression of MMP-9 mRNA significantly decreased when the AEG-1–overexpressing glioma cells were treated with either NF-κB inhibitor or AP-1 inhibitor (Supplementary Fig. S3), suggesting that the AEG-1–mediated MMP-9 upregulation might be through both NF-κB and AP-1 pathways.

***Upregulation of AEG-1 causes aggressive tumor invasion and induces MMP-9 expression* in vivo.** Finally, to determine whether overexpression of AEG-1 could stimulate glioma progression *in vivo*, U87MG/vector control cells or U87MG/AEG-1 cells were stereotactically implanted into the brains of mice, and the growing morphologies of implanted glioma tumors were examined. U87MG or U87MG-AEG-1–expressing cells were stereotactically implanted into individual nude mouse brains (n = 5). As shown in Fig. 6, U87MG/vector control cells formed noninvasive, oval-shaped

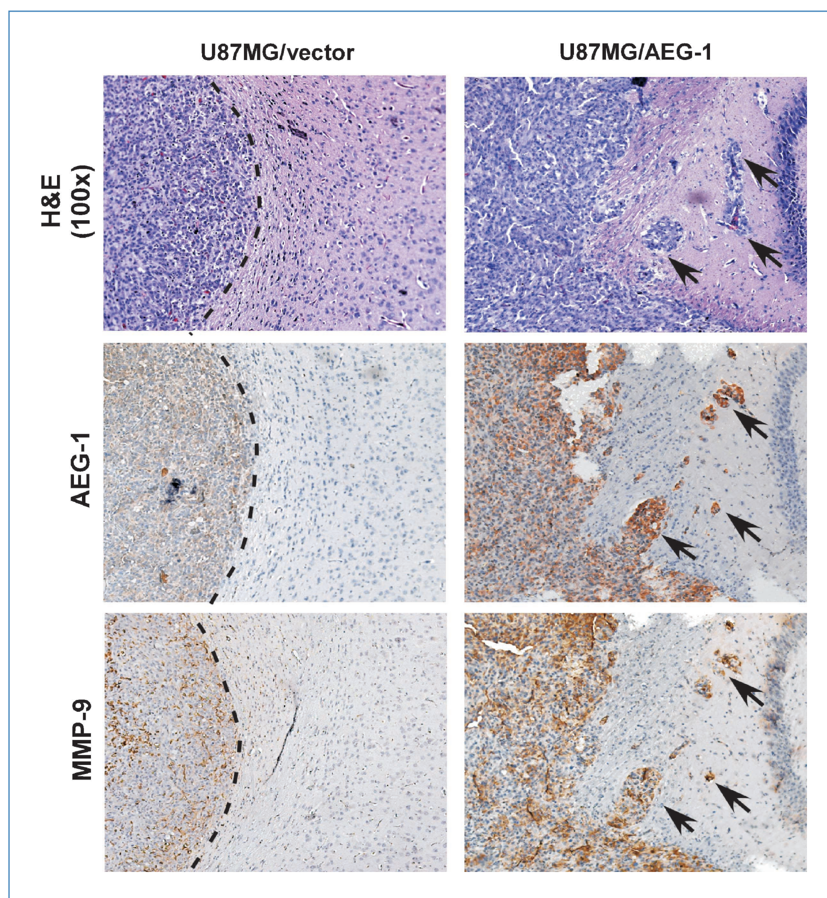

Figure 6. Overexpression of AEG-1 by U87MG cells induces glioma invasion in brains. Left, gliomas established by control U87MG/vector cells (n = 5 per group); right, invasive gliomas formed by U87MG/AEG-1 cells (n = 5 per group). Arrows, invasive tumor cells. The sections of U87MG/vector or U87MG/AEG-1 gliomas were stained with hematoxylin or immunostained with an anti–AEG-1 antibody and an anti–MMP-9 antibody. Original magnification, ×100.

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intracranial tumors in the brains of all nude mice, with sharp edges that expanded as spheroids (Fig. 6, left). In contrast, five mice that received U87MG/AEG-1 cells developed highly invasive gliomas, which invaded into the normal brain structures, displaying interspersed fibroblast-like structures (Fig. 6, right). It is noteworthy that the invasive phenotype displayed by U87MG/AEG-1 gliomas coincided with upregulation of MMP-9 (Fig. 6, right). Taken together, these results suggest that overexpressing AEG-1 caused aggressive tumor invasion and induced MMP-9 expression in the brain.

Discussion

Key findings of the current study provide new insights into the potential role of deregulated AEG-1 in promoting the aggressiveness of gliomas by showing that AEG-1, markedly overexpressed in both glioma cell lines and primary glioma tissues, contributes to the invasive phenotype of glioma cells through transcriptional upregulation of MMP-9 via interacting with its promoter. Immunohistochemical staining analysis revealed a significant correlation between the expressions of AEG-1 and MMP-9 in a cohort of clinical glioma samples.

Glioma, arising from glial cells, remains one of the most aggressive primary CNS tumors (1, 4, 30). The outstanding feature of invasive growth of gliomas has imposed impediments to thorough surgical removal of the tumor and thus might represent a key factor to which the poor prognosis of the disease is attributed. Previous findings, including those made by ourselves (15–28), indicating that AEG-1 contributes to promotion of cancer progression and activation of relevant signaling pathways promoted us to ask whether AEG-1 plays a role in the aggressiveness of gliomas and has a prognostic implication for glioma patients. To this end, experiments were done to examine the biological effect of AEG-1 on the invasive phenotype of glioma cells, in which both Transwell matrix penetration assay and three-dimensional spheroid invasion assay showed that upregulation of AEG-1 indeed enhanced the ability of glioma cells to invade, whereas depletion of endogenous AEG-1 drastically inhibited the invasiveness. Together with this result is an immunohistochemical analysis on 296 paraffin-embedded archival glioma specimens, which showed that, in addition to overall positive staining of AEG-1 in glioma cells, the AEG-1 expression significantly correlated with the WHO histologic grading ($P < 0.001$), strongly suggesting that AEG-1 might be involved in the progression of gliomas.

The biological process of glioma cells that invade and filtrate into the surrounding brain tissues involves proteolytic digestion of the connections between cells and ECM. The regulators of cell adhesion and invasion, such as MMP-2 and MMP-9, have been shown to closely correlate with the pathogenesis and clinical outcome of gliomas (6–10). MMP-9 facilitates the initiation and progression of multiple biological events required for glioma progression, such as invasion, migration, and dissemination of glioma cells, due to its capacity of digesting and degrading components of ECM (31, 32). Selective suppression of MMP-9 impairs the migration of glioma cells, and MMP-9–deficient mice show reduced metastasis of various cancers, such as melanoma (7, 33). It is of note that the activity of MMP-9 is modulated at three levels, namely, gene expression, proenzyme activation, and inhibition of the hydrolytic ability by specific inhibitors (34–36). MMP-9 expression has been found to be activated or enhanced by oncogenic proteins and elevated in many cancer types, including breast cancer, prostate cancer, melanoma, bladder cancer, pancreatic cancer, and gliomas, and to correlate with the prognosis of cancer patients (37–42). In addition to its effect on the invasive ability of tumor cells, MMP-9 also plays roles in tumor angiogenesis and is involved in a variety of signaling cascades leading to cancer progression (43, 44). Thus, accumulating evidence highlights MMP-9 as one of the major mediators for the functions of oncogenes and thereby a potential target for cancer therapy. Nonetheless, whether the enhanced MMP-9 expression in gliomas is mechanistically associated with upregulated AEG-1 had remained unknown. Along with this context, our current study provides the first demonstration that upregulation of AEG-1 can increase expression of MMP-9 in glioma cells at the transcription level and that depletion of endogenous AEG-1 represses MMP-9 expression. In support of this notion, a significant correlation between the expressions of AEG-1 and MMP-9 in clinical samples has also been identified by our present study.

At the molecular level, expression of MMP-9 expression is subject to transcriptional activation, and MMP-9 promoter contains multiple consensus binding sites for several transcriptional factors, including AP-1, AP-2, polyoma enhancer A–binding protein 3/Ets, NF-κB, and Sp-1 (45, 46). We have found that AEG-1 could bind to at least two regions in the MMP-9 promoter area (i.e., nucleotides −690 to −483 bp and nucleotides −164 to −3 bp), as revealed by the luciferase activity and ChIP assays. Because AEG-1 protein itself does not contain any DNA-binding domain, we hypothesize that AEG-1 might cooperate with other transcription factor(s) so that it can activate the transcription of the downstream gene. Interestingly, further sequence analysis showed that there is a potential NF-κB–binding site and an ABE between nucleotide positions −690 and −483 bp, as well as an ABE between nucleotides −164 and −3 bp, of the MMP-9 promoter. Moreover, knockdown of either NF-κB p65 or AP-1/c-Jun by RNAi decreased the binding efficiency of AEG-1 on MMP-9 promoter, and inhibition of the NF-κB activity or AP-1 activity by their specific inhibitors led to the reduction of MMP-9 mRNA or MMP-9 promoter-driven luciferase activity in AEG-1–overexpressing glioma cells, indicating that the AEG-1–mediated MMP-9 upregulation might be through both of NF-κB and AP-1 pathways. In agreement with our hypothesis, previous findings have suggested that NF-κB could be linked by AEG-1 to the cyclic AMP–responsive element binding protein–binding protein and other transcriptional activators by forming basal transcription machinery, where AEG-1 acts as a bridging factor, resulting in transcriptional activation of downstream genes of NF-κB (26). Whether AEG-1 does enhance the transcriptional activity of AP-1 family members, or interacts with other transcription

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factors, to activate MMP-9 promoter is of interest for further study.

In summary, we have shown that AEG-1, a potential oncogene, is evidently overexpressed in gliomas. Moreover, our finding that AEG-1 transcriptionally upregulates the expression of MMP-9 illustrates a new mode of action in the molecular mechanism underlying the invasiveness of gliomas. Further delineation of the mechanism that mediates the regulation of MMP-9 by AEG-1, particularly the direct binding partner(s) of AEG-1 along with this function, is needed.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Correction

Cancer Research

Correction: Online Publication Dates for *Cancer Research* April 15, 2010 Articles

The following articles in the April 15, 2010 issue of *Cancer Research* were published with an online publication date of April 6, 2010 listed, but were actually published online on April 13, 2010:

Garmy-Susini B, Avraamides CJ, Schmid MC, Foubert P, Ellies LG, Barnes L, Feral C, Papayannopoulou T, Lowy A, Blair SL, Cheresh D, Ginsberg M, Varner JA. Integrin $\alpha4\beta1$ signaling is required for lymphangiogenesis and tumor metastasis. Cancer Res 2010;70:3042–51. Published OnlineFirst April 13, 2010. doi:10.1158/0008-5472.CAN-09-3761.

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Astrocyte Elevated Gene-1 Upregulates Matrix Metalloproteinase-9 and Induces Human Glioma Invasion

Liping Liu, Jueheng Wu, Zhe Ying, et al.

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