

Apoptosis inhibitor 5 increases metastasis via Erk-mediated MMP expression

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Apoptosis inhibitor 5 (API5) has recently been identified as a tumor metastasis-regulating gene in cervical cancer cells. However, the precise mechanism of action for API5 is poorly understood. Here, we show that API5 increases the metastatic capacity of cervical cancer cells *in vitro* and *in vivo* via up-regulation of MMP-9. Interestingly, API5-mediated metastasis was strongly dependent on the Erk signaling pathway. Conversely, knock-down of API5 via siRNA technology decreased the level of phospho-Erk, the activity of the MMPs, *in vitro* invasion, and *in vivo* pulmonary metastasis. Moreover, the Erk-mediated metastatic action was abolished by the mutation of leucine into arginine within the heptad leucine repeat region, which affects protein-protein interactions. Thus, API5 increases the metastatic capacity of tumor cells by up-regulating MMP levels via activation of the Erk signaling pathway. [BMB Reports 2015; 48(6): 330-335]

INTRODUCTION

API5, also called AAC-11 (anti-apoptosis clone 11) and FIF (fibroblast growth factor 2-interacting factor), is a well-known nuclear protein whose expression prevents apoptosis due to the deprivation of serum and growth factors (1). Up-regulation of API5 has been associated with poor survival of patients with NSCLC (2). A recent study indicated that the antiapoptotic

function of API5 might be mediated by suppression of the apoptosis induced by the transcription factor E2F1 (3) and it also contributes to the activity of E2F1, which in turn contributes to the G1/S phase cell cycle transition (4). Pim-2 activates API5 to inhibit the apoptosis of hepatocellular carcinoma cells via the NF- κ B pathway (5). The anti-apoptotic function of API5 is accomplished by interaction with various binding partners such as fibroblast growth factor 2 (FGF-2) (6), Acinus, and ALC1.

Although the role of API5 has been revealed (7, 8), the molecular mechanism of API5 for regulating metastasis in cervical cancer is not yet well characterized. In this study, we demonstrated that API5 modulated metastasis of cervical cancer in an Erk-dependent manner. API5 consistently enhanced MMP-2 and MMP-9 secretion through the transcription factor, AP-1. Furthermore, after mutation of leucine residues in its heptad leucine repeat region, API5 could no longer enhance metastasis through the Erk signaling cascade, suggesting that the ability to mediate protein-protein interactions is one of the important functions of API5 for regulating metastasis in cervical cancer. Thus, this study demonstrates that API5 promotes the metastatic capacity of tumor cells by up-regulating MMP levels via Erk signaling.

RESULTS

API5 overexpression increases the *in vivo* and *in vitro* invasive potential of human cervical cancer cells.

To investigate whether API5 expression is associated with metastasis in cervical cancer, we transduced the API5 gene into API5^{low} CaSki cells and confirmed the expression level by western blotting (Fig. 1A). We observed a statistically significant increase in the invasive activity of API5-overexpressing cells compared to control cells (Fig. 1B). To further characterize the *in vivo* metastasis-promoting ability of API5, CaSki/no insert or CaSki/API5 cells were injected into nude mice, and the mice were examined for the formation of pulmonary metastatic nodules. Intravenous transplantation of CaSki/API5 cells

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<http://dx.doi.org/10.5483/BMBRep.2015.48.6.139>

Received 1 July 2014, Revised 4 August 2014,
Accepted 21 September 2014

Keywords: Apoptosis inhibitor 5, Cervical cancer, Metastasis, Matrix metalloproteinase

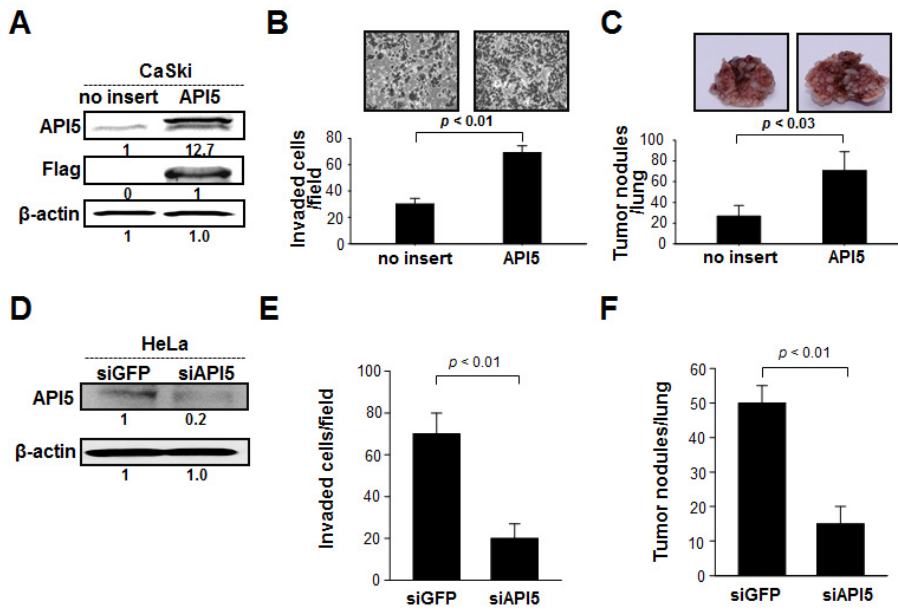


Fig. 1. Characterization of the metastatic function of API5 in CaSki tumor cells. (A) Western blot analysis to characterize the expression of API5 in CaSki/no insert and CaSki/API5 cells. (B) Representative photomicrograph of matrigel invasion assay using CaSki/No insert cells and CaSki/API5 cells (upper). Bar graph represents the total numbers of invaded cells (low). (C) Representative photographs of lung metastases by *in vivo* lung metastasis assay (upper). Bar graph (low) represents the total number of lung metastatic nodules from tumor-challenged mice intravenously inoculated with CaSki/No insert or CaSki/API5 cells. Error bars represent mean \pm SD. (D) Western blot analysis to characterize the expression of API5 and beta-actin in HeLa cells transfected with siGFP or siAPI5. (E) Matrigel invasion analysis to characterize the invasiveness of HeLa cells transfected with siGFP or siAPI5. (F) Bar graph represents the total number of lung metastatic nodules. Error bars represent mean \pm SD.

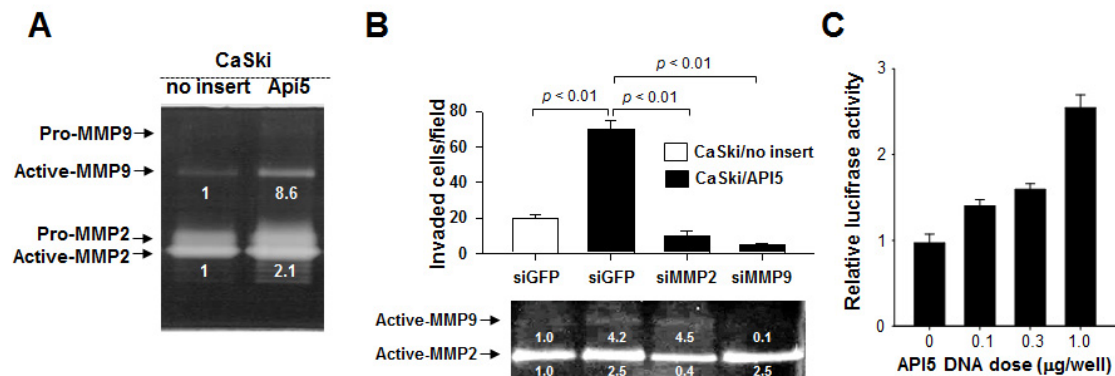


Fig. 2. Invasive capacity via MMP-9 expression in API5-overexpressing tumor cells. (A) Gelatin zymography to characterize the activation of MMP-2 and MMP-9 in CaSki/no insert and CaSki/API5 cells. (B) Matrigel invasion analysis to characterize the invasiveness of siGFP transfected CaSki/no insert, siGFP, siMMP-2, or siMMP-9 transfected CaSki/API5 cells (upper). Gelatin zymography to characterize the activation of MMP-2 and MMP-9 (lower) (C) Luminescence analysis to determine MMP-9 promoter luciferase activity induced by API5. Bar graph represents relative luciferase activity. Error bars represent mean \pm SD.

resulted in significantly more visible metastatic nodules in the lungs compared to the visible nodules after intravenous transplantation of CaSki/no insert cells (Fig. 1C). We also confirmed the expression of high levels of API5 in siAPI5 transfected HeLa cells (Fig. 1D) reduced cell invasion *in vitro* (Fig. 1E) and in tumor nodules *in vivo* (Fig. 1F). Both the *in vitro* invasion assay and the *in vivo* assay in nude mice suggest that API5 has the potential to promote metastasis of human cervical cancer cells.

MMP-9 is involved in invasion of CaSki/API5 cells

To further elucidate the molecular mechanism of API5 for en-

hancing metastasis, we examined the expression levels and activity of MMP2 and MMP9 (gelatinases A and B, respectively), which play essential roles in the degradation of the extracellular matrix during metastasis. Supernatants of CaSki cells expressing API5 or no insert were collected and used for gelatin zymography analysis. CaSki/no insert and CaSki/API5 cells secreted the pro-form of both MMP-2 and MMP-9, but only MMP-9 was upregulated by API5 overexpression (Fig. 2A). For further confirmation of the role of MMP9 in API5-mediated invasion, we performed an invasion assay after treatment of CaSki/API5 cells with MMP2 or MMP9 siRNA (siMMP2 or siMMP9, respectively). The suppression of MMP9 by siRNA

significantly inhibited the invasive activity of CaSki/API5 cells compared to that of MMP2 siRNA-transfected CaSki/API5 cells (Fig. 2B). This result suggests that MMP9 activity is required for the increased invasive capacity resulting from ectopic API5 expression. To determine whether API5 regulates MMP9 at the transcriptional level, we performed a reporter assay using a construct containing a luciferase gene driven by a MMP9 promoter. Transfection of the MMP9 reporter construct and the API5 gene into CaSki cells resulted in a significant dose-dependent increase in luciferase activity (Fig. 2C). This result suggests that MMP9 level is increased by API5 to promote the invasive activity of tumor cells.

MEK/Erk signaling pathway is involved in API5-induced MMP-9 activity

We performed western blot analysis to determine the expression of various signaling molecules that may play a role in the global control of metastasis. As shown in Fig. 3A, greater activation of Erk 1 (p-Erk) was observed in CaSki/API5 cells than in CaSki/no insert cells, while the phosphorylation of AKT and p38 were not altered. Based on this finding, we tried to determine whether Erk is a critical factor for API5-induced MMP-9 expression. After treatment with PD98059, which selectively blocks the activity of MEK1, the activation of MMP-9 was significantly down-regulated in CaSki/API5 cells (Fig. 3B). PD98059 could also inhibit the invasive ability of CaSki/API5

cells. To avoid the non-biological function of these pharmacological inhibitors, we examined the inhibition of the invasion activity of CaSki/API5 cells using p42 (Erk 2) specific siRNA (sip42). We transfected sip42 into CaSki/API5 cells and then examined the expression of p42 in CaSki/API5 cells by western blotting (Fig. 3C). The number of invading sip42-transfected CaSki/API5 cells was significantly decreased compared to the invading siGFP-transfected cells (Fig. 3D). To further confirm the dramatic decrease in the invasive potential of sip42-transfected CaSki/API5 cells *in vivo*, nude mice were injected with either siGFP- or sip42-transfected CaSki/API5 cells, and the mice were examined for the formation of pulmonary metastatic nodules. After intravenous transplantation of p42 siRNA-transfected CaSki/API5 cells, there was a significant decrease in the number of visible metastatic nodules in the lung compared to that observed after intravenous transplantation of siGFP-transfected CaSki/API5 cells (Fig. 3E). These results suggest that API5 positively induces the transcriptional activation of MMP-9 expression via the Erk signaling pathway.

API5 regulates MMP-9 expression through the transcription factor, AP-1

The AP-1 and NF- κ B elements of the MMP-9 promoter are essential for the induction of MMP-9 expression. To identify the cis-element responsible for the response to API5, we examined the promoter activity of MMP-9 by using a serial deletion con-

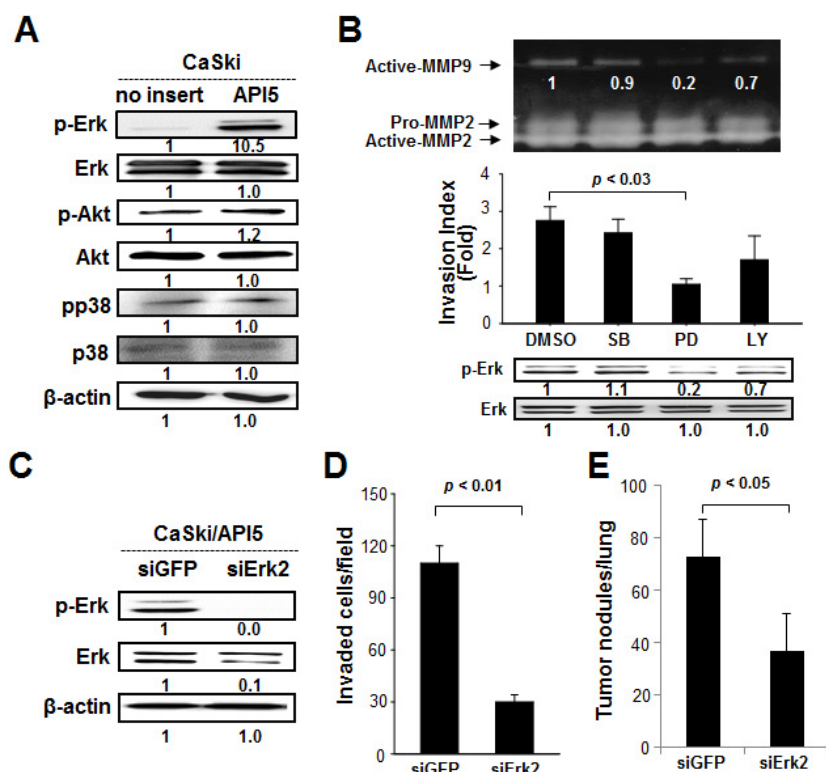


Fig. 3. Erk -dependent MMP-9 expression in API5-overexpressing tumor cells. (A) Western blot analysis to characterize the expression of total Erk, Thr 202/Tyr 204 pErk, total AKT, Ser 473 pAKT, total p38 MAP kinase, and Thr 180/Tyr 182 pp38 MAP kinase in the CaSki/no insert and CaSki/API5 tumor cells. (B) CaSki/API5 tumor cells were incubated with DMSO, p38 MAP kinase inhibitor SB203580, PI3K inhibitor LY294002, or Erk inhibitor PD98059 for 18 hours. Gelatin zymography to characterize the activation of MMP-2 and MMP-9 (upper). Matrigel invasion analysis to characterize the invasiveness of DMSO, p38 MAP kinase inhibitor SB203580, PI3K inhibitor LY294002, or Erk inhibitor PD98059 treated CaSki/API5 cells (middle). p-Erk and total Erk characterized by western blotting (low). (C) Western blot analysis to characterize the expression of total Erk and p-Erk in siGFP or siErk2 transfected CaSki/API5 cells. (D) Matrigel invasion analysis to characterize the invasiveness of siGFP or siErk2 transfected CaSki/API5 cells. (E) Bar graph represents the total number of lung metastatic nodules.

struct of the MMP-9 promoter. A genomic fragment containing 710 bp from the 5'-promoter region of the MMP-9 gene, which includes the putative TATA box, two AP-1 sites, and a NF- κ B site, as well as an AP-1 deletion mutant was subcloned into the pGL2-basic vector (WT-MMP9pro, Mut-AP-1-1, and Mut-AP-1-2). These plasmids were co-transfected with API5 into CaSki cells. Luciferase activity was barely observed when the pGL2-basic was co-transfected with API5 vector into CaSki cells (Fig. 4A). The luciferase activity of WT-MMP9pro, which was co-transfected with an API5 vector, was higher than that of

the pGL2-basic vector. On the other hand, API5-driven luciferase activity was reduced in the AP-1-1 and AP-1-2 mutants of the MMP-9 promoter, indicating that AP-1 binding sites are indispensable for the induction of MMP-9 expression by API5 in CaSki cells. This result indicates that API5-mediated MMP-9 expression critically depends on intact AP-1 binding sites within the MMP-9 promoter region. Furthermore, we determined whether API5 regulates transcriptional levels of the transcription factor AP-1 in cervical cancer cells via the Erk signaling cascade. As shown in Fig. 4B, the luciferase activity of

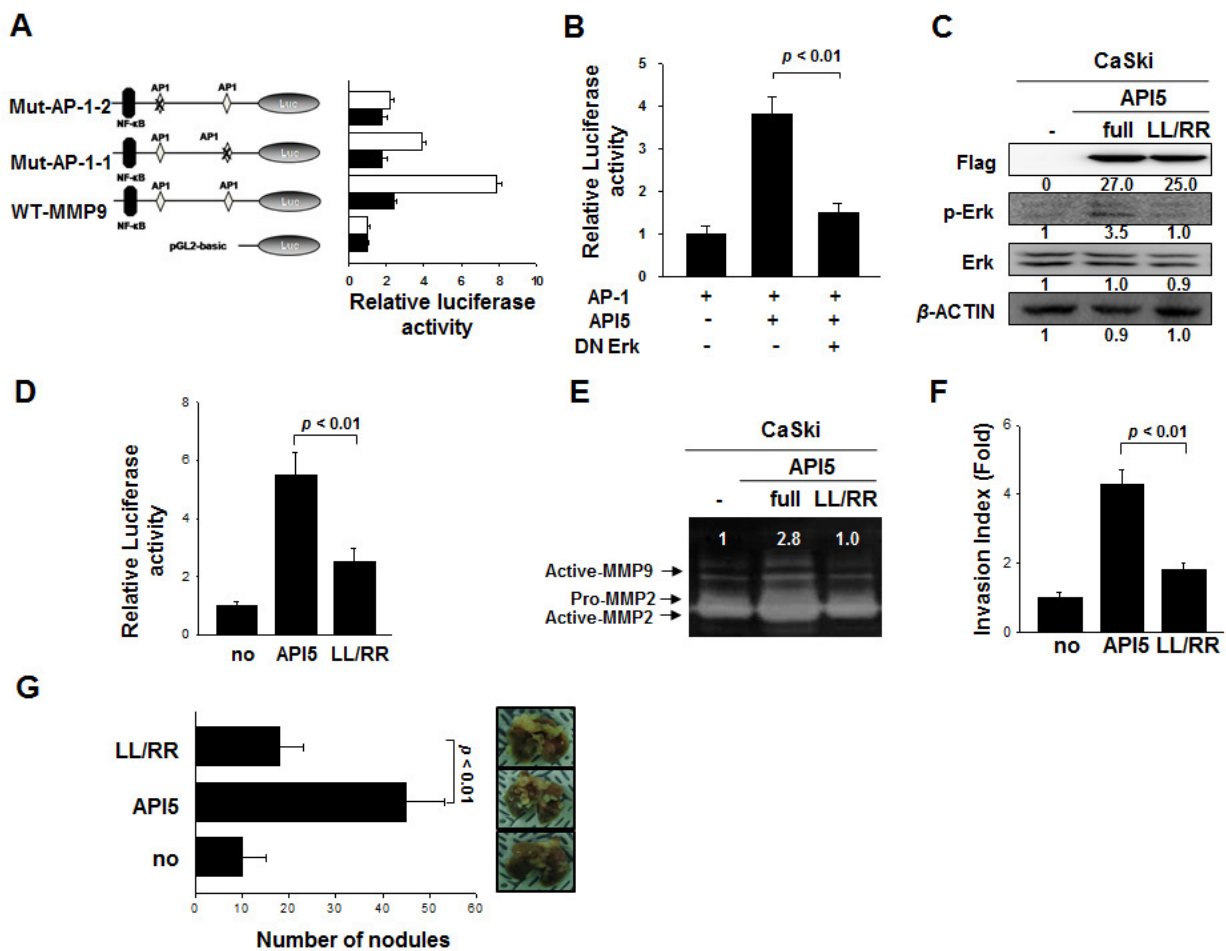


Fig. 4. API5 mediation of multiple protein complex dependent MMP-9 expression in cancer cells. (A) A schematic illustration of MMP-9 promoter reporter plasmid, AP-1-1 and AP-1-2 mutant of MMP-9 promoter reporter plasmid (left). Luminescence analysis to determine luciferase activity in CaSki cells co-transfected with API5 and MMP-9 promoter reporter plasmid, AP-1-1 or AP-1-2 mutant of MMP-9 promoter reporter plasmid. Bar graph represents relative AP-1 promoter luciferase activity. Error bars represent mean \pm SD. (B) Luminescence analysis to determine AP-1 promoter luciferase activity in CaSki cells transfected with or without AP-1 promoter, API5, and DN- Erk gene. Bar graph represents relative AP-1 promoter luciferase activity. Error bars represent mean \pm SD. (C) Western blot analysis to characterize the expression of API5, p-Erk, total Erk, and beta-actin in CaSki cells transduced with no insert, API5, API5LL/RR mutant. (D) Luminescence analysis to determine MMP-9 promoter luciferase activity in CaSki/no insert, CaSki/API5, and CaSki/API5LL/RR cells. (E) Gelatin zymography to characterize the activation of MMP-2 and MMP-9 in CaSki/no insert, CaSki/API5, and CaSki/API5LL/RR cells. (F) Matrigel invasion analysis to characterize the invasiveness of CaSki/no insert, CaSki/API5, and CaSki/API5 LL/RR cells. (G) Bar graph (left) represents the total number of lung metastatic nodules. Representative photographs of lung metastases by in vivo lung metastasis assay (right). Error bars represent mean \pm SD.

AP-1 in API5 transfected cells was higher than in control cells. However, the luciferase activity of AP-1 was reduced in DN-Erk-transfected cells. This result indicates that API5 induced the activation of Erk, which in turn induced the activity of AP-1, thereby regulating MMP-9 expression through the transcriptional factor AP-1.

Heptad leucine repeat region of API5 might be important for activation of the Erk-MMP9 axis

The heptad leucine repeat region of API5 might affect protein-protein interactions between API5 and other API5-interacting partners, such as Acinus (10, 11). We therefore considered the possibility that mutation of leucine residues (Leu-384 and Leu-391) in the heptad leucine repeat region could affect Erk-mediated MMP-9 expression induced by API5. To address this question, we generated an API5 LL/RR mutant (LL/RR) and transduced it into CaSki cells. As shown in Fig. 4C, API5 LL/RR transduced cells did not display Erk activation, unlike wild type (wt) API5 transduced cells. This result indicated that the heptad leucine repeating region of API5 may be important for Erk activation in cervical cancer cells. Furthermore, we analyzed MMP9 promoter activity using gelatin zymography in the API5 LL/RR-transduced cells. As shown in Fig. 4D and E, the activity and expression of MMP-9 were lower in the API5 LL/RR-transfected cells compared to the wtAPI5-transfected cells. We further performed an invasion assay, which showed that the LL/RR mutant transfected cells exhibited decreased invasion compared to API5 transfected cells (Fig. 4F). These results indicate that the heptad leucine repeat region of API5 may be required for Erk-mediated MMP-9 expression. To confirm the decrease in the invasive potential of the LL/RR mutant transfected cells *in vivo*, nude mice were injected with API5 transfected cells and the mice were examined for the formation of pulmonary metastatic nodules. Inoculation with API5 LL/RR-transfected cells caused a significant decrease in visible metastatic nodules in the lungs compared to inoculation with wtAPI5 transfected cells (Fig. 4G). Our data suggest that interactions between API5 and its binding partners through the heptad leucine repeat region of API5 can affect the metastasis of cervical cancer cells via Erk-induced MMP9 expression.

DISCUSSION

In this study, we demonstrated that API5 mediates the activation of the Erk cascade, which leads to the induction of AP-1. The promoter region of the MMP-9 gene contains binding sites for AP-1 and NF- κ B in MMP-9 gene activation, and the important roles of AP-1 and NF- κ B have been reported (12, 13). MAPK activation can lead to MMP-9 induction through the regulation of an essential transcription factor such as c-fos (14). Expression of this immediate early gene depends on MAPK activation; further phosphorylation of c-fos by MAPK enhances MMP-9 expression. Thus, initial activation may serve to enhance c-fos transcriptional activity and AP-1-dependent ex-

pression of the MMP-9 gene. Based on this observation, activation of Erk is relayed through a well characterized signaling pathway involving transactivation and DNA binding of the AP-1 complex. Therefore, we hypothesized that an API5 signaling cascade during Erk activation may induce MMP-9 expression through the activation of AP-1. In this study, we demonstrated that API5-induced MMP-9 expression is critically dependent on an intact AP-1 binding site within the MMP-9 promoter region. AP-1 binding site mutation could affect MMP-9 expression in API5 transfected cells. This result may support our hypothesis that API5 regulates MMP9 expression via Erk-dependent AP-1 activation.

API5 has the ability to form a protein complex with binding partners such as FGF2 and Acinus, and it prevents drug-induced apoptotic cell death through physiological binding because API5 possesses HEAT and ARM-like repeats, which are a kind of protein-protein interaction modules. In the present study, API5 LL/RR failed to increase the invasive potential caused by the activation of the Erk-MMP9 axis (11). The interaction between API5 and its binding partners through the heptad leucine repeat region of API5 may therefore regulate Erk activity, and subsequently contribute to the invasion-promoting ability of API5. Further studies are needed to identify the API5 binding partner and explain the regulatory mechanism of Erk activation induced by API5.

In conclusion, API5 induces the Erk pathway, and in turn, the activation of the Erk pathway leads to the activation of the transcription factor AP-1 and finally to an increase in MMP-9 expression. Based on these data, we propose that API5 plays a role in the induction of MMP-9 via activation of the Erk signaling pathway.

MATERIALS AND METHODS

DNA construct, Cells, siRNA transfection, Promoter assay, and Zymographic assay are described in the online data supplement, available at <http://www.bmbreports.org/>.

Mice

Six- to 8-week-old female C57BL/6 mice and nude mice were purchased from DaehanBiobank (Chungbuk, Korea). All animal procedures were performed in accordance with the recommendations for the proper use and care of laboratory animals.

***In vitro* invasion assay**

The invasion ability of tumor cells was examined using 24-well culture insert-based assays (BD Biosciences, Franklin Lakes, NJ). The culture insert, with an 8 μ m pore size, was pre-coated to a density of 2-3 mg/ml insert of 20 μ l Matrigel Basement Membrane Matrix (BD Biosciences). Cells were suspended in serum free medium, and 1×10^5 cells were added onto the insert. After incubating for 24 hours at 37°C, the cells that invaded or migrated through the Fluoro-Blok membrane were stained with crystal-violet, and images of the stained cells

were taken. The invasive cells were then counted using a q20X objective microscope. The samples were plated in triplicate or quadruplicate, and the experiment was repeated at least three times.

Pulmonary metastasis assay

Female BALB/c nude (nu/nu) mice (5-week-old) were purchased from Charles River Laboratory (Japan), and housed under pathogen-free conditions. All animal procedures were performed according to approved protocols and in accordance with recommendations for the proper use and care of laboratory animals. Mice were injected with 1×10^6 cells in 0.1 ml Opti-MEM through the tail vein. The overall health and total body weight of the mice were then monitored. At 4 weeks after injection, the mice were sacrificed. The lung tissues were observed with the naked eye and the number of visible tumors on the lung surface was counted. Each experimental group included 6 to 10 mice.

Statistical analysis

All data are representative of at least three separate experiments. The data from *in vivo* lung metastasis experiments were evaluated by ANOVA. Comparisons between individual data points were made using Student's *t*-test. All *P*-values < 0.05 were considered statistically significant.

ACKNOWLEDGEMENTS

This work was funded by the National Research Foundation of Korea (2013M3A9D3045881 and 2012R1A2A2A01007527) and the Korea Healthcare Technology R&D Project (A080816 and H111C-0052-030013).

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