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Suppression of Asparaginyl Endopeptidase Inhibits Polyomavirus Middle T Antigen-Induced Tumor Formation and Metastasis

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Elevated circulating asparaginyl endopeptidase (AEP), a novel lysosomal protease, has been found in breast cancer, and AEP is thus considered to be a prognostic factor in this disease. However, the pathological functions of circulating AEP in the development of breast cancer and the potential of AEP-targeted therapy remain unclear. We used MMTV-PyVmT transgenic mice, which spontaneously develop mammary tumors. Western blotting showed overexpression of AEP in both primary tumor tissue and lung metastases compared to their normal counterparts. Moreover, the concentration of circulating AEP gradually increased in the serum during the development of mammary tumors. Purified AEP protein injected through the tail vein promoted tumor growth and mammary tumor metastasis and shortened survival, whereas AEP-specific small compound inhibitors (AEPIs) effectively suppressed tumor progression and prolonged host survival. Further analysis of the molecular mechanism revealed that AEP was important for PI3K/AKT pathway activation. Thus, an elevated serum AEP level was closely related to mammary cancer progression and metastasis, and AEP is a potential target for breast cancer therapy in the clinic.

Key words: Asparaginyl endopeptidase (AEP); Breast cancer; Transgenic mice; Metastasis

INTRODUCTION

Understanding the molecular basis of tumorigenesis, tumor growth, and metastasis from a primary site to other tissues is a major challenge. Spontaneous breast tumor models are powerful tools for studying the reproducible development of spontaneous tumors, the occurrence of invasion and metastasis, and the presence of an intact immune system. More importantly, tumor models resemble human disease with regard to progression through various developmental stages of cancer[1,2]. Mammary tumors, which can be followed by palpation, are especially useful for therapeutic and prevention investigations because tumor localization removes the need to sacrifice the animal to determine a clinical response. Induction of mammary tumors by mammary gland-specific expression of the polyomavirus middle T antigen (MMTV-PyVmT) oncogene results in the widespread transformation of the mammary epithelium and the rapid production of multifocal mammary adenocarcinomas[3]. The transgenic mouse model is ideal for metastatic studies, as the majority of the tumor-bearing transgenic mice develop secondary metastatic tumors in the lung. Indeed, the functions of many molecules and signaling pathways in pathology have been elucidated by taking advantage of this model.

Asparaginyl endopeptidase (AEP), which is highly specific for asparaginyl bonds and is currently the only known AEP encoded by the mammalian genome, has been found to be highly expressed in a variety of solid tumors and in acute lymphoblastic leukemia; by contrast, only a limited quantity of AEP is detectable in normal tissue[4–9].

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Moreover, AEP expression is positively related to clinicopathological and biological variables in breast cancer and colorectal cancer[5,8]. AEP has also been found to influence hepatocellular growth and inhibit the cell cycle at the G_1/S phase[10] and to activate the zymogene MMP2 and cathepsins[11–13]. In a previous study, we demonstrated that TRAF6 ubiquitinates AEP, promoting its stability and secretion, steps that are vital for breast cancer progression[8]. However, the pathological function of circulating AEP in breast cancer development remains unknown.

Potent and specific inhibitors of AEP (AEPIs) could be developed into new drugs for treating cancer and other diseases. To date, many different classes of AEPIs have been developed, including reversible and irreversible transition-state inhibitors. Aza-Asn epoxides have high specificity toward AEP[14]. However, the dose and efficiency of Aza-Asn epoxides in mammary cancer treatment are unclear. In this study, we investigated both the function of circulating AEP in breast cancer progression in a transgenic mouse model and the relevant signaling pathway correlating with AEP regulation.

MATERIALS AND METHODS

Cell Lines

HEK293T cells and a human breast carcinoma cell line were cultured in Dulbecco's modified Eagle's medium (HyClone, Logan, UT, USA) supplemented with 10% FBS in a 5% CO_2 humidified atmosphere at 37°C.

Plasmids and Antibodies

HA-tagged AEP was cloned into pcDNA3.1. All PCR products were confirmed by sequencing. The antibodies used were as follows: sheep anti-mouse AEP (R&D Systems, Abingdon, Oxford, UK), anti-p-AKT, -AKT, -p-PI3K, and -PI3K (Cell Signaling Technology, Danvers, MA, USA).

Synthesis of AEP-Specific Small Compound Inhibitors

Proton and carbon NMR spectra were recorded using a 500-MHz spectrometer. NMR chemical shifts are reported in δ (ppm) using the δ 2.50 signal of DMSO (^{1}H NMR) and the δ 39.5 signal of DMSO (^{13}C NMR) as internal standards. Mass spectra were measured in ESI mode with LCMS MSD (Hewlett Packard, Palo Alto, CA, USA).

A mixture of 30% HBr/AcOH (88 ml) was added dropwise with stirring to D-(−)-tartrate (1.30 g) in an ice bath. After the mixture was stirred overnight at RT, ice water (200 ml) was added to quench the reaction. The aqueous phase was extracted with ether (200 ml×3), and the combined organic layer was washed with water and brine and dried over Na_2SO_4. The solvent was then removed to afford compound 2 as a pale yellow oil (42 g, 93%).

A mixture of 30% HBr/AcOH (7.1 ml) was added dropwise to a solution of compound 2 (19.4 g, 62.6 mmol) in EtOH (80 ml). The reaction was continued under reflux for 4 h, and ice water (50 ml) was added to quench the reaction. The aqueous phase was extracted with ether (200 ml×3), and the combined organic layer was washed with water and brine. After drying over Na_2SO_4, the solvent was evaporated to afford compound 3 as a pale yellow oil (14.5 g, 87%).

A piece of metal sodium (0.27 g) was dissolved in anhydrous EtOH (15 ml) in a flask cooled in an ice bath. A solution of compound 3 (2.67 g, 9.95 mmol) in anhydrous EtOH (8 ml) was added. After stirring at RT for 2 h, AcOH (1 ml) was added. After concentrating, ice water (20 ml) was added. The aqueous phase was extracted with ether (200 ml×3), and the combined organic layer was washed with water and brine and dried over Na_2SO_4. After solvent removal, compound 4 was obtained as a pale yellow oil (1.5 g, 80%).

A solution of compound 4 (1.5 g, 7.9 mmol) in EtOH (10 ml) was added to a solution of KOH (460 mg, 7.9 mmol) in EtOH (3 ml) with stirring at 0°C. After continuous stirring at RT for 2 h, the solvent was removed by evaporation at RT, and the residue was diluted with ether (50 ml). The undissolved solid was filtered, and the solvent was concentrated to dryness to produce a solid. To acidify the solid, 5% $KHSO_4$ was then added, and the resulting mixture was extracted with EA (30 ml×3). The combined organic layer was washed with water and brine and dried over Na_2SO_4. After solvent removal, compound 5 was obtained as a pale yellow oil (1.0 g, 80%).

Anhydrous hydrazine (320 mg, 100 mmol) was added to a solution of compound 6 (3.08 g, 10 mmol) in MeOH (60 ml) at RT, and the resulting mixture was then stirred at RT for 16 h. Excess hydrazine and solvent were removed by evaporation. The resulting residue was washed with ethanol and ether to afford compound 7 as a white solid (2.1 g, 68% yield).

2-Bromoacetamide (1.16 g, 8.44 mmol) was added dropwise to a stirring solution of compound 7 (2 g, 6.49 mmol) and NMM (0.85 g, 8.44 mmol) in DMF cooled at −10°C. The resulting solution was stirred for 30 min at −10°C, and the mixture was allowed to react at RT for 36 h. The DMF was evaporated, and the residue was purified on a silica gel column using 1:9 MeOH/CH_2Cl_2 as the eluent to afford compound 8 as a yellow solid (2.0 g, 85%).

Compound 8 (500 mg, 1.37 mmol) was added to a mixture of compound 5 (329 mg, 2.05 mmol), ECI (520 mg, 2.74 mmol), and HOBt (373 mg, 2.74 mmol) in DMF (30 ml). The mixture was stirred at RT overnight. The mixture was diluted with EA (100 ml), washed with H_2O (100 ml×4) and brine (100 ml), and dried over Na_2SO_4.

The solvent was removed, and the residue was purified by preparative HPLC to afford compound 9 as a white solid (249 mg, 36%). ^{1}H NMR (500 MHz DMSO) δ 10.76 (^{1}H, br), 8.19 (^{1}H, br), 7.54 (^{1}H, s), 7.33 (^{6}H, m), 5.01 (^{2}H, q, J = 12.5 Hz), 4.16 (^{6}H, m), 3.60 (^{2}H, m), 2.00 (^{9}H, m); ^{13}C NMR (125 MHz DMSO) δ 172.5, 172.4, 168.4, 166.7, 155.6, 137.0, 128.3, 127.8 (×2), 72.4, 65.4, 61.5, 51.5, 51.1, 49.7, 17.9, 17.0, 13.8, MS (ESI, m/e): 508.1 (M+1)+; HPLC purity (detected at 214 and 254 nm): 100%.

Western Blot Analysis

Extraction of proteins from cells using a modified buffer was followed by immunoprecipitation and immunoblotting with appropriate antibodies, as described previously[8].

ELISA

AEP concentrations in conditioned medium or serum were measured using an ELISA, as previously described[8].

In Vivo Treatments and Analysis of Tumor Metastasis Formation

For experimental metastasis, cells were injected into tail veins, and mice were randomized and treated with saline, purified AEP (4 μg/per mouse/per time), a mouse anti-human AEP antibody (2 μg/per mouse/per time; MAB2199; R&D), or AEPI twice a week for 10 weeks. At the end point, all mice were euthanized with CO_2, and the lungs were removed and fixed in Bouin's solution. Lung metastases in the five lobes of the lung were counted using an anatomy microscope, with all micrometastases being larger than 0.5 mm (diameter). Every group included six to eight mice, with three repetitions.

Statistics

Differences in the level of AEP protein in sera from breast cancer patients and healthy volunteers were analyzed by the Mann–Whitney *U*-test. The two-tailed Student's *t*-test was used to analyze differences between groups with protein overexpression or knockdown. Before applying the two-tailed paired or unpaired Student's *t*-test, one-way analysis of variance was initially performed to determine the existence of an overall statistically significant change. A multiple test-adjusted value of $p < 0.05$ was considered statistically significant.

RESULTS

AEP Is Upregulated in Mammary Tumors in Transgenic PyVmT Mice

Although AEP has been implicated in the biology of cancer development, the expression and functions of AEP have not been systematically studied in tumor progression. We examined AEP expression in primary tumors and lung metastases in PyVmT mice. Consistently, AEP was highly expressed in both the primary tumor and lung metastasis (Fig. 1a and b). Moreover, the level of circulating AEP gradually increased during tumor progression (Fig. 1c).

Aza-Asn Epoxides Effectively Inhibit the Activity of AEP

As a new class of irreversible cysteine protease inhibitors, Aza-Asn epoxides are specific and selective for AEP proteins. To evaluate the optimal concentration of reaction, AEP protein was reacted with inhibitors (AEPI) at 0, 0.1, 0.2, and 1 μM. As shown in Figure 2b, the inhibitory efficiency of AEPIs was positively correlated with the concentration, with a maximal inhibitory efficiency at 1 μM.

AEP Protein Injected Through the Tail Vein Promotes Mammary Tumor Development in Transgenic PyVmT Mice

Because we found that AEP is a secreted protein, we injected purified AEP protein and AEPIs through the tail vein to enhance or block AEP function. The mice injected with the purified AEP protein had more and larger mammary tumors than the control group treated with saline. Conversely, the number and size of breast tumors decreased when AEPIs were injected twice a week for 10 weeks (Fig. 3a–d). The tumors from the AEP-treated mice were the heaviest; by contrast, the control mice had a medium tumor weight, whereas the AEPI-treated mice had the lowest tumor weight (Fig. 3e).

AEP Inhibitors Restrict Mammary Tumor Metastasis in Transgenic PyVmT Mice

Because metastasis to the lung is the major route for breast cancer, we examined the function of AEP in mammary tumor metastasis. Tumors that metastasized to the lung in the three groups are shown in Figure 4a–c. The average incidence of metastasis was notably higher in the AEP group compared with the control group, whereas the AEPI group showed fewer lung metastases compared with the control group. The AEP group exhibited extensive metastases, whereas the AEPI-treated group had very few metastases. We also used immunohistochemistry to analyze AEP expression in the three groups (Fig. 4d). The AEP group exhibited the highest AEP expression in lung metastasis tissue, whereas the AEPI-treated group exhibited low AEP expression. These results strongly indicated that AEP promotes tumor progression.

AEP Affects Survival in Tumor-Burdened Mice

Kaplan–Meier analyses were used to assess whether expression of AEP has a substantial influence on the cumulative survival rate. Among the three groups, we found AEP to be negatively associated with the

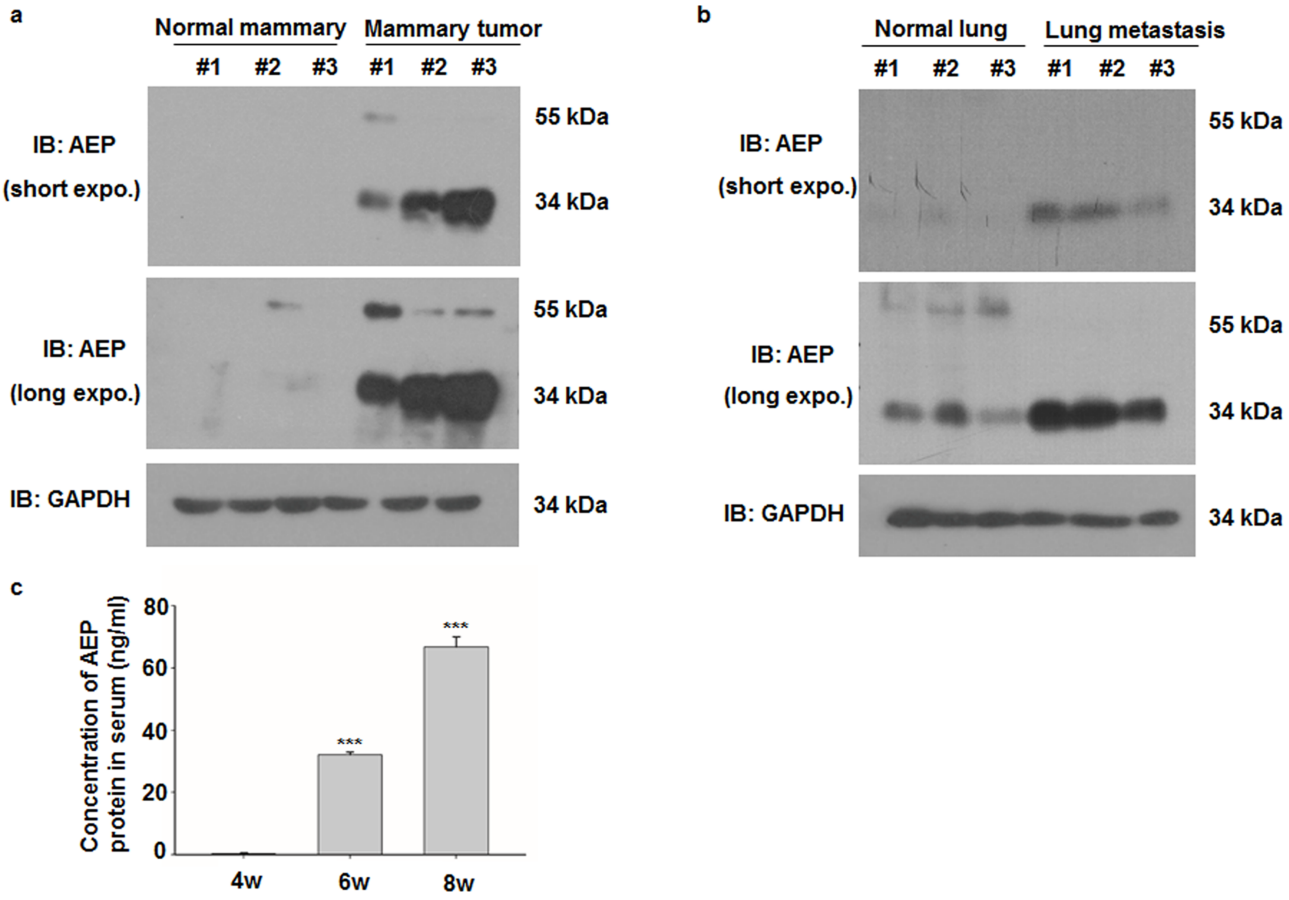


Figure 1. AEP is highly expressed in primary mammary tissue and lung metastasis tissue. (a) Western blot analysis was performed using three paired normal mammary and tumor tissue specimens. The 55-kDa band is pro-AEP, and the 34-kDa band is active AEP; GAPDH was used as an internal control for equal loading. (b) AEP expression in normal lung tissue and lung metastasis tissue was detected by Western blotting. (c) Concentration of circulating AEP was assessed by ELISA assay over different time periods.

cumulative survival rate (Fig. 4e): the AEP-treated group had a shorter survival time compared with the AEPI-treated mice.

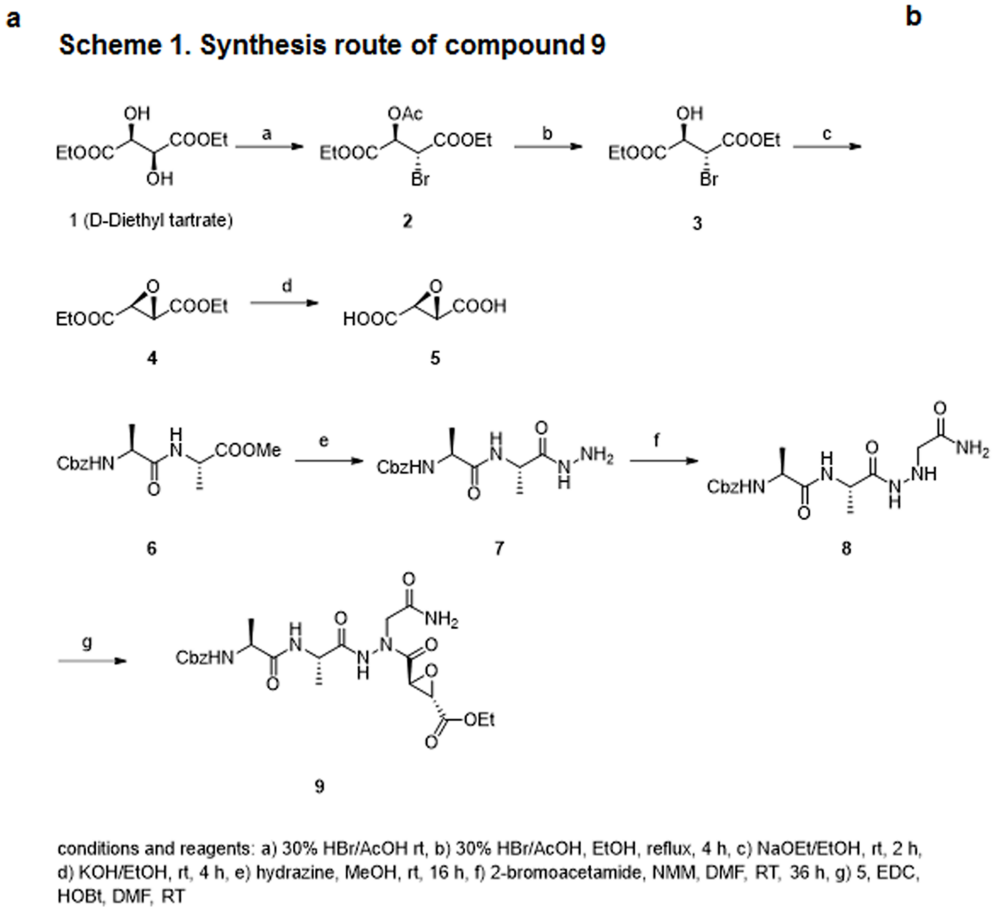
AEP Regulates PI3K/AKT Pathway Activation

The PI3K/AKT pathway plays a critical role in multiple biological processes, including proliferation, migration, and invasion, and previous studies have found that AKT activation is regulated by AEP in gastric cancer cells. To explore whether the PI3K/AKT pathway is influenced by AEP in mammary cancer, Western blotting was used to analyze the relative proteins of this pathway in mammary cancer and metastasis tissues. As shown in Figure 5a, the level of PI3K and AKT phosphorylation increased significantly in the AEP-treated mammary cancer tissue compared with the control group, even though the expression of total PI3K and AKT protein did not change. Additionally, the levels of these phosphorylated proteins decreased when the mice were treated with AEPIs. Similar results were found in the metastatic tissue (Fig. 5b), revealing that AEP may promote tumor progression via the PI3K/AKT pathway.

DISCUSSION

Previous studies have revealed that overexpression of AEP in human breast neoplasms correlates with a poor prognosis. Serum AEP also promotes tumor invasion and metastasis in an athymic mouse model, but the opposite effect occurs with AEP inhibition[8,15]. To mimic the clinical situation in a mouse breast cancer model to further analyze the function of circulating AEP in mammary cancer development, we generated a spontaneous breast cancer model in MMTV-PyVmT mice. We found that AEP was highly expressed in both primary tumors and lung metastases and that the level of serum AEP increased during tumor progression. Furthermore, an increased level of serum AEP facilitated migration and metastasis, whereas such activity was weakened when AEP was inhibited.

As a peptidase with strict specificity for asparagine bonds, AEP plays a crucial role in the processing and



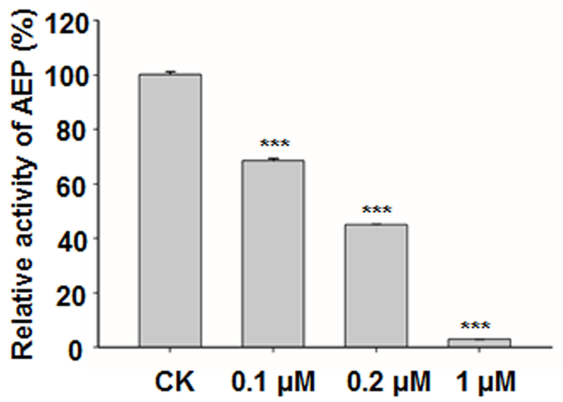


Figure 2. Synthesis of Aza-Asn epoxides and activity testing. (a) Synthetic steps are shown. (b) Activated AEP was reacted with AEPIs at 0, 0.1, 0.2, and 1 μM, and the rate of inhibition was measured.

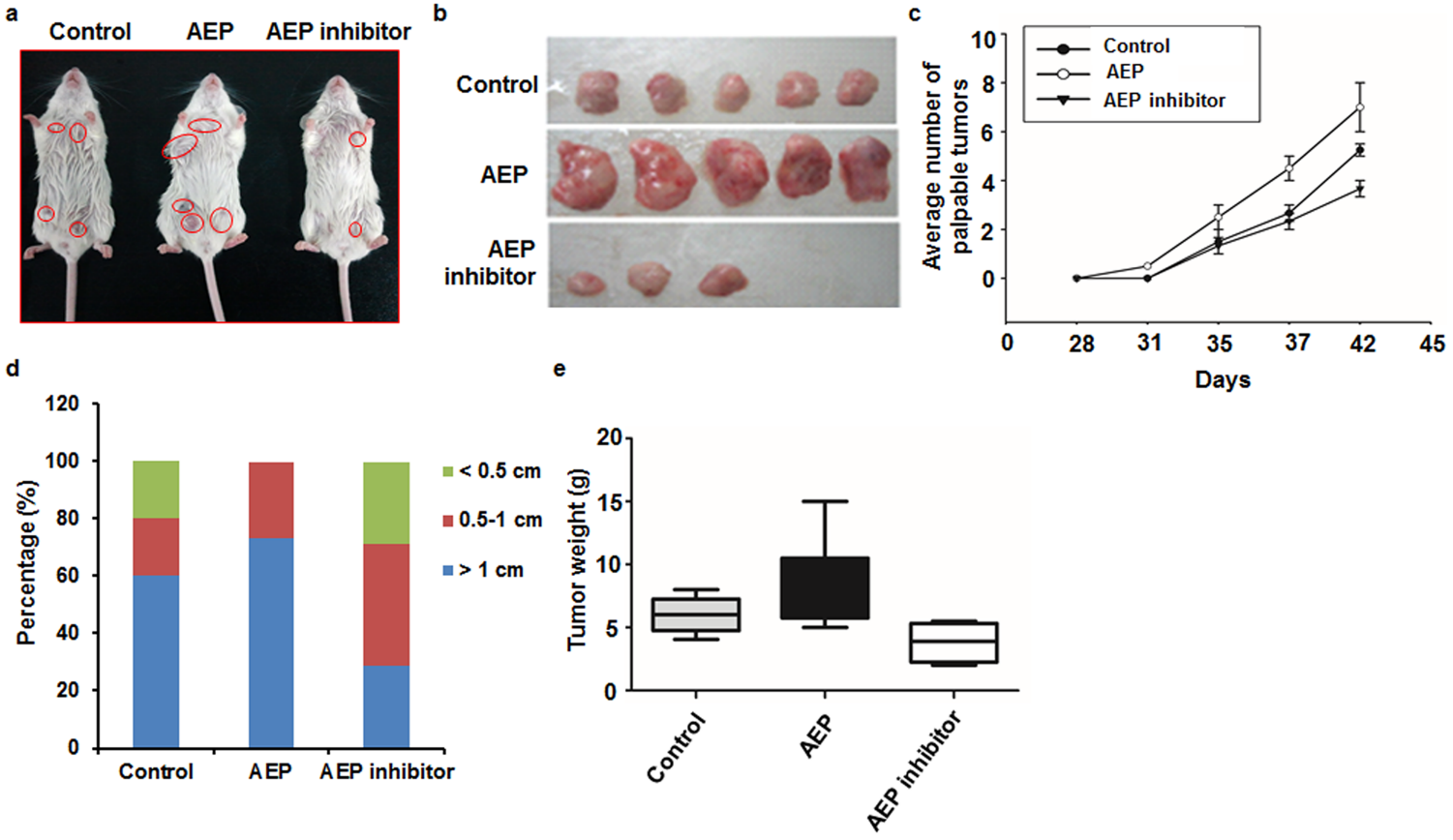


Figure 3. Functional role of AEP in tumor progression. (a) Spontaneous mammary tumors from the control group, AEP-treated group, and AEP inhibitor group were imaged. (b) Mammary tumor tissues of the three groups were imaged. (c) The average number of palpable tumors in the three groups was counted. (d) The volume of the tumors was divided into three different sizes (greater than 0.5 cm, between 0.5 and 1 cm, less than 0.5 cm in diameter) and analyzed. (e) The weight of the tumors in the three groups was analyzed.

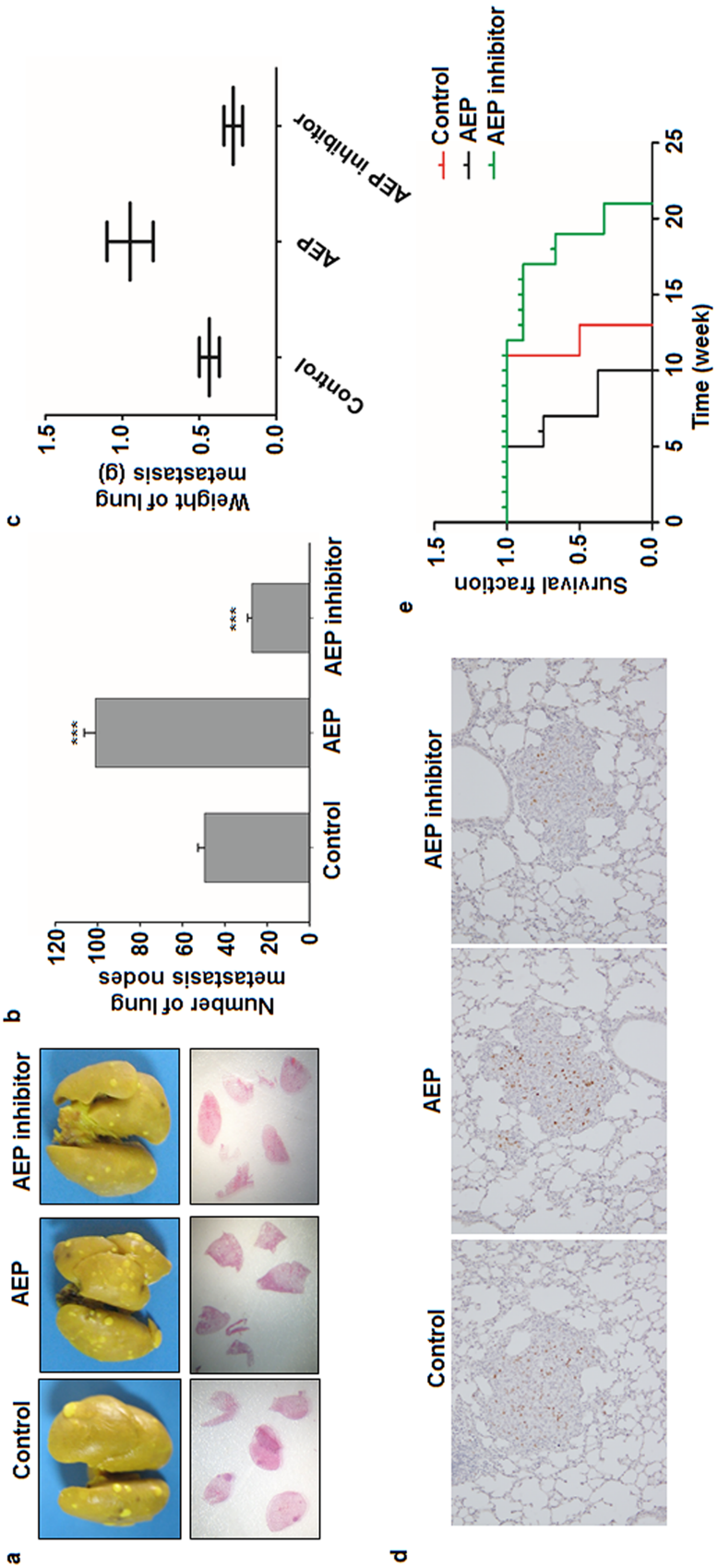


Figure 4. AEP is associated with metastasis in mice mammary cancer. (a) Lung metastasis derived from mammary cancer was imaged (top); mice were randomized and treated with saline, purified AEP protein, or AEP inhibitor twice a week for 10 weeks. Hematoxylin and eosin staining analysis and photos of lung metastases are shown (bottom). (b) The number of lung metastasis nodes was counted using an anatomy microscope. (c) The weight of the lung metastases in the three groups was analyzed. Values are mean±standard deviation. (d) Immunohistochemical staining for AEP in lung metastasis. The control group has medium AEP staining, the purified AEP protein-treated group has strong AEP staining, and the AEPI-treated group has weak AEP staining. (e) Kaplan–Meier curves were used to analyze the association between AEP and the cumulative survival rate.

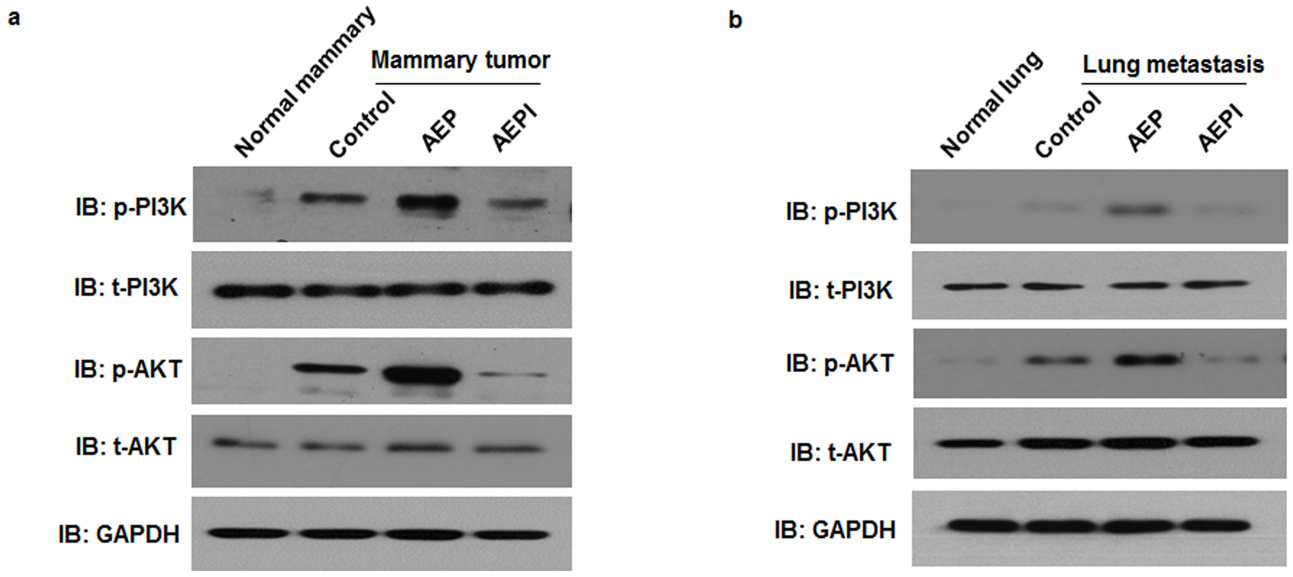


Figure 5. AEP regulates AKT/PI3K pathway activation. (a) The expression of phosphor-PI3K, total PI3K, phospho-AKT, and total AKT in the AKT signaling pathway in normal mammary tissue and mammary tissue (grouped by control, AEP treated, AEPI treated) was analyzed by Western blotting. (b) The above proteins in normal lung and metastasis tissues were also analyzed. These results showed that AEP may participate in PI3K/AKT pathway activation.

presentation of antigens, promoting angiogenesis factor release, activating matrix metalloproteinases, modulating fibronectin, participating in tumor-associated macrophage functions, and regulating the epithelial-to-mesenchymal transition (EMT)[6,8,12,16–19]. Thus, AEP is a risk factor closely associated with cancer prognosis. AEP is rarely expressed in normal mammary tissue but is highly expressed in mammary cancer tissue. In this study, tumor progression was effectively suppressed when MMTV-PyVmT mice were treated with an AEPI, indicating that AEP-targeted therapy might be an effective treatment for breast cancer in the clinic.

AKT is a serine/threonine kinase, and the PI3K/AKT pathway is involved in regulating cell growth, adhesion, and migration in response to bioactive substances[20,21]. AKT activation or overexpression can be considered to be a biomarker for predicting hematogenous metastasis of breast cancer in humans[22]. In a previous study, Cui et al.[6] found that knockdown of AEP markedly inhibited the activation via phosphorylation of proteins in the AKT signaling pathway, resulting in reduced EMT in gastric cancer. EMT allows epithelial cells to acquire a mesenchymal-like phenotype, which usually correlates with tumor migration and metastasis[23–25]. Emerging evidence shows that the PI3K/AKT pathway modulates the expression of E-cadherin, which is consistently involved in EMT in breast cancer[26]. Our in vivo study revealed that inhibition of AEP reduced AKT activation in both primary and metastatic sites. However, we were not able to confirm a direct interaction in vitro between PI3K/AKT and AEP in mammary cancer, and we are currently assessing whether AEP regulates the process of EMT via the PI3K/AKT pathway.

In conclusion, we are the first to show that AEP is overexpressed in both primary tumor tissue and in lung metastases tissue. High expression of AEP was found to be closely related to mammary cancer progression and metastasis, and these processes were reversed by AEP inhibitors. Further analysis of the molecular mechanism revealed that AEP is important for PI3K/AKT pathway activation.

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