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

Cheng Xu,*1 Lu Cao,*1 Jianhua Liu,†1 Zhongrun Qian,‡ Yu Peng,§ Wenjing Zhu,§ Yongming Qiu,‡ and Yingying Lin‡¶

*Department of Radiation Oncology, Ruijin Hospital, Shanghai, P.R. China
†Institute of Medical Science, Shanghai Jiao Tong University School of Medicine, Shanghai, P.R. China
‡Department of Neurosurgery, Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, P.R. China
§Department of Urology, Yueyang Hospital of Integrated Traditional Chinese and Western Medicine, Shanghai University of Traditional Chinese Medicine, Shanghai, P.R. China
¶Shanghai Institute of Head Trauma, Shanghai, P.R. China

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.4,6

1These authors provided equal contribution to this work.

Address correspondence to Yingying Lin, Ph.D., Department of Neurosurgery, Ren Ji Hospital, Shanghai Jiao Tong University School of Medicine, Room 118, Building 11, 1630 Dongfang Road, Pudong District, Shanghai 200127, P.R. China. Tel: (86) 15800727180; Fax: (86) 021-58394262; E-mail: yylin@sibs.ac.cn or Yongming Qiu, M.D., Ph.D., Department of Neurosurgery, Ren Ji Hospital, Shanghai Jiao Tong University School of Medicine, Room 118, Building 11, 1630 Dongfang Road, Pudong District, Shanghai 200127, P.R. China. Tel: (86) 18221750005; Fax: (86) 021-58394262; E-mail: quzhoub@hotmail.com
Moreover, AEP expression is positively related to clinicopathological and biological variables in breast cancer and colorectal cancer\(^5\). AEP has also been found to influence hepatocellular growth and inhibit the cell cycle at the G1/S phase\(^10\) and to activate the zymogene MMP2 and cathepsins\(^1\) in a previous study, we demonstrated that TRAF6 ubiquitinates AEP, promoting its stability and secretion, steps that are vital for breast cancer progression\(^6\). 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\(^4\). 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 400-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 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\(_2\)SO\(_4\), the solvent was evaporated to afford compound 3 as a pale yellow oil (14.5 g, 87%).

A solution of compound 4 (1.5 g, 7.9 mmol) in EtOH (15 ml) was added to a solution of KOH (460 mg, 8.44 mmol) in EtOH (3 ml) with stirring at 0°C. After continuous 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\(_2\)SO\(_4\). After solvent removal, compound 4 was obtained as a pale yellow oil (1.5 g, 80%).

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\(_2\)SO\(_4\), 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\(_2\)SO\(_4\). 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 unidissolved 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\(_2\)SO\(_4\). 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\(_2\)Cl\(_2\) 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\(_2\)O (100 ml × 4) and brine (100 ml), and dried over Na\(_2\)SO\(_4\).
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) $\delta$ 10.76 (1H, br), 8.19 (1H, br), 7.54 (1H, s), 7.33 (6H, m), 5.01 (2H, q, J = 12.5 Hz), 4.16 (6H, m), 3.60 (2H, m), 2.00 (9H, m); $^{13}$C NMR (125 MHz DMSO) $\delta$ 172.5, 172.4, 168.4, 166.7, 155.6, 137.0, 128.3, 127.8 (x2), 72.4, 65.4, 61.5, 51.5, 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.

**ELISA**

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

**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 micro-metastases 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
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. 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 AEP inhibitor-treated group has weak AEP staining. (e) Kaplan–Meier curves were used to analyze the association between AEP and the cumulative survival rate.
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.\(^5\) 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 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.

ACKNOWLEDGMENTS: This research was funded by the National Natural Science Foundation of China (No. 81402042), Shanghai Science and Technology (Nos. 14140903400 and 14YF1402600), State Key Laboratory of Oncogenes and Related Genes (No. 90-14-01), Shanghai Municipal Population and Family Planning Commission (No. 2013SY024), Key Specialty Construction Project and Science Technology Development Project of Pudong Health and Family Commission of Shanghai (Nos. PWZ-2013-18 and PW2013A-19), and Training Plan for Scientific Research of Ren Ji Hospital (No. RJZZ13-021). The authors declare no conflicts of interest.

REFERENCES
1. Menezes ME, Das SK, Emdad L, Windle JJ, Wang XY, Sarkar D, Fisher PB. Genetically engineered mice as experimental tools to dissect the critical events in breast cancer. Adv Cancer Res. 2014;121:331–82.
2. Hutchinson JN, Muller WJ. Transgenic mouse models of human breast cancer. Oncogene 2000;19:6130–7.
3. Guy CT, Cardiff RD, Muller WJ. Induction of mammary tumors by expression of polyomavirus middle T oncogene.
A transgenic mouse model for metastatic disease. Mol Cell Biol. 1992;12:954–61.

4. Chen JM, Dando PM, Rawlings ND, Brown MA, Young NE, Stevens RA, Hewitt E, Watts C, Barrett AJ. Cloning, isolation, and characterization of mammalian legumain, an asparaginyl endopeptidase. J Biol Chem. 1997;272:8090–8.

5. Murthy RV, Arbman G, Gao JF, Roodman GD, Sun XF. Legumain expression in relation to clinicopathologic and biological variables in colorectal cancer. Clin Cancer Res. 2005;11:2293–9.

6. Cui Y, Wang Y, Li H, Li Q, Yu Y, Xu X, Xu B, Liu T. Asparaginyl endopeptidase promotes the invasion and metastasis of gastric cancer through modulating epithelial-to-mesenchymal transition and analysis of their phosphorylation-signaling pathways. Oncotarget 2016;7:16227–47.

7. Gawenda J, Traub F, Lück HJ, Kreipe H, von Wasielewski R. Legumain expression as a prognostic factor in breast cancer patients. Breast Cancer Res Treat. 2007;102:1–6.

8. Lin Y, Qiu Y, Xu C, Liu Q, Peng B, Kaufmann GF, Chen X, Lan B, Wei C, Lu D, Zhang Y, Guo Y, Lu Z, Jiang B, Edgington TS, Guo F. Functional role of asparaginyl endopeptidase ubiquitination by TRAF6 in tumor invasion and metastasis. J Natl Cancer Inst. 2014;106:dju012:1–12.

9. Patel N, Krishnan S, Ofman MN, Krol M, Moss CX, Leighton C, van Delft FW, Holland M, Liu J, Alexander S, Dempsey C, Ariffin H, Essink M, Eden TOB, Watts C, Bates AP, Saha V. A dyad of lymphoblastic lysosomal cysteine proteases degrades the antileukemic drug L-asparaginase. J Clin Invest. 2009;119:1964–73.

10. Andrade V, Guerra M, Jardim C, Melo F, Silva W, Ortega JM, Robert M, Nathanson MH, Leite F. Nucleoplasmic calcium regulates proliferation of hepatocytes through legumain. J Hepatol. 2011;55:626–35.

11. Liu C, Sun C, Huang H, Janda K, Edgington T. Over-expression of legumain in tumors is significant for invasion/metastasis and a candidate enzymatic target for prodrug therapy. Cancer Res. 2003;63:2957–64.

12. Chen JM, Fortunato M, Stevens RA, Barrett AJ. Activation of progelatinase A by mammalian legumain, a recently discovered cysteine protease. Biol. Chem. 382:2001;777–83.

13. Briggs JJ, Haugen MH, Johansen HT, Riker AL, Abrahamson M, Fodstad Ø, Maelandsmo GM, Solberg R. Cystatin E/M suppresses legumain activity and invasion of human melanoma. BMC Cancer 2010;10:1–13.

14. Götz MG, James KE, Hansell E, Dворак J, Seshadri A, Sojka D, Kopáček P, McKerrow JH, Caffrey CR, Powers JC. Aza-peptidyl Michael acceptors. A new class of potent and selective inhibitors of asparaginyl endopeptidases (legumains) from evolutionarily diverse pathogens. J Med Chem. 2008;51:2816–32.

15. Wu M, Shao GR, Zhang FX, Wu WX, Xu P, Ruan ZM. Legumain protein as a potential predictive biomarker for Asian patients with breast carcinoma. Asian Pac J Cancer Prev. 2014;15:10773–7.

16. Sepulveda FE, Maschhalidi S, Colisson R, Heslop L, Ghirelli C, Sakkas E, Lennon-Duménil AM, Amigorena S, Cabanie L, Manoury, B. Critical role for asparaginyl endopeptidase in endocytic Toll-like receptor signaling in dendritic cells. Immunity 2009;31:737–48.

17. Morita Y, Araki H, Sugimoto T, Takeuchi K, Yamane T, Maeda T, Yamamoto Y, Nishi K, Asano M, Shirahama-Noda K, Nishimura M, Uzu T, Hara-Nishimura I, Koya D, Kashiwagi A, Ohkubo I. Legumain/asparaginyl endopeptidase controls extracellular matrix remodeling through the degradation of fibronectin in mouse renal proximal tubular cells. FEBS Lett. 2007;581:1417–24.

18. Chen JM, Fortunato M, Stevens RA, Barrett AJ. Activation of progelatinase A by mammalian legumain, a recently discovered cysteine protease. Biol Chem. 2011;382:777–83.

19. Lin Y, Wei C, Liu Y, Qiu Y, Liu C, Guo F. Selective ablation of tumor-associated macrophages suppresses metastasis and angiogenesis. Cancer Sci. 2013;104:1217–25.

20. Coffey JC, Wang JH, Smith MJ, Laing A, Boucher-Hayes D, Cotter TG, Redmond HP. Phosphoinositide 3-kinase accelerates postoperative tumor growth by inhibiting apoptosis and enhancing resistance to chemotherapy-induced apoptosis. Novel role for an old enemy. J Biol Chem. 2005;280:20968–77.

21. Kermorgant S, Aparicio T, Dessirier V, Lewin MJ, Lehy T. Hepatocyte growth factor induces colon cancer cell invasiveness via enhanced motility and protease overproduction. Evidence for PI3 kinase and PKC involvement. Carcinogenesis 2001;22:1035–42.

22. Yang H, Guan L, Li S, Jiang Y, Xiong N, Li L, Wu C, Zeng H, Liu Y. Mechanosensitive caveolin-1 activation-induced PI3K/Akt/mTOR signaling pathway promotes breast cancer motility, invadopodia formation and metastasis in vivo. Oncotarget 2016;7:16227–47.

23. Kalluri R. EMT: When epithelial cells decide to become mesenchymal-like cells. J Clin Invest. 2009;119:1417–9.

24. Thiery JP, Acloque H, Huang BY, Nieto MA. Epithelial-mesenchymal transitions in development and disease. Cell 2009;139:871–90.

25. Zeisberg M, Neilson EG. Biomarkers for epithelial-mesenchymal transitions. J Clin Invest. 2009;119:1429–37.

26. Rafael D, Doktorovová S, Florindo HF, Gener P, Abasolo I, Schwartz SJ, Videira MA. EMT blockage strategies: Targeting Akt dependent mechanisms for breast cancer metastatic behaviour modulation. Curr Gene Ther. 2015;15:300–12.