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Suppression of asparaginyl endopeptidase attenuates breast cancer-induced bone pain through inhibition of neurotrophin receptors

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Abstract

Objective: Cancer-induced bone pain is a common clinical problem in breast cancer patients with bone metastasis. However, the mechanisms driving cancer-induced bone pain are poorly known. Recent studies show that a novel protease, asparaginyl endopeptidase (AEP) plays crucial roles in breast cancer metastasis and progression. We aim to determine the functions and targeted suppress of AEP in a mouse model of breast cancer-induced bone pain.

Methods: Breast cancer cells with AEP knocked-down or overexpression were constructed and implanted into the intramedullary space of the femur to induce pain-like behavior in mice. AEP-specific inhibitors or purified AEP proteins were further used in animal model. The histological characters of femur and pain ethological changes were measured. The expressions of AEP and neurotrophin receptors (p75NTR and TrkA) in dorsal root ganglion and spinal cord were examined.

Results: Femur radiographs and histological analysis revealed that cells with AEP knocked-down reduced bone destruction and pain behaviors. However, cells with AEP overexpression elevated bone damage and pain behaviors. Further, Western blot results found that the expressions of p75NTR and TrkA in dorsal root ganglions and spinal cords were reduced in mice inoculated with AEP knocked-down cells. Targeted suppression of AEP with specific small compounds significantly reduced the bone pain while purified recombinant AEP proteins increased bone pain.

Conclusions: AEP aggravate the development of breast cancer bone metastasis and bone pain by increasing the expression of neurotrophin receptors. AEP might be an effective target for treatment of breast cancerinduced bone pain.

Keywords

cancer-induced bone pain, breast cancer, asparaginyl endopeptidase, neurotrophin receptors

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Introduction

Bone is one of the most common sites of metastasis for malignancies arising from breast, prostate, and lung.[1–3] Patients with metastatic breast cancer survive on an average of 1.5 to 3 years, during which time they have a number of comorbidities, including intractable pain.[1,4–6] Cancer-induced bone pain (CIBP) is a growing health concern, as it is inadequately managed with current stand of care therapy.[7,8] Moreover, the development of dose-limiting side effects and combined with tumor progression limit analgesic efficacy in nearly 42% of cancer pain patients.[8,9] Thus, there is an urgent need to elucidate the multifaceted etiology of CIBP and identify innovative targets for CIBP treatment.

Asparaginyl endopeptidase (AEP), which is highly specific for asparaginyl bond, is currently the only known AEP encoded by the mammalian genome. AEP has been found to be highly expressed in a variety

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of solid tumors and in acute lymphoblastic leukemia; while only a limited quantity of AEP is detectable in normal tissue.[10–12] Moreover, AEP expression is positively associated with clinicopathological and biological variables in breast cancer and colorectal cancer.[13,14] AEP has also been found to influence hepatocellular cell growth and to activate the zymogene MMP2 and Cathepsins.[15,16] TRAF6 ubiquitinates and promotes AEP protein stability and secretion, which eventually promotes breast cancer progression.[17] Moreover, the non-enzymatic 17 kDa C-terminal fragment of AEP is also biologically active and inhibits osteoclast differentiation through binding to an uncharacterized receptor.[18,19] Further, by genetic and pharmacological manipulation, AEP inhibits osteoblast differentiation and in vivo bone formation through degradation of the bone matrix protein fibronectin indicating that AEP might contribute to the decreased bone mass in postmenopausal osteoporosis.[20] However, the pathological function of AEP in breast CIBP remains unknown.

Previous studies reported that nerve growth factor (NGF) and associated receptors, p75 neurotrophin receptor (p75NTR) and tropomyosin receptor kinase A (TrkA), are important mediators of pain sensitization.[21,22] These factors are overexpressed in overwhelming majority of human solid cancers.[23–25] Blockade of NGF or these receptors has been shown to reduce pain.[22,26–28] In this study, we found that AEP plays important function in breast CIBP through regulation of the expressions of receptors (p75NTR and TrkA) in dorsal root ganglion (DRG) and spinal cord. The expressions of these receptors were reduced in DRGs and spinal cords of mice inoculated with AEP knocked-down cells or treated with AEP selective inhibitors, indicating that AEP might be a novel target for breast CIBP treatment.

Materials and methods

Cell culture

Human breast cancer cell line MDA-MB-231 was 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.

Lentivirus-mediated AEP knockdown, rescue, or overexpression in MDA-MB-231 cells

Lentiviral vectors for human AEP-shRNA were constructed by Hanyin Co. (Shanghai, China). AEP shRNA sequence was 5′-GATGGTGTTCTAC ATTGAA-3′ (AEP-SH). The recombinant AEP knockdown and the negative control (NC) lentivirus (Hanyin Co., Shanghai, China) were prepared and tittered to 10^9 TU/ml (transfection unit). To obtain the stable AEP knocked-down cell line, MDA-MB-231 cells were cultured in six-well dish at a density of $2–3 \times 10^5$ cells per well. Cells were then infected with the identical titer virus with 8 μg/ml polybrene on the next day. About 72 h after viral infection, the culture medium were changed with fresh medium containing 4 μg/ml puromycin. These cells were further cultured for about 14 days. The puromycin-resistant cells were amplified in medium containing 2 μg/ml puromycin for about nine days, and further cultured in a medium without puromycin. The cells were named as AEP-SH or NC cells.

For rescue or overexpression of AEP, lentivirus-containing AEP expression vectors with synonymous mutation at targeted knock-down sequences were constructed by Hanyin Co. (Shanghai, China). MDA-MB-231, AEP-SH, or NC cells were infected by the lentivirus. The clones were designated as AEP-SH, RES, or OE cells. The knock-down or overexpression efficiency was confirmed via quantitative real-time polymerase chain reaction and Western blot.

Western blot analysis

Extraction of proteins from cells using a modified buffer was followed by immunoblotting with appropriate antibodies, as described previously.[17] The antibodies used were as follows: Goat anti-AEP (AF2199, R&D systems, UK), anti-TrkA (ab109010, Abcam, USA), and anti-p75 (ab8874, Abcam, USA).

Establishment of breast CIBP mouse model

All procedures were approved by China Medical University Animal Care and Use Committee and conformed to the Guidelines by the National Institutes of Health and the International Association for the Study of Pain. Adult female Balb/c nude mice (15–18 g) were maintained in a climate-controlled room on a 12-h light–dark cycle and allowed food and water ad libitum. Cells were implanted into the femur intramedullary space as described.[25] The condyles of the right distal femurs were exposed and a hole was drilled to create a space for injection of 5×10^5 cells in 5 μL sterile phosphate-buffered saline into the intramedullary space.

A digital Faxitron machine was used to acquire live radiographs of mice anesthetized with ketamine/xylazine on day 49. Animals were assessed for spontaneous pain presurgery and on postsurgery at day 49 weekly after treatment including flinching, paw withdrawal latency, and paw withdrawal threshold as previously described.[28] All testing was performed by a blinded observer during the animals' light cycle.

Observation of spontaneous foot-constriction frequency. The mice were placed in transparent plexiglass boxes where they could walk freely. The spontaneous foot-constriction frequency of left hind paw within 5 min was then observed.

Determination of paw withdrawal thermal latency. The thermalgia threshold was determined by using the BME-410A Thermalgia Instrument (Institute of Bioengineering, Chinese Academy of Medical Sciences). The mice were placed in a transparent organic glass cage for observation, and when they kept quiet for 30 min, the thermal radiation source was focused on the middle bottom of left toe, the latency from the beginning of irradiation to till the rat lifted its foot or hind away was set as the thermalgia threshold; the measurement was repeated three times, with the interval of 10 min, and the mean value was then used as the final value. To prevent the burns, the maximal irradiation time was 20 s each time.

Determination of paw withdrawal mechanical threshold. The mice was placed in a quiet environment, and the von Frey wire (Stoelting) was used for the determination of paw withdrawal mechanical threshold (PWMT) by the ''up and down'' method as reported by Chaplan et al.[29] The mice were placed on a metal net and covered with one transparent plexiglass box. They were let to adapt to the environment for 30 min, and then a series of standard von Frey wires was used to stimulate the middle skin of rat's left toe in a certain order, until the wire slightly bent into S-shape; this stimulus was continued for 6–8 s, and it was observed whether the foot-constriction reaction occurred. If the quick foot-constriction reaction of the rat immediately appeared in the stimulation time or at the time when the von Frey wire was removed, this phenomenon was recorded as positive reaction. However, the body movement-caused foot constriction was not recorded as a positive reaction, and the test was performed for a total of 10 times, with the stimulus interval of 10 min.

Bone histology

Animal femurs were inoculated with breast cancer cells. After behavioral testing on postsurgery day 49, animals were anesthetized (ketamine 80 mg/kg:xylazine 12 mg/kg, i.p.) and perfused transcardially with 0.1 M phosphate-buffered saline followed by 4% neutral-buffered formalin and 12.5% picric acid (Sigma). Femurs were collected, postfixed overnight at 4°C, and decalcified in 10% ethylenediaminetetraacetic acid (RDO-Apex, Aurora, IL) for 14 days, and then paraffin embedded. Femurs were cut in the frontal plane into 5-μm sections and stained with hematoxylin and eosin (H&E).

Immunohistochemistry

Tissue slides were routinely deparaffinizated and rehydrated. The antibodies against AEP, p75, or TrkA were used as a primary antibody. For antigen retrieval, the slides were heated at 98°C in a citrate buffer (pH 9.0) for a total of 20 min and cooled to room temperature. Sections were then incubated in 0.3% hydrogen peroxide for 20 min to eliminate endogenous peroxides. Sections were blocked with 5% normal horse serum in phosphate-buffered saline for 30 min and incubated with the primary antibody at 4°C overnight, then stained using a streptavidin-biotin-peroxidase detection system and counterstained with hematoxylin. A NC was introduced using pre-immune IgG instead of the primary antibody.

Synthesis of AEP-specific small compound inhibitors

The AEP-specific small compound inhibitors were synthesized as previously described.[30]

Recombinant AEP protein or AEP inhibitor treatment

Animals received recombinant AEP proteins or AEP inhibitors dissolved in vehicle solutions of 0.9% saline. Repeated dosing studies consisted of once-weekly recombinant AEP proteins (4 μg/100 μL saline, tail vein injection, week 2–5), AEP inhibitors (100 μg/100 μL saline, tail vein injection, week 2–5), or vehicle (100 μL saline, tail vein injection) after femoral inoculation.

Statistics

The two-tailed Student's t test was used to analyze differences between groups with protein overexpression or knock-down. Before applying the two-tailed paired or unpaired Student t test, one-way analysis of variance was initially performed to determine the existence of an overall statistically significant change. A multiple test-adjusted p-value of <0.05 was considered statistically significant.

Discussion

Breast cancer metastasis to bone is frequently accompanied by pain.[1] However, the etiology of breast CIBP is complex and far from known. It is suggested that bone destruction induced by imbalanced osteoclastogenesis in the tumor bone microenvironment is one of the reason for CIBP. Besides, breast cancer-induced pathological sprouting and reorganization of sensory nerve fibers provide insight into the mechanism.[4] In this study, we found that a novel protease-AEP played crucial functions in CIBP. Breast cancer cells with high AEP expression induced severe bone pain while reducing AEP expression in these cells alleviated bone pain, indicating that AEP might be an important factor in CIBP.

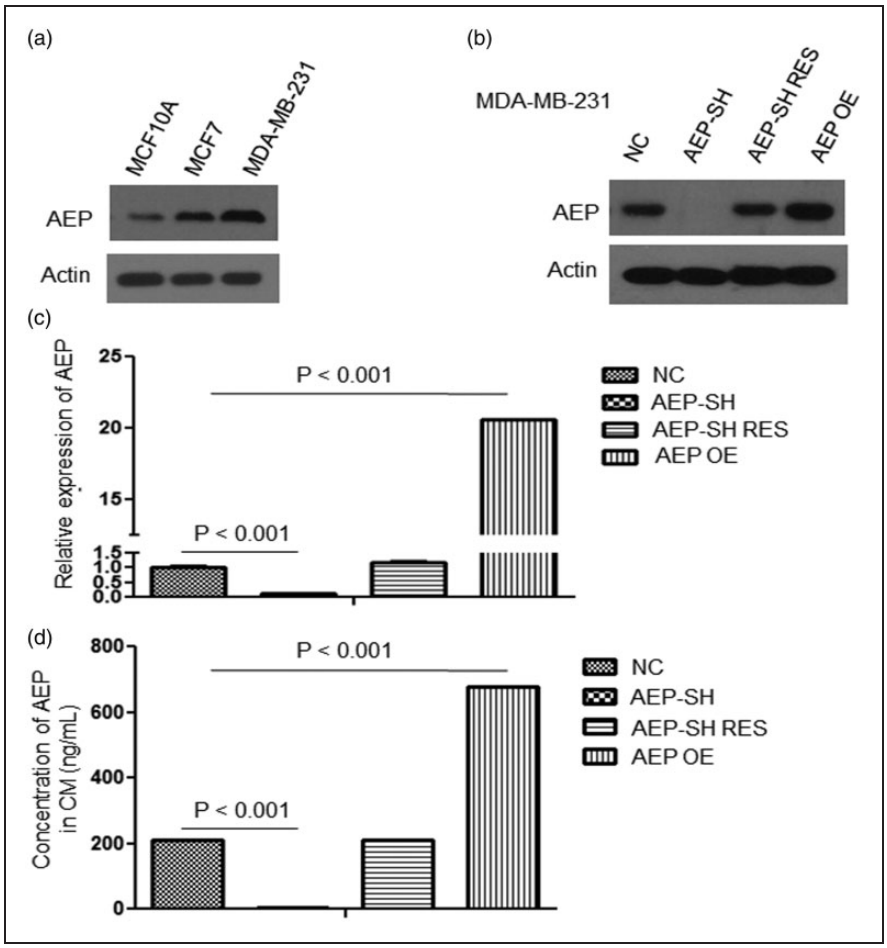

Figure 1. Knock-down or overexpression of AEP in MDA-MB-231 cells. (a) Western blot analysis of AEP expression in normal mammary epithelial cells (MCF10A) and breast cancer cells (MCF7 and MDA-MB-231). (b) Western blot analysis of AEP expression in MDA-MB-231 cells with AEP knock-down, rescue, or overexpression. (c) Real-time polymerase chain reaction analysis of AEP expression in MDA-MB-231 cells with AEP knock-down, rescue, or overexpression. (d) Enzyme-linked immunosorbent assay analysis of AEP concentration in conditioned medium (CM) of MDA-MB-231 cells with AEP knock-down, rescue, or overexpression.

Though the mechanisms of AEP in breast cancer metastasis have been investigated, the functions and underlying mechanisms of AEP in CIBP are unknown. Meanwhile, AEP has been found as an important mediator of neurodegenerative diseases through its enzymatic activity.[31–34] AEP acts as an innovative trigger for neurodegenerative diseases. Inhibition of AEP is thought to provide a disease-modifying treatment for neurodegenerative diseases including Alzheimer's disease.[28–30] In this study, we found for the first time that AEP expression was correlated with TrkA and p75NTR expression in DRG and spinal cord. How AEP influence the TrkA and p75NTR expression in DRG and spinal cord needs further investigation.

Blocking the TrkA receptor inhibits pain behavior in rat models of osteoarthritis.[23] Low-affinity p75 inhibitory antibody reduces pain behavior and CGRP expression in DRG in the mouse sciatic nerve crush model.[24] Preventive or late administration of anti-NGF therapy attenuates tumor-induced nerve sprouting, neuroma formation, and cancer pain. Besides, targeted suppression of AEP through small compound might be a new therapeutic strategy for CIBP. Potent and specific inhibitors of AEP (AEPIs) could be developed into new drugs for treating cancer and associated 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.[35] The efficiency of Aza-Asn epoxides in CIBP treatment might be promising.

Results

Induction of bone pain via AEP knock-down or overexpressing MDA-MB-231 cells

We analyzed the expression of AEP in normal breast epithelial cells (MCF10A), benign breast cancer cells

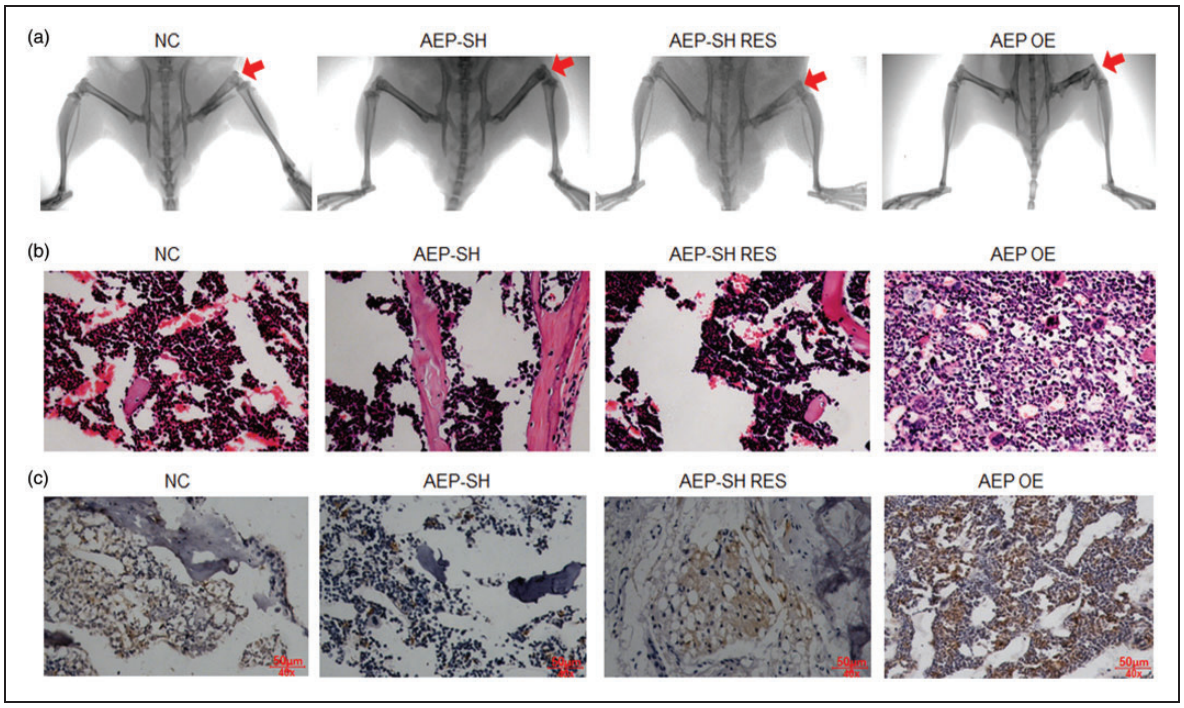

Figure 2. Breast cancer-induced bone pain model was established by MDA-MB-231 cells with different AEP levels. (a) Radiographs of mice inoculated with MDA-MB-231 cells with AEP knock-down, rescue, or overexpression (n = 8). (b) H&E analysis of femur of mice inoculated with MDA-MB-231 cells with AEP knock-down, rescue, or overexpression. (c) Immunohistochemical analysis of AEP expression in femur of mice inoculated with MDA-MB-231 cells with AEP knock-down, rescue, or overexpression.

(MCF7), and malignant breast cancer cells (MDA-MB-231). The Western blot results showed that AEP was highly expressed in MDA-MB-231 cells compared to normal or benign breast cancer cells (Figure 1(a)). We thus chose MDA-MB-231 cells for further study. The lentivirus-mediated rescue of AEP expression in knock-down cells and AEP overexpressing MDB-MB-231 cells were constructed. Both the Western blot and real-time polymerase chain reaction results verified the efficiency of AEP suppression, rescue, and overexpression (Figure 1(b) and (c)). Since AEP has been found to be secreted by breast cancer cells, we examined the levels of AEP in conditioned medium (CM) as well. The enzyme-linked immunosorbent assay results showed that the concentration of AEP was much lower in CM collected from AEP knock-down cells while significantly higher in CM derived from AEP overexpressing cells (Figure 1(d)). Altogether, MDA-MB-231 cells with different levels of AEP were efficiently constructed.

We further inoculated these cells into the femur of six-week-old nude mice. Radiography was taken after seven weeks. As shown in Figure 2(a), femur inoculated with MDA-MB-231 cells developed bone damage. However, femur inoculated with MDA-MB-231–AEP knock-down cells (AEP-SH) developed much less bone damage which were worse again in mice inoculated with rescued AEP expression cells (AEP-SH RES). On the contrary, femur inoculated with MDA-MB-231–AEP overexpressing cells (AEP-OE) developed much severer bone damage (Figure 2(a)). The H&E staining verified the breast cancer formed by inoculated cells (Figure 2(b)). The expression of AEP in these tumors was certificated by immunohistochemistry (Figure 2(c)). Thus, the breast cancer cells with different AEP levels-induced bone pain model was established.

Suppression of AEP alleviated CIBP while overexpression of AEP promoted CIBP

We monitored the bone pain every week. The paw flinches in mice inoculated with MDA-MB-231 cells–NC cells increased with time (Figure 3(a)). However, in mice inoculated with MDA-MB-231–AEP-SH cells reduced paw flinches. The paw flinches were worse in mice inoculated with MDA-MB-231–AEP-OE cells (Figure 3(a)). The mean paw withdrawal threshold at base line (day 0 post-inoculation) was comparable among groups (Figure 3(b)). A gradual decline in PWMT was observed in mice inoculated with MDA-MB-231–NC cells as well as MDA-MB-231–AEP RES cells but the decline was reversed in mice inoculated with AEP knock-down cells (Figure 3(b)). On the contrary, a significant decline in PWMT was observed in mice inoculated with MDA-MB-231–AEP-OE cells (Figure 3(b)),

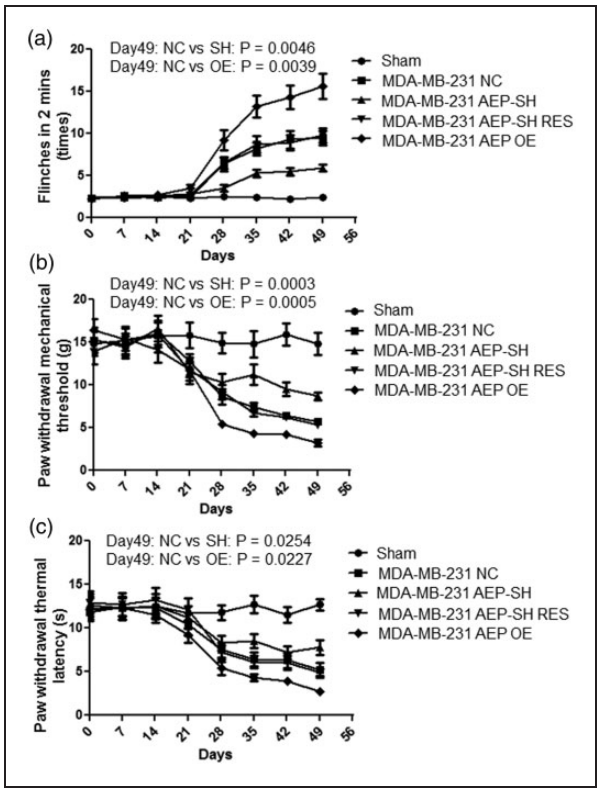

Figure 3. Suppression of AEP alleviated breast cancer cells-induced bone pain while overexpression of AEP suffered severe bone pain. (a) Flinches of paw in 2 min of mice injected with MDA-MB-231 cells with AEP knock-down, rescue, or overexpression ($n = 8$). (b) Paw withdrawal mechanical threshold of mice injected with MDA-MB-231 cells with AEP knock-down, rescue, or overexpression ($n = 8$). (c) Paw withdrawal thermal latency of mice injected with MDA-MB-231 cells with AEP knock-down, rescue, or overexpression ($n = 8$).

indicating that AEP was highly associated with breast CIBP. Consistently, the paw withdrawal thermal latency was declined in mice inoculated with MDA-MB-231–NC cells as well as MDA-MB-231–AEP RES cells, but recovered in mice inoculated with MDA-MB-231–AEP-SH group (Figure 3(c)). The paw withdrawal thermal latency was much declined in mice inoculated with MDA-MB-231–AEP-OE cells (Figure 3(c)). These results showed that down-regulation of AEP alleviated breast CIBP pain while up-regulating AEP severed the bone pain.

AEP increased the expression of p75NTR and TrkA in DRG and spinal cord

We further examined the expression of neurotrophin receptors (p75NTR and TrkA) in DRG and spinal cord. The Western blot results found that the expressions of p75NTR and TrkA were reduced in DRGs and spinal cords in mice inoculated with AEP knock-down cells (Figure 4(a)–(f)). However, the expressions of p75NTR and TrkA were elevated in DRGs and spinal cords in mice inoculated with AEP overexpressing cells (Figure 4(a)–(f)). Consistently, immunohistochemical analysis showed that the staining of p75NTR and TrkA was weaker in DRGs and spinal cords in mice inoculated with AEP knock-down cells while stronger in mice inoculated with AEP overexpressing cells (Figure 4(g) and (h)). These results suggest that the neurotrophin receptors (p75NTR and TrkA) in DRGs and spinal cords are involved in AEP-induced CIBP.

Recombinant AEP protein-induced severer CIBP with increased TrkA and P75NTR expression

Since AEP is a secreted protein, we injected mice with recombinant AEP proteins to mice inoculated with MDA-MB-231 cells. Radiography observed severer damage in mice treated with recombinant AEP proteins compared to control (Figure 5(a)). The paw flinches were increased in mice treated with recombinant AEP proteins (Figure 5(b)). Significant declines in PWMT were observed in mice treated with recombinant AEP proteins (Figure 5(c)). Consistently, the paw withdrawal thermal latencies were much declined in mice treated with recombinant AEP proteins (Figure 5(d)).

We examined the expressions of neurotrophin receptors (p75NTR and TrkA) in DRGs and spinal cords as well. The Western blot results found that the expressions of p75NTR and TrkA were elevated in DRGs and spinal cords in mice treated with recombinant AEP proteins (Figure 6(a)–(f)). The IHC results found that the staining of p75NTR and TrkA was stronger in DRGs and spinal cords in mice treated with recombinant AEP proteins (Figure 6(g) and (H)).

Selective inhibition of AEP-reduced CIBP with reduced TrkA and P75 expression

Meanwhile, we injected AEP-specific inhibitors to mice inoculated with MDA-MB-231 cells. Radiography observed much less bone damage in mice treated with AEP inhibitors compared to control (Figure 5(a)). The paw flinches were decreased in mice treated with AEP inhibitors (Figure 5(b)). Significant recoveries in PWMT were observed in mice treated with AEP inhibitors (Figure 5(c)). The paw withdrawal thermal latencies were also reversed in mice treated with AEP inhibitors (Figure 5(d)).

The Western blot results found that the expression of p75NTR and TrkA was suppressed in DRGs and spinal cords in mice treated with AEP inhibitors (Figure 6(a) and (b)). The IHC results found that the staining of p75NTR and TrkA was weak in DRGs and spinal

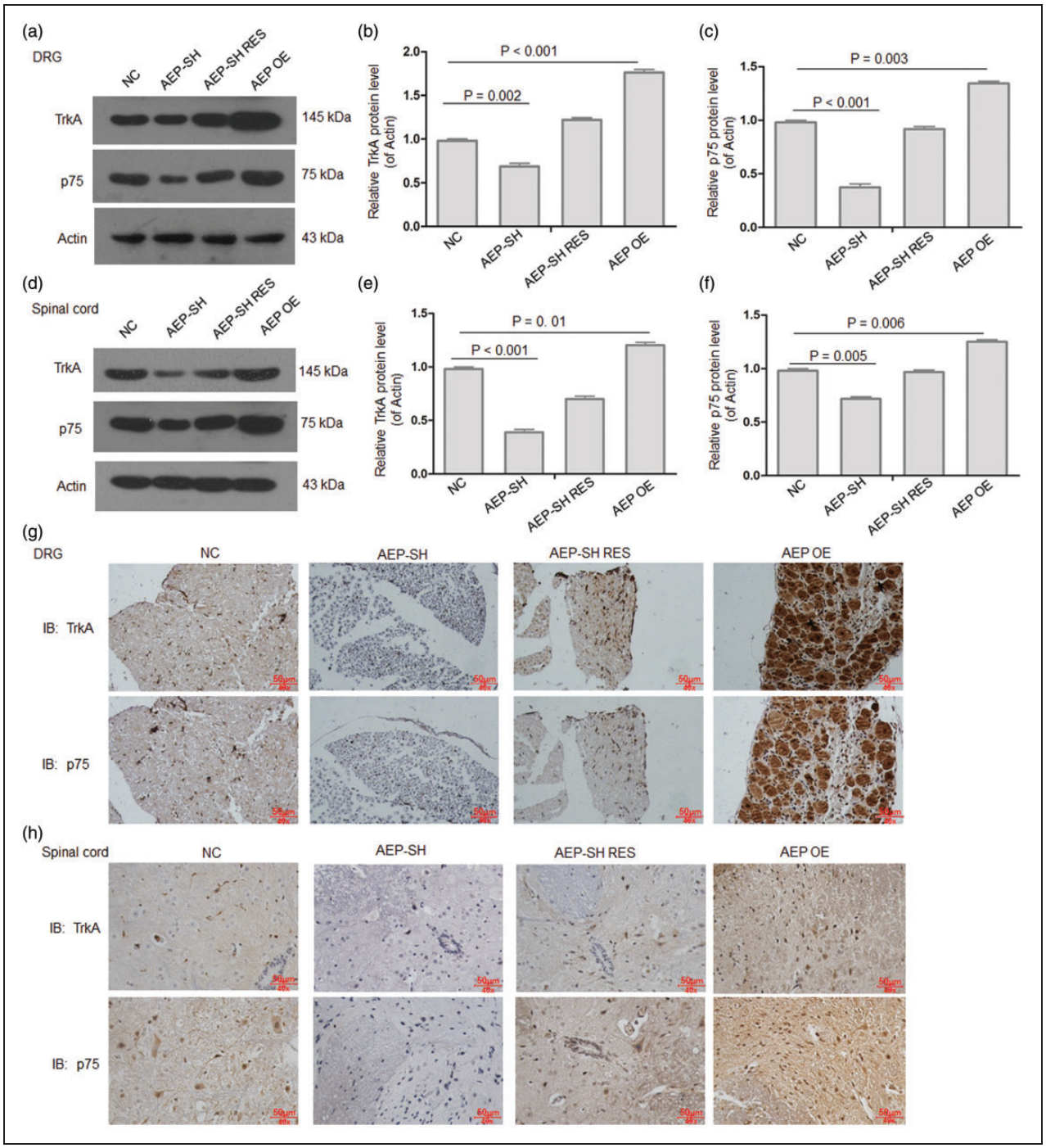

Figure 4. Expressions of P75 and TrkA were reduced in DRGs and spinal cords of mice injected with AEP knock-down cells while increased in mice injected with AEP overexpression cells. (a–c) Western blot analysis of P75 and TrkA expression in DRGs of mice injected with MDA-MB-231 cells with AEP knock-down, rescue, or overexpression. (d–f) Western blot analysis of P75 and TrkA expression in spinal cords of mice injected with MDA-MB-231 cells with AEP knock-down, rescue, or overexpression. (g) Immunohistochemical analysis of P75 and TrkA expression in DRGs of mice injected with MDA-MB-231 cells with AEP knock-down, rescue, or overexpression. (h) Immunohistochemical analysis of P75 and TrkA expression in spinal cords of mice injected with MDA-MB-231 cells with AEP knock-down, rescue, or overexpression.

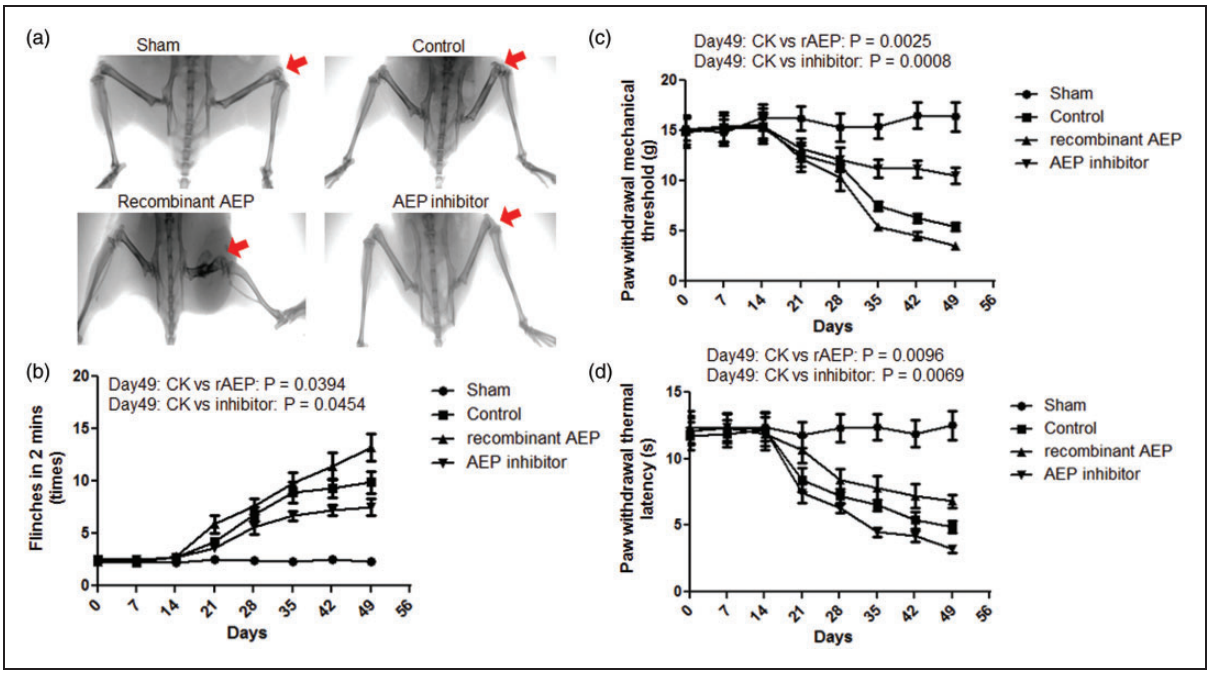

Figure 5. Selective inhibition of AEP reduced breast cancer cells induced bone pain while AEP protein injection increased bone pain. (a) Flinches of paw in 2 min of mice injected with MDA-MB-231 cells treated with AEP inhibitors, recombinant AEP proteins, or saline control (n = 8). (b) Paw withdrawal mechanical threshold of mice injected with MDA-MB-231 cells treated with AEP inhibitors, recombinant AEP proteins, or saline control (n = 8). (c) Paw withdrawal thermal latency of mice injected with MDA-MB-231 cells treated with AEP inhibitors, recombinant AEP proteins, or saline control (n = 8).

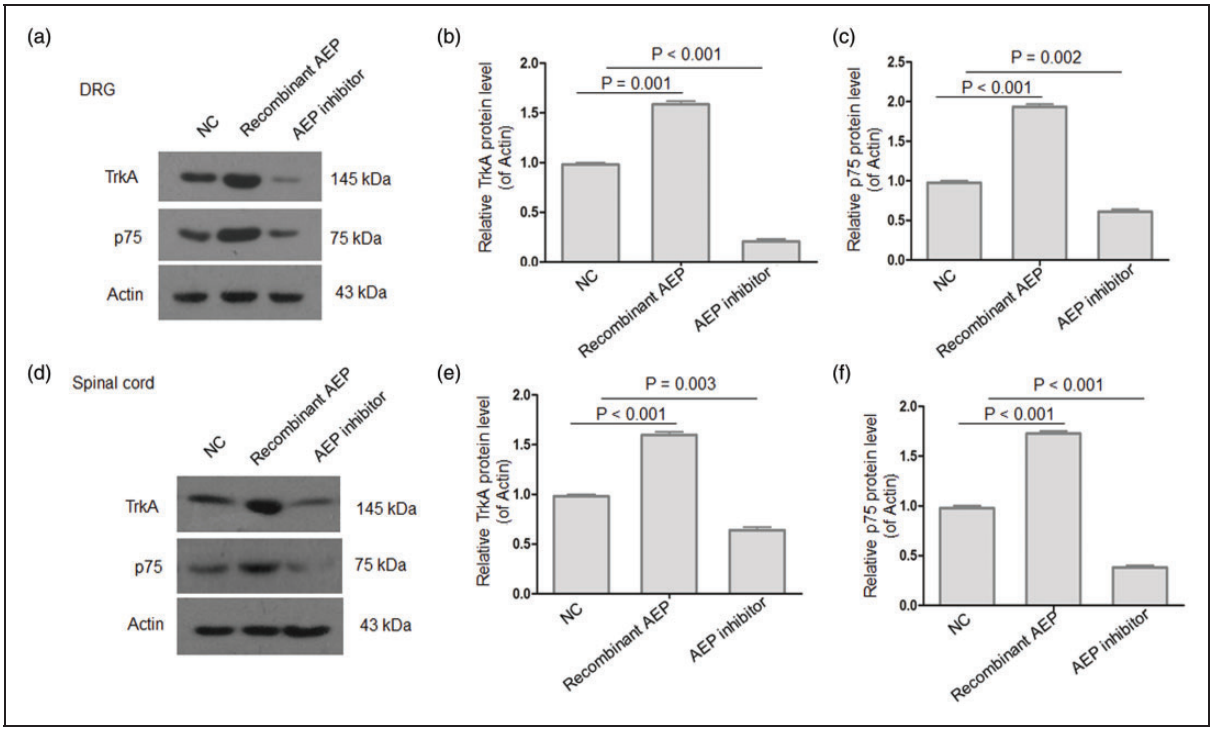

Figure 6. Expressions of P75 and TrkA were suppressed in DRGs and spinal cords of mice injected with AEP inhibitors while increased in mice injected with AEP proteins. (a–c) Western blot analysis of P75 and TrkA expression in DRGs of mice injected with MDA-MB-231 cells treated with AEP inhibitors, recombinant AEP proteins, or saline control. (d–f) Western blot analysis of P75 and TrkA expression in spinal cords of mice injected with MDA-MB-231 cells treated with AEP inhibitors, recombinant AEP proteins, or saline control. (g) Immunohistochemical analysis of P75 and TrkA expression in DRGs of mice injected with MDA-MB-231 cells treated with AEP inhibitors, recombinant AEP proteins, or saline control. (h) Immunohistochemical analysis of P75 and TrkA expression in spinal cords of mice injected with MDA-MB-231 cells treated with AEP inhibitors, recombinant AEP proteins, or saline control.

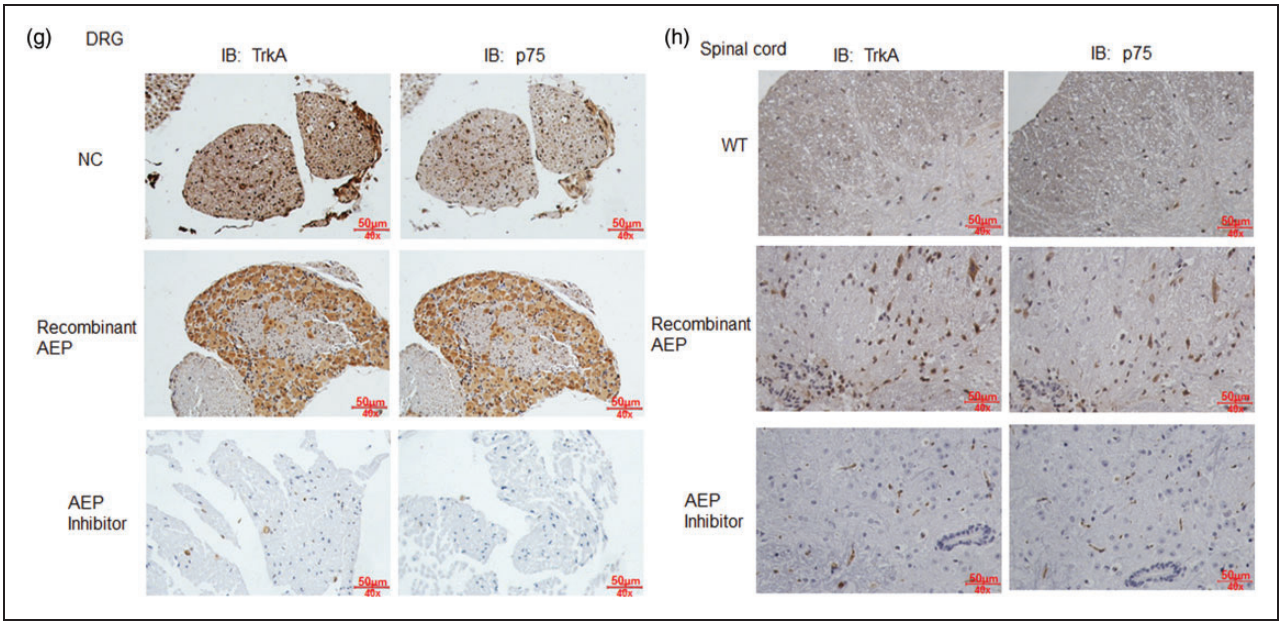

Figure 6. Continued.

cords in mice treated with AEP inhibitors (Figure 6(c) and (d)).

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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