Neutral Endopeptidase 24.11/CD10 Suppresses Progressive Potential in Ovarian Carcinoma *In vitro* and *In vivo*

Hiroaki Kajiyama, Kiyosumi Shibata, Mikio Terauchi, Takanori Morita, Kazuhiko Ino, Shigehiko Mizutani, and Fumitaka Kikkawa

Department of Obstetrics and Gynecology, Nagoya University Graduate School of Medicine, Tsurumai-cho, Showa-ku, Nagoya, Japan

ABSTRACT

Recently, numerous studies have shown that endothelin-1 (ET-1) is expressed in ovarian carcinoma and that ET-1 selectively acts as an autocrine or paracrine growth factor through the endothelin A receptor (ET_AR), and is involved in cell proliferation, invasiveness, neovascularization, and prevention of apoptosis. Neutral endopeptidase 24.11 (NEP) is a cell surface aminopeptidase with a ubiquitous expression and is capable of degrading a number of bioactive peptides including ET-1. Our previous report showed that stromal NEP expression in ovarian carcinoma was down-regulated as the histologic grade advanced. Here, we confirmed that NEP was expressed in tumor cells as well as stromal tissues in ovarian carcinoma, and investigated the functions of NEP in this carcinoma. We showed that there was a significant decrease in cell proliferation and invasiveness with a reduction in the concentration of ET-1 in the conditioned medium on the overexpression of NEP in ovarian carcinoma cells. In addition, the overexpression of NEP enhanced susceptibility to paclitaxel, resulting in an increased occurrence of apoptotic morphologic change. Furthermore, tumorigenesis was reduced *in vivo* with the overexpression of NEP, down-regulation of both matrix metalloproteinase-2, and vascular endothelial growth factor expression. This evidence suggests that NEP functionally suppresses the progression of ovarian carcinoma and further study of this enzyme may reveal an effective way to target ET-1 for the treatment of this carcinoma.

INTRODUCTION

Ovarian carcinoma is the leading cause of death from gynecologic malignancy. Owing to ovarian carcinoma frequently remaining clinically silent, the majority of patients with this disease have advanced i.p. metastatic disease at diagnosis (1).

However, the regulatory mechanisms responsible for the malignant transformation and progression of this tumor remain unclear. Recently, an extensive characterization of the growth factors and corresponding receptors that regulate the progression of ovarian carcinoma was conducted in an attempt to develop a more effective treatment for this disease.

Endothelin-1 (ET-1) is one such mediator, and is produced by endothelial cells, vascular smooth muscle cells, or tumor cells. ET-1 receptors, ET_A and ET_B, belong to the family of G-protein-coupled receptors and bind ET-1 with equal affinity (2, 3). Recently, several studies have shown that ET-1 is expressed in ovarian carcinoma and selectively acts as an autocrine or paracrine growth factor through the ET_A receptor (ET_AR; refs. 4–6). Moreover, in this tumor, ET-1 not only induced cell proliferation but the engagement of ET_AR by ET-1 also triggered activation of signal pathways linked to neovascularization, invasiveness, and apoptosis protection (7–9).

Neutral endopeptidase 24.11 (NEP, Neprilysin, EC 3.4.24.11) is a cell surface aminopeptidase which was originally characterized as a T-cell differentiation antigen (CD10/common acute lymphoblastic leukemia antigen; ref. 10). NEP has been reported to be present on epithelial cells of various tissues such as the lung, kidney, intestine, prostate, endometrium, and placenta (11–13), and plays an important role in the maintenance of homeostasis in normal tissues. NEP is capable of efficiently degrading a number of bioactive peptides and cytokines, including ET-1, enkephalins, atrial natriuretic peptides, and bombesin-like peptides (14, 15). Thus, the biological activities of such substrates are regulated by this enzyme by reducing local concentrations available for receptor binding and signal transduction (11).

Recent reports have shown that NEP is involved in neoplastic transformation and tumor progression in certain human malignancies including lung, breast, and prostate carcinomas by inactivating ET-1 or bombesin, which are autocrine growth factors for these tumors (16–19). We previously reported that NEP was also expressed in the stroma of borderline and malignant ovarian tumors and that stromal NEP expression was down-regulated as the histologic grade advanced. This implies that this peptidase plays a role in the biology of neoplastic transformation or tumor progression in ovarian carcinoma through the degradation and modulation of specific peptide substrates such as ET-1.

Therefore, in the present study, we investigated the cellular functions of NEP in ovarian carcinoma cells and examined the effects of NEP on the progression of ovarian carcinoma *in vitro* and *in vivo*. A possible role for this enzyme as a suppressor of ovarian carcinoma is proposed.

MATERIALS AND METHODS

Cell Culture. We used six human ovarian carcinoma cell lines: NOS2, OVCAR3, RMG-I, RMG-II, SKOV3, and TAOV. SKOV3 cells were generously donated by the memorial

Sloan-Kettering Cancer Research Laboratory. RMG-I and RMG-II cells were kindly provided by Dr. Shiro Nozawa (Keio University, Tokyo, Japan). NOS2 and TAOV cells were established in our institute. OVCAR3 cells were purchased from the American Type Culture Collection (Rockville, MD). These cell lines were maintained in RPMI 1640 supplemented with 10% FCS and 100 units/mL of penicillin and streptomycin, and were incubated at 37°C in a humidified atmosphere of 5% CO_2.

Plasmid Construction and Transfection. Full-length cDNA for NEP was kindly provided by Dr. David M. Nanus (Cornell University, New York). The eukaryotic expression vector pcDNA 3.1(+) (Invitrogen, San Diego, CA) was used to drive the expression of the inserted NEP cDNA. Transfections were carried out using Lipofectamine according to the instructions of the manufacturer (Invitrogen). SKOV3 cells were transfected with pcDNA3.1(+), SKpcDNA, or pcDNA3.1(+) with NEP cDNA inserted. Stable transfectants were selected by growth in media supplemented with 400 μg/mL of G 418 (Sigma-Aldrich, St. Louis, MO). Several hundred clones resistant to G 418 were obtained and two independent monoclonal cells (SKNEP3 and SKNEP16) from these transfectants were used in the subsequent experiments.

Enzyme Activity Assay. NEP enzyme activity was measured spectrophotometrically using succinyl-Ala-Ala-Phe-*p*-nitroanilide (Suc-Ala-Ala-Phe-pNA; Bachem Bioscience, Inc., King of Prussia, PA) as a substrate. Whole cell suspensions were prepared in test tubes, then washed with PBS. Thereafter, 5×10^4 cells were resuspended in 200 μL of PBS contained in each well of 96-well microtiter plates. Then the substrate and aminopeptidase M (0.5 units/mL) were added (final concentration, 0.9 mmol/L Suc-Ala-Ala-Phe-pNA), and incubated at 37°C for 60 minutes. NEP enzyme activity was measured at 405 nm with a microplate reader (Labsystems, Multiskan Bichromatic, Helsinki, Finland) every 10 minutes during incubation at 37°C. We used the NEP inhibitor phosphoramidon (Peptide Institute, Osaka, Japan) as a specific inhibitor of NEP enzyme activity.

Flow Cytometric Analysis. Fluorescence-activated cell sorting (FACS) was done to quantify the expression of NEP on the cell surface of various clones of the NEP transfectant. Then, the cells were incubated with phycoerythrin-conjugated monoclonal antibody specific for NEP (PharMingen, San Diego, CA) for 30 minutes at 4°C and washed thrice with PBS. FACS data were acquired on a FACS Calibur (Becton Dickinson, San Jose, CA), and analyzed using CELL Quest software (Becton Dickinson).
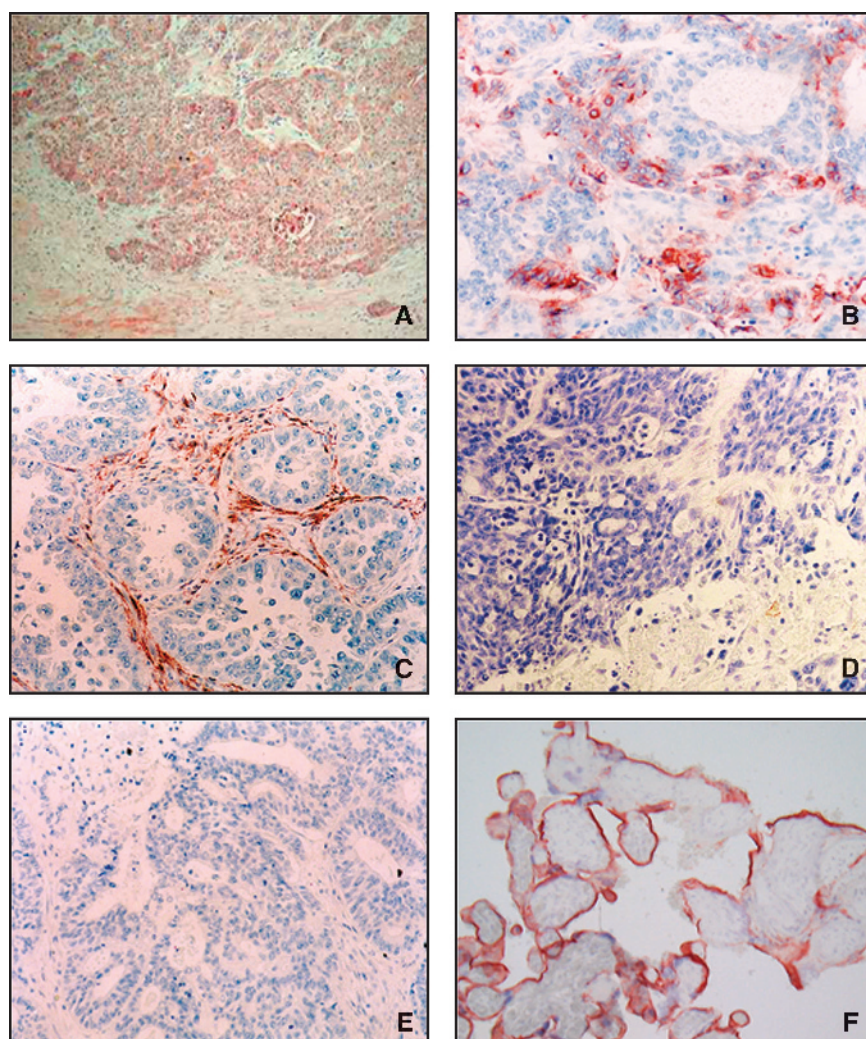
Immunohistochemical Staining of Ovarian Carcinoma Tissues. Tissue samples of ovarian carcinoma were obtained from patients who were surgically treated at Nagoya University Hospital with informed consent. All samples were fixed in 10% formalin and embedded in paraffin, and sections were cut at a thickness of 4 μm. For heat-induced epitope retrieval, deparaffinized sections in 0.01 mol/L citrate buffer were treated thrice at 90°C for 5 minutes at 750 W using a microwave oven. Immunohistochemical staining for NEP was done using the avidin-biotin immunoperoxidase technique (Histofine SAB-PO kit, Nichirei, Tokyo, Japan) according to the instructions of the manufacturer as previously described (20). As a primary antibody for NEP staining, anti-NEP/CD10

monoclonal antibody (NCL-CD10-270, Novocastra, Newcastle, United Kingdom) was used at a dilution of 1:100. For the negative control, mouse immunoglobulin G replaced the primary antibody. We used placental chorion as a positive control for NEP expression.

Western Blot Analysis. Immunoblot analysis of NEP, ET_AR, and endothelin converting enzyme-1 (ECE-1) was done as described previously (21). Briefly, cells were grown to 70% to 80% subconfluence and treated with lysis buffer containing 1% Triton-X in PBS and protease inhibitor cocktail tablets (Roche, Barcelona, Spain). Total cell lysate (20 μg) was electrophoresed on a 10% (NEP) or 15% (ET_AR and ECE-1) SDS polyacryl-amide gel and transferred electrophoretically to an Immobilon membrane (Millipore, Bedford, MA). After blocking in blocking solution (5% nonfat dry milk/0.1% Tween 20/PBS), the membranes were incubated with a recommended dilution of primary antibodies overnight. We used the following antibodies: anti-NEP/CD10 monoclonal antibody (NCL-CD10-270, Novocastra), anti-ET_AR polyclonal antibody (R19, Santa Cruz Biotechnogy, Santa Cruz, CA), and anti-ECE-1 polyclonal antibody (Zymed Laboratories, San Francisco, CA). The primary antibodies were washed in 0.1% Tween 20/PBS, and then incubated with horseradish peroxidase–conjugated secondary antibody. Proteins were visualized using enhanced chemiluminescence (ECL) reagent (Amersham Bioscience, Buckinghamshire, United Kingdom) followed by exposure to X-ray film.

***In vitro* Cell Proliferation Assay.** SKpcDNA, SKNEP3, and SKNEP16 cells were plated in triplicate at a density of 1,500 cells per 200 μL of culture medium containing 10% FCS in 96-well plates and cultured for 1 to 4 days. Cell viability was assayed every 24 hours using a modified tetrazolium salt [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay with a CellTiter 96 AQueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI) according to the instructions of the manufacturer. Absorbance was measured at 492 nm with a microplate reader (Labsystems, Multiskan Bichromatic). Furthermore, we assessed the effect of recombinant ET-1 (Calbiochem) and/or TAK-044, an ET_AR antagonist (Takeda Chemical Industries, Osaka, Japan), on cell proliferation. SKpcDNA and SKNEP3 cells were plated in triplicate at a density of 2,000 cells per 200 μL of culture medium containing 10% FCS in 96-well plates and incubated for 24 hours; after which the medium was replaced with a serum-free medium (RPMI 1640) for serum starvation. After 12 hours of incubation, the medium was replaced again with fresh serum-free medium with or without 100 nmol/L of recombinant ET-1, 100 nmol/L of TAK-044, or 10 μmol/L of phosphoramidon. After an additional 36 hours, cell viability was assayed as described above. Experiments were done in triplicate.

***In vitro* Invasion Assay.** Cell invasion was evaluated using 24-well Matrigel invasion chambers (Becton Dickinson Labware). SKOV3 parental and vector or NEP-transfected cells were suspended in the upper chamber at a final concentration of 75×10^4 /mL in 200 μL of RPMI 1640 supplemented with or without 100 nmol/L of ET-1 or 10 μmol/L of phosphoramidon. The lower chamber contained 700 μL of RPMI 1640 supplemented with chemoattractants. After 16 hours of incubation, the tumor cells remaining on the upper surface of the filters
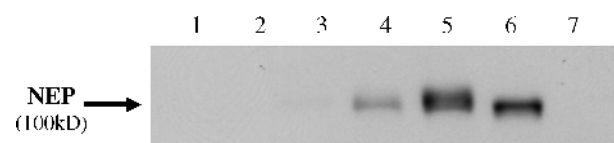
Fig. 1 NEP expression in ovarian carcinoma tissues and cell lines. *A-D,* immunohistologic staining of NEP in surgically resected ovarian carcinoma tissues using NEP-specific antibody. *A,* NEP was expressed intensively in tumor cells but only slightly in stromal cells. *B,* NEP was partially expressed in tumor cells. *C,* NEP was intensively expressed in stromal cells but almost undetectable in tumor cells. *D,* NEP staining was not detected in tumor cells or stromal cells. *E,* negative control in ovarian carcinoma tissue. *F,* positive control in human placental chorion.

were removed by wiping with cotton swabs, and the cells on the lower surface were stained with May-Grünwald Giemsa reagent. The number of cells on the lower surface of the filters was counted under a microscope at a magnification of 200. Four individual experiments were done in triplicate.

***In vitro* Migration Assay.** Cell migration was assayed in 24-well Transwell cell culture chambers (Costar). Cells were suspended in the upper chamber at a final concentration of 50 $\times 10^4$ /mL in 200 μL of RPMI 1640 supplemented with 0.1% bovine serum albumin. The subsequent procedures were the same as those used for the invasion assay except that the incubation time was 12 hours.

Quantification of Endothelin-1 by ELISA. SKpcDNA, SKNEP3, and SKNEP16 cells were seeded into 24-well culture dishes and incubated in the culture medium. After become confluent, the cells were washed with a serum-free medium and incubated for 24 hours in the presence or absence of phosphoramidon (10 μmol/L; Peptide Institute). After the incubation, the culture supernatants were tested using ET-1 ELISA kits (G-T, Minneapolis, MN) according to the directions of the manufacturer. Experiments were done in triplicate.

Measurement of Matrix Metalloproteinase-2 Protein by ELISA. SKpcDNA, SKNEP3, and SKNEP16 cells were seeded into 24-well culture dishes, and incubated in the culture medium. Once confluent, the cells were washed with a serum-free medium, and incubated for 24 hours in the presence or absence of recombinant ET-1 (100 μmol/L). After the incubation, the culture supernatants were tested using matrix metalloproteinase (MMP)-2 ELISA kits (R & D Systems, Minneapolis, MN) according to the directions of the manufacturer. Experiments were done in triplicate.

Fig. 2 Immunoblot analysis of NEP expression in various ovarian carcinoma cell lines. Lane 1, negative control (no protein loaded); lane 2, NOS2; lane 3, OVCAR3; lane 4, RMG-1; lane 5, RMG-II; lane 6, SKOV3; lane 7, TAOV. In NOS2, OVCAR3, and TAOV cells, NEP expression was limited or undetectable. In contrast, NEP was expressed in RMG-I, RMG-II, and SKOV3 cells.
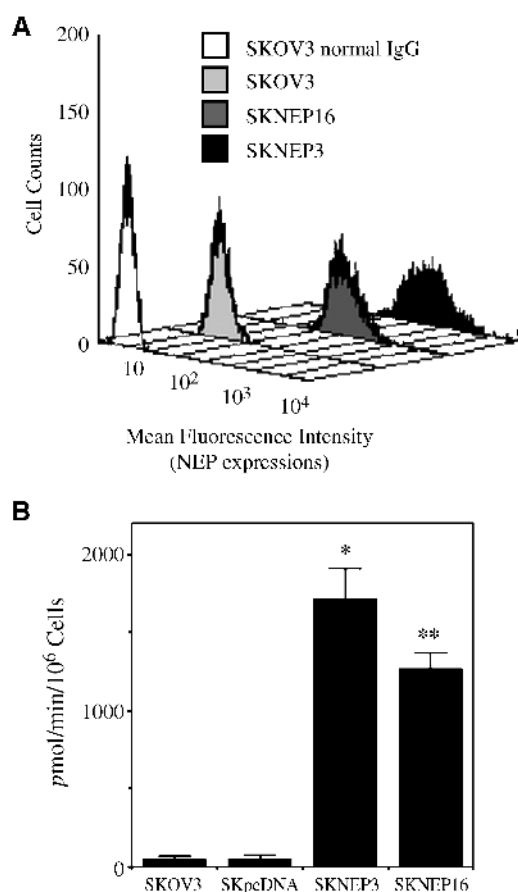
Fig. 3 Generation of NEP-overexpressing ovarian carcinoma cells (SKOV3 cells). *A*, analysis of cell surface expression of NEP by FACS. White peak, SKOV3 cells (control, mouse normal immunoglobulin G); light gray peak, SKOV3 cells; dark gray peak, SKNEP16 cells; and black peak, SKNEP3 cells. Mean fluorescence intensities were 3.8, 8.4, 140, and 350, respectively. *B,* increase in NEP enzyme activity in NEP-overexpressing ovarian carcinoma cells. Enzyme activity was determined by quantification of the enzymatic cleavage of the synthetic substrate succinyl-Ala-Ala-Phe-*p*-nitroanilide. NEP activities of parental SKOV3, mock, and NEP transfected cells are shown as the increase in absorbance at 405 nm. The activity levels of NEP-transfected cells were significantly higher than those of parental and mock cells (*, $P < 0.001$: SKNEP3 versus SKOV3 or SKpcDNA; **, $P < 0.001$: SKNEP16 versus SKOV3 or SKpcDNA). Data on enzyme activity were consistent with mean fluorescence intensity data from the FACS analysis.

Gelatin Zymography. The activities of MMPs in the conditioned medium of SKpcDNA, SKNEP3, and SKNEP16 cells were assayed by zymography as described previously (22).

Paclitaxel Chemosensitivity Assay. SKpcDNA and SKNEP3 cells were seeded in triplicate in 96-well plates at a density of 5,000 cells in 200 μL of culture medium containing 10% FCS. After incubation for 24 hours at 37°C, the medium was replaced with fresh medium with or without various concentrations of paclitaxel (Bristol Myers, Tokyo, Japan). After 36 hours, cell viability was measured as described above (Proliferation Assay). Data were expressed as a percentage of the control (without paclitaxel).

In vivo Experiments. Female nude mice (BALB/c), 5 weeks of age, were obtained from Japan SLC (Nagoya,
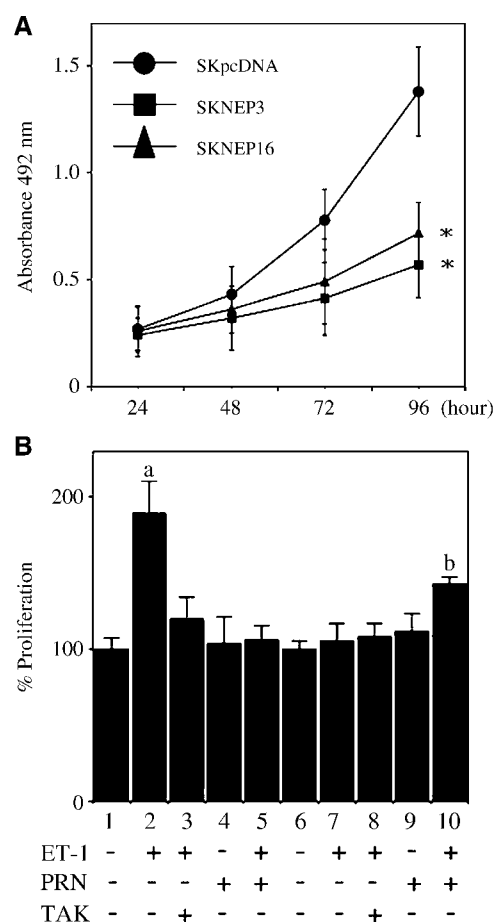
Fig. 4 *A*, effect of NEP on cell proliferation examined using NEP-transfected ovarian carcinoma cells in a 4-day modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The proliferation rate was analyzed as described in Materials and Methods. Points, mean (*n* = 3); bars, SD. Cell proliferation was significantly slower in SKNEP3 and SKNEP16 cells than in mock cells (*, $P < 0.05$). *B,* effect of recombinant ET-1 on cell proliferation in mock and SKNEP3 cells in the presence or absence of the ET_AR antagonist, TAK-044. Lane 1, SKpcDNA cells (mock) alone; lane 2, SKpcDNA cells with 100 nmol/L of ET-1; lane 3, SKpcDNA cells with ET-1 (100 nmol/L) and TAK-044 (100 nmol/L); lane 4, SKpcDNA cells with phosphoramidon (*PRN*, 10 μmol/L); lane 5, SKpcDNA cells with ET-1 (100 μmol/L) and phosphoramidon (10 μmol/L); lane 6, SKNEP3 cells alone; lane 7, SKNEP3 cells with 100 nmol/L of ET-1; lane 8, SKNEP3 cells with ET-1 (100 nmol/L) and TAK-044 (100 nmol/L); lane 9, SKNEP3 cells with phosphoramidon (10 μmol/L); lane 10, SKNEP3 cells with ET-1 (100 μmol/L) and phosphoramidon (10 μmol/L). Addition of recombinant ET-1 significantly stimulated the proliferation of mock cells (*a*: lane 1 versus lane 2, $P < 0.005$) which was almost inhibited by the addition of TAK-044. In contrast, the addition of recombinant ET-1 did not significantly affect proliferation in SKNEP3 cells. Furthermore, this growth-suppressive effect of NEP was significantly reversed by the addition of phosphoramidon in SKNEP3 cells (*b*: lane 6 versus lane 10, $P < 0.05$).

Japan). The treatment protocol followed the guidelines of animal experimentation adopted by Nagoya University. Mice were given injections s.c. on the bilateral dorsal flank with 1.0×10^6 viable SKpcDNA or SKNEP3 cells resuspended in 100 μL of PBS. After 7 days, established tumors were

detectable. Tumor volume was measured with calipers and calculated using the formula $\pi / 6 \times$ (larger diameter) $\times$ (smaller diameter)^2. One experiment was terminated after 40 days to allow the harvesting of tumor xenografts for immunohistochemical analysis. Each group consisted of six mice. At the end of the experiment, mice underwent euthanasia with diethyl ether. Tumor tissue was excised, fixed in 4% paraformaldehyde, and embedded in paraffin sections (5 mm). Furthermore, SKpcDNA and SKNEP3 cells (1×10^7 cells /0.5 mL of medium/mouse) were injected i.p. to examine their peritoneal metastatic potential. Mice were sacrificed 30 days after the injection of carcinoma cells, and the i.p. dissemination was evaluated. In addition, the total weight of disseminated tumors was compared between the two groups. Each group consisted of six mice.

Statistical Analysis. For the data from *in vitro* and *in vivo* experiments, statistical comparisons among groups were done using the Student's *t* test and ANOVA with Bonferroni corrections. Differences between groups were considered statistically significant at $P < 0.05$. Data are expressed as the mean $\pm$ SD.

RESULTS

Neutral Endopeptidase 24.11 Expression in Ovarian Carcinoma Tissues and Cell Lines. We first confirmed expression of NEP in surgically resected ovarian carcinoma tissues. NEP staining was found in tumor cells as well as stromal tissues (Fig. 1*A*-*C*) although NEP was mainly expressed in stromal tissues as shown in our previous study (20). We examined 55 ovarian cancer tissue samples including all histologic types of ovarian carcinoma. Of these samples, seven (12.7%) were positive for NEP immunohistochemical staining in tumor cells, 29 (52.7%) were positive in stromal cells but not in tumor cells, and 19 (34.5%) were negative in both tumor and stromal cells. The intensity of immunohisto-chemical staining varied among the tissues. No expression of NEP was found in ovarian surface epithelial cells. However, NEP was partially expressed in follicular granulosa cells or the corpus luteum (data not shown).

Figure 2 shows the extent of NEP expression in ovarian carcinoma cell lines as assessed by Western blot analysis. NEP was expressed in RMG-I, RMG-II, and SKOV3 cells but
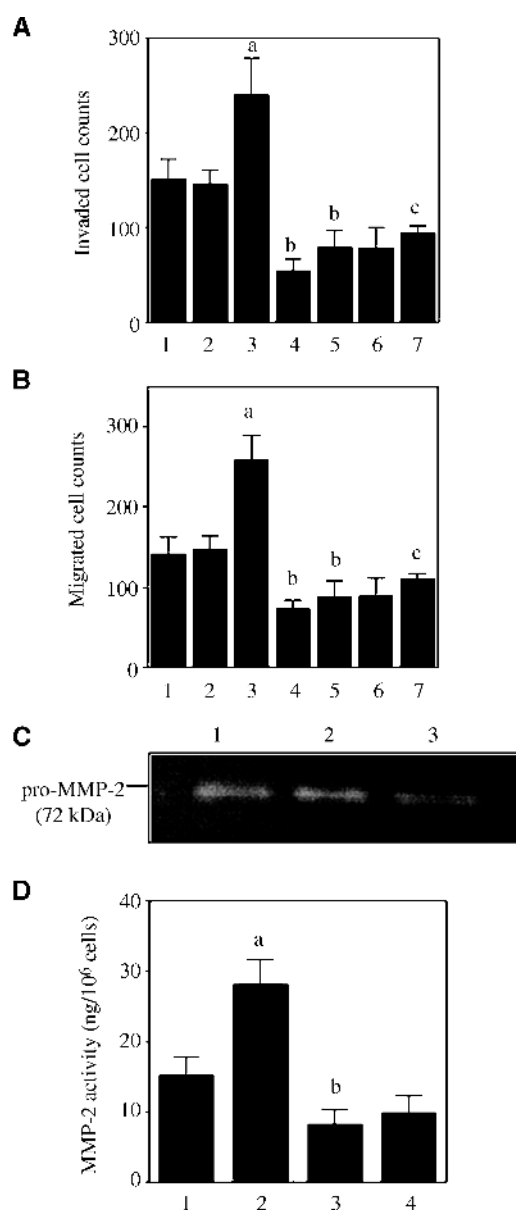
Fig. 5 A, effect of NEP transfection on invasive potential (Matrigel invasion assay) in the absence or presence of phosphoramidon or recombinant ET-1. Lane 1, SKOV3 parental cells; lane 2, SKpcDNA cells; lane 3, SKpcDNA cells with ET-1 (100 nmol/L); lane 4, SKNEP3 cells; lane 5, SKNEP16 cells; lane 6, SKNEP3 cells with ET-1 (100 nmol/L); lane 7, SKNEP3 cells with ET-1 (100 nmol/L) and phosphoramidon (10 µmol/L). The number of invading cells was significantly lower in both SKNEP3 and SKNEP16 cells compared with mock cells (*b*: lane 2 versus lane 4 or 5, $P < 0.05$). This reduction could be significantly restored by the addition of 10 µmol/L of phosphor-amidon in SKNEP3 cells (*c*: lane 4 versus lane 7, $P < 0.05$). The number of invading cells was significantly larger with ET-1 than without ET-1 in SKpcDNA cells (*a*: lane 2 versus lane 3, $P < 0.01$). Additionally, invasive potential was enhanced by ET-1 in SKNEP3 cells but not in SKpcDNA cells (lane 4 versus lane 6, not significant). *B,* effect of NEP transfection on migratory potential (migration assay) in the absence or presence of phosphoramidon or recombinant ET-1. Lane 1, SKOV3 parental cells; lane 2, SKpcDNA cells; lane 3, SKpcDNA cells with ET-1 (100 nmol/L); lane 4, SKNEP3 cells; lane 5, SKNEP16 cells; lane 6, SKNEP3 cells with ET-1 (100 nmol/L); lane 7, SKNEP3 cells with ET-1 (100 nmol/L) and phosphoramidon (10 µmol/L). A similar result was obtained in the migration assay (*a*: lane 2 versus lane 3, $P < 0.01$; *b*: lane 2 versus lane 4 or 5, $P < 0.05$; *c*: lane 4 versus lane 7, $P < 0.05$). *C,* effect of NEP transfection on gelatin zymography. Lane 1, SKOV3 cells; lane 2, SKpcDNA cells; lane 3, SKNEP3 cells. Pro-MMP-2 activity (72 kDa) was remarkably reduced in SKNEP3 cells, whereas SKpcDNA cells showed the same level of pro-MMP-2 activity as parental SKOV3 cells. *D,* effect of ET-1 on MMP-2 secretion in SKpcDNA and SKNEP3 cells. MMP-2 was measured in conditioned medium from cells treated with or without ET-1 for 24 hours using an ELISA kit. Lane 1, SKpcDNA cells; lane 2, SKpcDNA cells with ET-1 (100 µmol/L); lane 3, SKNEP3 cells; lane 4, SKNEP cells with ET-1 (100 µmol/L). ET-1 stimulated MMP-2 secretion from SKpcDNA cells. In contrast, ET-1 could not stimulate it from SKNEP3 cells (lane 3 versus lane 4, not significant). Bars, SD. a: lane 1 versus lane 2, $P < 0.05$; b: lane 1 versus lane 3, $P < 0.05$.
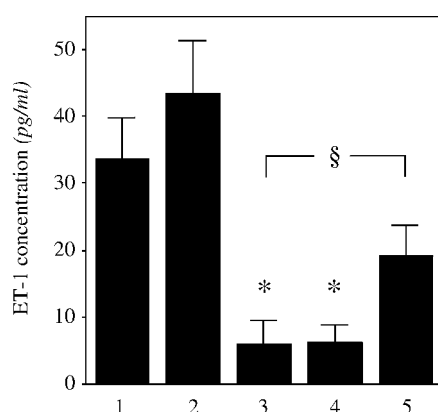
Fig. 6 Change in soluble ET-1 levels in culture medium after NEP transfection in SKOV3 cells. Lane 1, conditioned medium from SKOV3 cells; lane 2, conditioned medium from SKpcDNA cells; lane 3, conditioned medium from SKNEP3 cells; lane 4, conditioned medium from SKNEP16 cells; lane 5, conditioned medium from SKNEP3 cells in the presence of 10 μmol/L of phosphoramidon. The ET-1 concentration was significantly lower in SKNEP3 and SKNEP16 cells than in mock cells (*, $P < 0.001$). Furthermore, the addition of 10 μmol/L of phosphoramidon significantly restored the decrease in the concentration of ET-1 in the conditioned medium (§, $P < 0.05$).

not in the other cell lines. However, no apparent correlation between the expression profile of NEP and proliferative or invasive potential was found in the current study (data not shown).

Overexpression of Neutral Endopeptidase 24.11 in Ovarian Carcinoma Cells. To investigate the functional effect of NEP on ovarian carcinoma cells, we transfected NEP cDNA into ovarian carcinoma SKOV3 cells. A previous report showed that SKOV3 cells overexpress ET_AR and secrete high levels of ET-1 (5). Whereas parental SKOV3 cells expressed a limited amount of NEP, NEP-transfected monoclonal cells (SKNEP3 and SKNEP16) expressed a remarkably high level of NEP as determined by FACS analysis (Fig. 3*A*). To confirm the enzyme activity of NEP overexpressed by the transfection, we also checked for aminopeptidase activity. The NEP activity of SKNEP3 and SKNEP16 was ~ 30 times higher than that of the parental SKOV3 cells and SKpcDNA cells in terms of absorbance ($P < 0.001$; Fig. 3*B*).

Functional Analysis of Neutral Endopeptidase 24.11 Using Neutral Endopeptidase 24.11 – Overexpressing Ovarian Carcinoma Cells. We first investigated the effect of NEP on cell proliferation using SKpcDNA, SKNEP3, and SKNEP16 cells as described in MATERIALS AND METHODS. As shown in Fig. 4*A*, cell proliferation rates were lower for SKNEP3 and SKNEP16 cells than for SKpcDNA cells ($P < 0.05$). Next, we assessed the effect of recombinant ET-1 on cell proliferation in SKpcDNA and SKNEP3 cells in the presence or absence of an ET_AR antagonist, TAK-044, and an NEP inhibitor, phosphoramidon. As shown in Fig. 4*B*, 100 nmol/L of recombinant ET-1 significantly stimulated the proliferation of SKpcDNA cells ($P < 0.005$) and this effect was almost completely inhibited by the addition of TAK-044 (100 nmol/L). In contrast, the addition of recombinant ET-1 did not significantly affect the proliferation of SKNEP3 cells.

Furthermore, this growth-suppressive effect of NEP was partially restored by the addition of phosphoramidon in SKNEP3 cells ($P < 0.05$).

We next examined the effect of NEP overexpression on migratory and invasive potential *in vitro*. As shown in Fig. 5*A*, the number of cells that had invaded the Matrigel was significantly small for both SKNEP3 and SKNEP16 cells compared with parental SKOV3 or SKpcDNA cells ($P < 0.05$). This reduction was partially restored by the addition of 10 μmol/L of phosphoramidon in the upper chamber. Additionally, invasive potential was enhanced by recombinant ET-1 in SKpcDNA cells but not in SKNEP3 cells (Fig. 5*A*). A similar result was obtained in the migration assay (Fig. 5*B*). Gelatin zymography revealed that pro-MMP-2 activity was remarkably reduced in SKNEP3 cells, whereas SKpcDNA cells showed the same level of pro-MMP-2 activity as parental SKOV3 cells (Fig. 5*C*). Moreover, we measured the effect of exogenous ET-1 on the secretion of MMP-2 in SKpcDNA or SKNEP3 cells using the human MMP-2 ELISA kit. As shown in Fig. 5*D*, ET-1 enhanced the secretion in SKpcDNA cells but not in SKNEP3 cells.

We next examined soluble ET-1 levels in the culture medium to clarify whether the concentration of ET-1 was affected by overexpression of NEP. Concentrations of soluble ET-1 were significantly lower in SKNEP3 and SKNEP16 cells than in SKpcDNA cells (Fig. 6; $P < 0.001$). Additionally, the addition of 10 μmol/L of phosphoramidon partially restored the decrease in the concentration of ET-1 in the conditioned medium ($P < 0.05$). Furthermore, we examined ET_AR or ECE-1 expression in mock and NEP-transfected cells by Western blotting (Fig. 7). In this analysis, levels of both the proteins were found to be slightly elevated in NEP-expressing cell lines by quantitating these bands (data not shown).

Paclitaxel Chemosensitivity Assay. A previous report showed that ET-1 protected ovarian carcinoma cells against paclitaxel-induced apoptosis (7). In the present study, we examined whether the ET-1-degrading enzyme NEP influenced the susceptibility of ovarian carcinoma cells to paclitaxel. Cell death with morphologic changes associated with apoptosis was more frequently observed in SKNEP3 cells than in SKpcDNA cells 12 hours after exposure to 100 ng/mL paclitaxel (Fig. 8*A-D*). Moreover, similar morphologic change was observed when the cells were cultured in serum-free conditions for more than 72 hours (Fig. 8*E* and *F*). Therefore, NEP-overexpressing cells were more sensitive to stress such as exposure to paclitaxel or serum starvation. We then treated these cells with 30 to 100 ng/mL of paclitaxel for 36 hours and measured the effect on cell viability
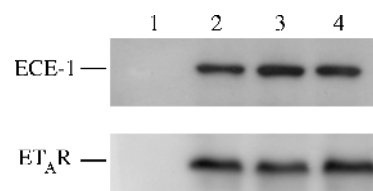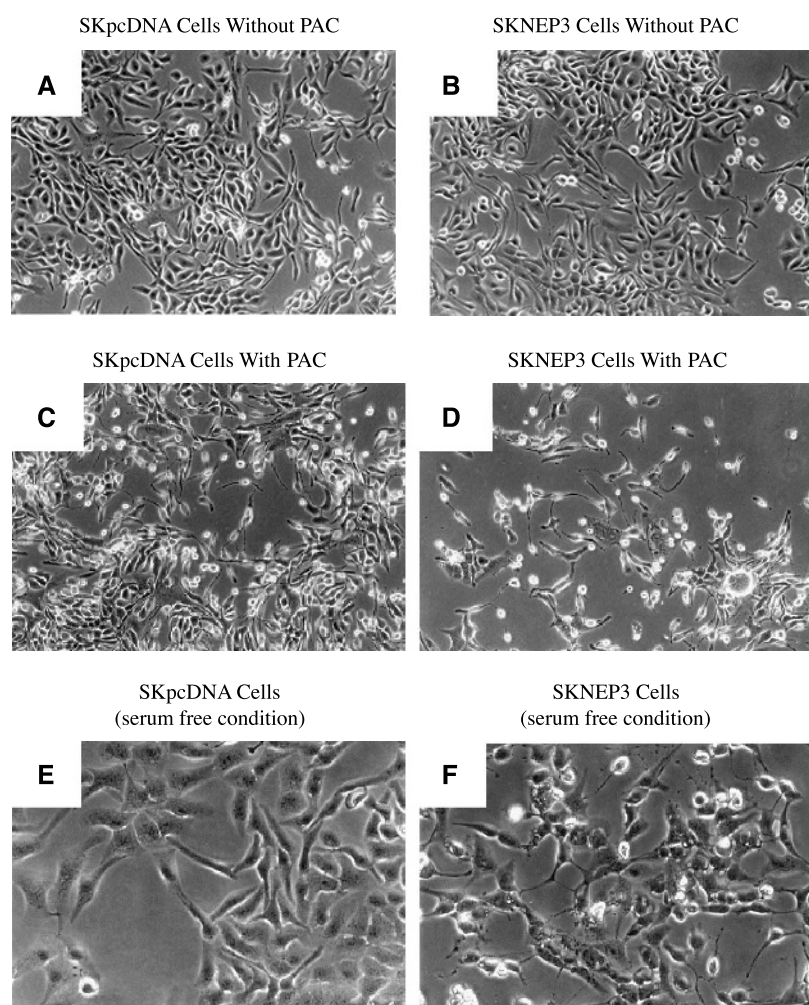

Fig. 7 Western blot analysis of ECE-1 and ET_AR after transfection of NEP cDNA into SKOV3 cells using specific antibodies. Lane 1, negative control (no protein loaded); lane 2, SKpcDNA cells; lane 3, SKNEP3 cells; lane 4, SKNEP16 cells.

SKpcDNA Cells Without PAC   SKNEP3 Cells Without PAC

SKpcDNA Cells With PAC   SKNEP3 Cells With PAC

Fig. 8 Morphologic change in mock (SKpcDNA) and NEP-transfected (SKNEP3) lines under paclitaxel (*PAC*) treatment or serum-starvation. *A,* mock cells without paclitaxel; *B,* SKNEP3 cells without paclitaxel; *C,* mock cells with paclitaxel; *D,* SKNEP3 cells with paclitaxel; *E,* mock cells without FCS; *F,* SKNEP3 cells without FCS. Cell treatment was done as described in Materials and Methods.

SKpcDNA Cells
(serum free condition)   SKNEP3 Cells
(serum free condition)

(Fig. 9). In SKNEP3 cells, a markedly enhanced sensitivity was displayed at 10 to 100 ng/mL of paclitaxel compared with SKpcDNA cells ($P < 0.001$).

Effect of Neutral Endopeptidase 24.11 on Tumor Progression *In vivo*. We investigated whether NEP suppressed the formation of tumors in s.c. xenografts using nude mice. Subcutaneous tumorigenesis was observed approximately 1 week after the inoculation of SKpcDNA or SKNEP3 cells into the mice. Figure 10*A* shows the general appearance of the mice 29 days after the inoculation with SKpcDNA (*left*) or SKNEP3 cells (*right*). Significantly, tumors were macroscopically larger following the inoculation of SKpcDNA cells than SKNEP3 cells (Fig. 10*A*). The comparison of the time course of tumor-growth curves by ANOVA was statistically significant ($P < 0.001$; Fig. 10*B*). Furthermore, immunohistochemical evaluation of the expression of ET-1, ET_AR, MMP-2, and vascular endothelial growth factor (VEGF) done on tumor xenografts revealed a marked reduction in the percentage of ET-1-, MMP-2-, and VEGF-positive cells among SKNEP3 cells (Fig. 11). However, ET_AR expression was not altered in tumor xenografts by the inoculation of SKNEP3 cells compared with that in control xenografts (mock injection; data not shown). These results are consistent with
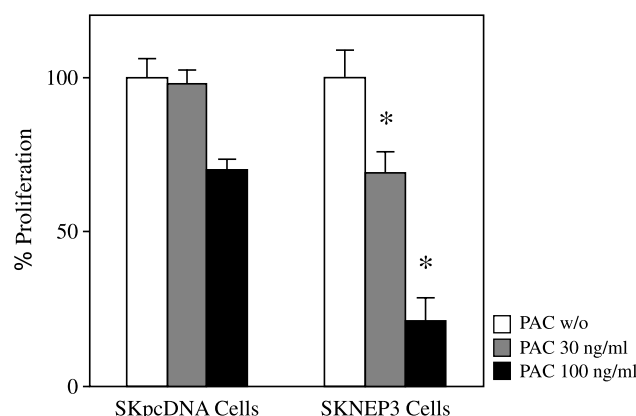
Fig. 9 Change in sensitivity to paclitaxel following NEP transfection in SKOV3 cells. NEP transfectants (SKNEP3 cells) displayed markedly increased sensitivity to 30 to 100 ng/mL of paclitaxel as compared with mock cells ($P < 0.001$). White bar, without paclitaxel; gray bar, 30 ng/mL of paclitaxel; black bar, 100 ng/mL of paclitaxel. The absorbance values are expressed as percentage of the control (without paclitaxel).
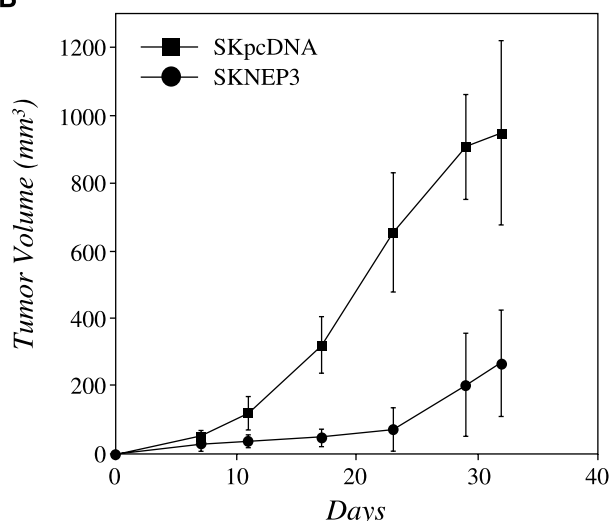
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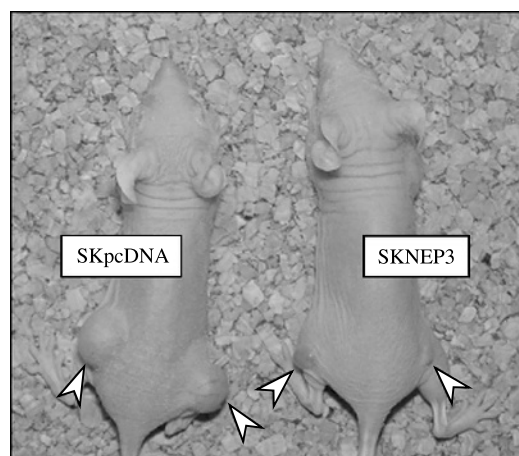
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Fig. 10 *A*, representative example of nude mice 29 days after tumor implant showing a mouse inoculated with SKpcDNA cells (*left*) and SKNEP3 cells (*right*). Mice were given injections s.c. into the bilateral dorsal flank with 1.0×10^6 viable mock SKpcDNA or SKNEP3 cells resuspended in 100 μL of PBS. *B,* comparison of the time course of tumor-growth curves. ■, tumor xenografts on the inoculation of SKpcDNA cells; ●, tumor xenografts on the inoculation of SKNEP3 cells. Each group consisted of six mice. Bars, SD.

the finding made *in vitro* that NEP inhibits the growth and invasion of SKOV3 cells. We also investigated *in vivo* whether NEP suppressed the of peritoneal metastasis of ovarian carcinoma. SKpcDNA or SKNEP3 cells were injected i.p. to examine their metastatic potential. Mice were sacrificed 30 days after the injection and the i.p. dissemination was evaluated. The mice injected with SKpcDNA cells displayed a number of disseminated and enlarged tumor nodules throughout the peritoneal cavity (Fig. 12*A* and *B*). In contrast, the mice injected with SKNEP3 cells had a small number of tumors. As shown in Fig. 12*C*, the total weight of the disseminated tumors was smaller in SKNEP3-injected than in SKpcDNA-injected mice ($P < 0.0001$).

DISCUSSION

Peritoneal dissemination is the main metastatic process in cases of ovarian carcinoma besides direct extension of the carcinoma into adjacent tissues and lymphatic dissemination. In the initial stage of peritoneal dissemination, carcinoma cells detach from the primary lesions and are released in the ascites. Once the carcinoma cells attach to mesothelial cells, they invade local tissues with increased neovascularization. During each of these steps, bioactive peptides such as ET-1 and bombesin act as autocrine growth factors on specific receptors to stimulate the progression of ovarian carcinoma cells (4, 5, 8).

Cell-surface peptidases play a key role in controlling the growth, differentiation, and signal transduction of many cellular systems by modulating the activity of peptide factors and regulating their access to receptors (11). NEP was also reported to be involved in carcinoma cell differentiation and apoptosis by regulating the local concentration of possible bioactive peptides including ET-1 (13, 23). In ovarian carcinoma, our previous reports showed that NEP was specifically expressed in the stroma of borderline and malignant ovarian tumors but not in benign adenomas and that stromal NEP expression was down-regulated as the histologic grade advanced (20). We had previously thought that NEP was expressed only in stromal cells in ovarian carcinoma but found that it was also expressed in tumor cells. Chu and Arber (24) reported the expression of NEP in 505 nonhematopoietic neoplasms and found that in 3 of 27 (11%) ovarian carcinomas, NEP was expressed in tumor cells. Thus, NEP was expressed in both tumor cells and stromal cells in ovarian carcinoma although the staining intensity varied among the tissues. However, a functional analysis of NEP in ovarian carcinoma is yet to be conducted. Accordingly, it would be beneficial to establish a subline overexpressing NEP to clarify the role of NEP in ovarian carcinoma.

In this study, we showed that there was a significant decrease in cell proliferation and invasiveness in NEP-transfected ovarian carcinoma cells. In addition, recombinant ET-1 promoted the proliferation of SKOV3 cells that were completely inhibited by TAK-044, an ET_AR antagonist. In contrast, no evident effects of recombinant ET-1 or TAK-004 on proliferation were found in SKNEP3 cells. Moreover, we showed that the concentration of ET-1 was markedly lower in the conditioned medium of NEP-overexpressing SKOV3 cells than in cells. In this respect, the addition of phosphoramidon also reversed the reduction, although only partially. On the other hand, it is possible that the overexpression of NEP affects ET_AR or ECE-1 expression in SKOV3 cells. In the Western blot analysis, both proteins were slightly enhanced in the cell lines overexpressing NEP. Although details remain unclear, one of the molecular mechanisms may be a negative feedback system from ET-1 lacking due to NEP overexpression at the cell surface. These results suggest that NEP may directly influence local concentrations of ET-1 via its enzyme activity. Therefore, we hypothesize that NEP could degrade ET-1 enzymatically on the cell surface and negatively regulate the mitogenic effects of ET-1 on SKOV3 cells.

We also showed that the invasive potential of SKOV3 cells was markedly reduced by transfection of NEP with a decrease in MMP-2 activity and that the reduction was in part
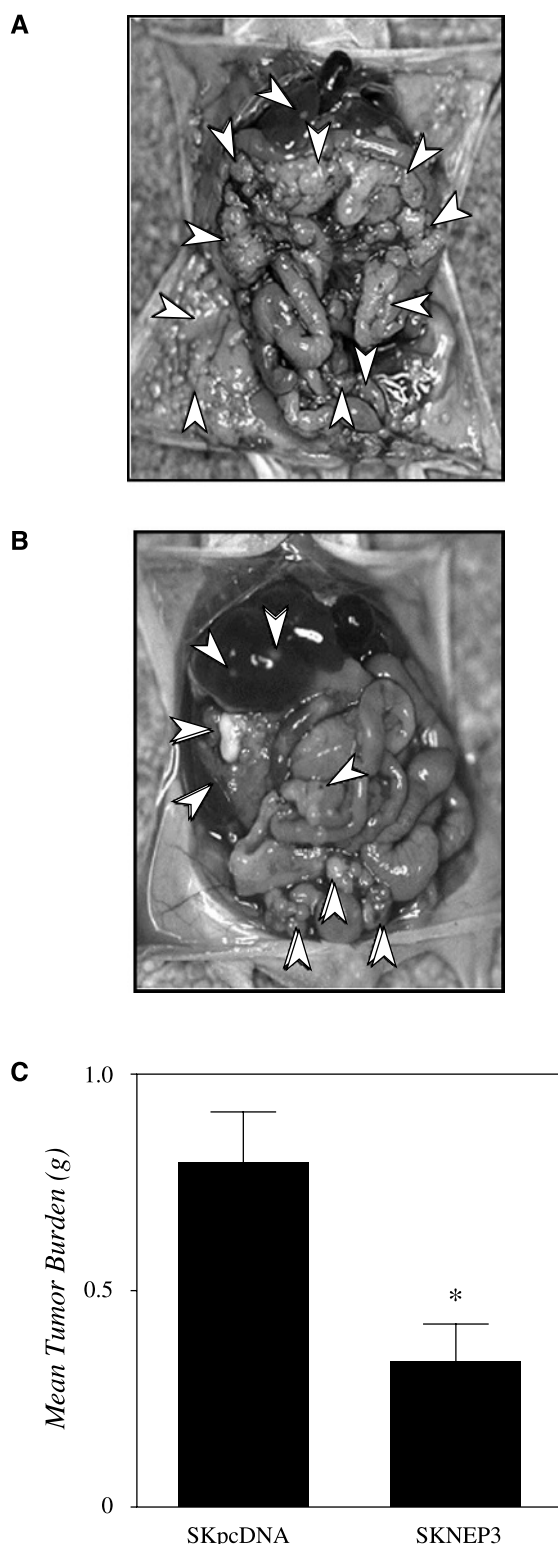
SKpcDNA Cells Inoculated   SKNEP3 Cells Inoculated
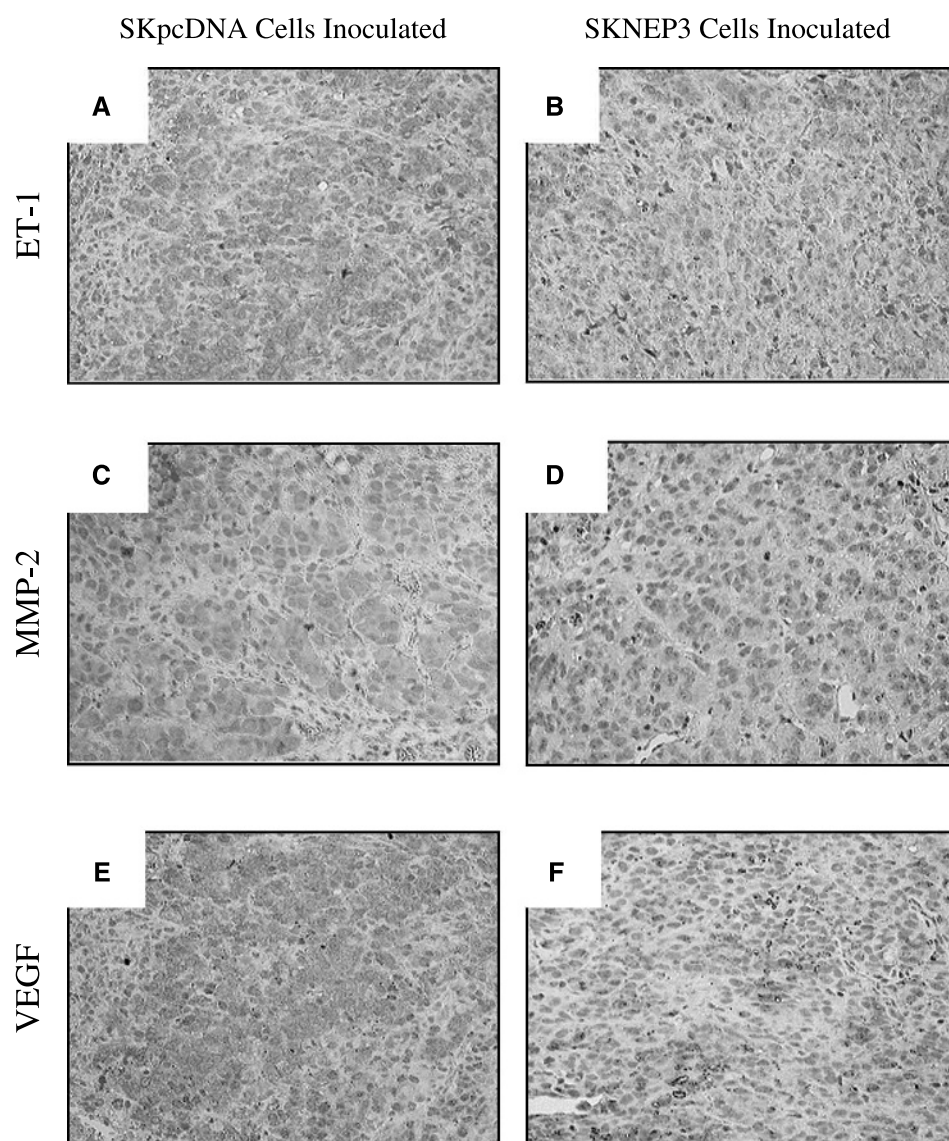
Fig. 11 Immunohistochemical evaluation of the expression of ET-1, ET$_A$R, MMP-2, and VEGF done on tumor xenografts following the inoculation of SKpcDNA and SKNEP3 cells. Forty days after the inoculation, tumor tissue was excised, fixed in 4% paraformaldehyde, and embedded in paraffin sections (5 mm). The panels show marked reductions in the percentage of ET-1-, MMP-2-, and VEGF-positive cells in the xenografts on the inoculation of SKNEP3 cells.

reversed by the addition of NEP inhibitors. Rosano et al. (8) that ET-1 induced activation of MMP-2, -9, -3, -7, and -13 through ET$_A$R in ovarian carcinoma, leading to the degradation of all known extracellular matrix components and thus enabling tumor cells to break down the basement membrane. This is consistent with our current data and, in this regard, ovarian carcinoma cells presumably enhance their metastatic ability by secreting endogenous ET-1 as an autocrine factor. Thus, we assume that increased NEP activity might, at least in part, contribute to the suppressive effect on invasiveness and metastasis in ovarian carcinoma. However, other mechanisms independent of enzyme activity may result in the partial suppression of invasive potential by the NEP inhibitor. Sumitomo et al. (25) reported that NEP inhibited the phosphorylation of focal adhesion kinase and cell migration by blocking the association of focal adhesion kinase with phosphatidylinositol 3-kinase in prostate cancer cells independently of the enzyme activity of NEP.

The current study showed that overexpression of NEP in SKOV3 cells enhanced susceptibility to paclitaxel, resulting in an increased occurrence of apoptotic morphologic change. Figure 9 shows that NEP transfectants (SKNEP3 and SKNEP16) displayed markedly increased sensitivity to paclitaxel compared with mock cells. Del Bufalo et al. (7) provided data that the addition of ET-1 markedly inhibited serum withdrawal and paclitaxel-induced apoptosis in a concentration-dependent manner via the phosphatidylinositol 3-kinase-Akt pathway, and that the blockade of ET$_A$R by ET$_A$R antagonists reversed the ET-1-induced protective effect in ovarian carcinoma cells. According to this, we can assume that the relative decrease in ET-1 due to degradation by NEP may lead to a down-regulation of Akt phosphorylation and, thus, apoptosis signaling may be activated through a Bcl-2-dependent pathway.

A recent report described the ET$_A$R antagonist as a new target in the treatment of ovarian carcinoma treatment. Rosano et al. (26) showed that ABT-627, a new ET$_A$R antagonist, had

A

B

C

Fig. 12 Effect of NEP on i.p. disseminated metastasis in nude mice. The i.p. appearance in sacrificed mice injected with 1×10^7 SKpcDNA cells (*A*) or SKNEP3 cells (*B*) 30 days after inoculation i.p. Arrowheads, disseminated tumors. *C,* total weight of i.p. tumors in SKpcDNA or SKNEP3-inoculated mice. The total weight of the disseminated tumors was smaller in SKNEP3-injected mice than in SKpcDNA-injected mice ($P < 0.0001$). Bars, SD. Each group consisted of six mice.

therapeutic effects in nude mice and synergistic effects in combination with paclitaxel. In this study, *in vivo* tumor formation was reduced when NEP was overexpressed with the down-regulation of both MMP-2 and VEGF expressions. Moreover, peritoneal metastatic potential was decreased by transfection of NEP. Owing to the majority of patients with ovarian carcinoma having advanced i.p. metastatic disease at diagnosis, the 5-year survival rates for disseminated cases remain poor. Our evidence suggests the potential of NEP for treating i.p. metastatic ovarian carcinoma although extensive work is required before this enzyme can become available for clinical use.

In summary, the current study indicates a possible functional role of NEP in ovarian carcinoma through its association with ET-1. However, NEP also degrades peptides and cytokines other than ET-1, which promotes tumor proliferation and invasion. Thus, the result of the current study may also be due to the decrease in other NEP substrates or another molecular mechanism independent of enzyme activity. Furthermore, it is possible that NEP expression in stromal fibroblasts acts similarly considering that a loss of stromal NEP was reported in poorly differentiated ovarian carcinoma tissues (20). The potential of NEP for treating ovarian carcinoma including gene therapy is promising although extensive study is required to elucidate the molecular mechanisms of its activity in tumor cell biology.

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Hiroaki Kajiyama, Kiyosumi Shibata, Mikio Terauchi, et al.

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