Int J Clin Exp Pathol 2019;12(6):1932-1946
www.ijcep.com /ISSN:1936-2625/IJCEP0093897

Original Article

Epinephrine increases malignancy of breast cancer through p38 MAPK signaling pathway in depressive disorders

Xueyan Ouyang[1*], Zhen Zhu[4*], Chao Yang[2], Li Wang[2], Gang Ding[5], Feng Jiang[2,3]

[1]Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, Shanghai 200092, China; [2]Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, Chongming Branch, Shanghai 202150, China; [3]Xinhua Translational Institute for Cancer Pain, Shanghai 202150, China; [4]Shanghai University, Shanghai 200444, China; [5]Shanghai International Medical Center, Shanghai 201318, China. [*]Equal contributors.

Received March 15, 2019; Accepted April 23, 2019; Epub June 1, 2019; Published June 15, 2019

Abstract: Objective: To uncover the possible mechanism and the effects of epinephrine on tumor growth in depression. Materials and methods: A chronic mild stress (CMS) model was employed to test the change of serum epinephrine levels in mice with depression. Tumor tissues and cell lines were analyzed to identify the molecular and cellular events influenced by depression *in vitro*. Results: The level of epinephrine was up-regulated in CMS mice serum. We found that β-adrenergic receptors (β-ARs) were expressed on MCF-7 and MDA-MB-231 breast cancer cells and that epinephrine enhanced the proliferation, migration, and invasion of breast cancer cells. Treatment of epinephrine increased the phosphorylation of p38 MAPK in cells and enhanced the growth of tumor in *vivo*. These two effects were significantly attenuated by propranolol (a β-adrenergic receptor antagonist). Conclusions: These findings suggest that activation of epinephrine-induced p38 MAPK signaling pathway enhances the malignancy of breast cancer in depressive disorders. More effective psychological intervention might improve prognosis of cancer patients.

Keywords: Depression, breast cancer, epinephrine, β-adrenergic receptors, p38 MAPK

Introduction

Depression is now considered an independent risk factor for mortality in breast cancer [1] and is expected to become the second most common mental illness in the world by 2020. Depression can lead to insomnia, weight loss, even suicide and development of cancer [2]. Extensive research indicates that there is a significant correlation between physiological stress, such as depression, and clinical diseases [3, 4], especially in breast cancer patients [5, 6]. Breast cancer is the most common cancer in women and the most common cause of cancer death among women aged 15-59 according to the latest statistics released by the National Cancer Center of China [7]. Clinical research has already found that cancer patients with depression have poorer prognosis and shorter survival times than patients who are not depressed [8], but most anti-tumor treatment programs only focus on eliminating and alleviating physical symptoms without addressing anxiety, fear, or depression.

Recently, a 10-year meta-analysis of the relationship between psychological disorders and breast cancer showed that people with trauma and poor mental status, especially depression, had increased risk of breast cancer [9]. Interestingly, retrospective analyses of breast cancer patients in three independent epidemiologic studies revealed that β-blockers significantly reduced metastasis formation and consequently prolonged patient survival time [10-12]. The effect was also observed in melanoma [13], ovarian cancer [14], and prostate cancer [15]. As for breast cancer, there was a report that depression leads to changes in a variety of neurotransmitters and hormones such as serotonin, epinephrine and β-adrenaline receptors (β-ARs) [16]. But it is still unknown how depression has an effect on breast cancer.

Here we evaluated the expression of epinephrine in mice with symptoms of depression, then we studied the effect of epinephrine on proliferation, migration, and invasion of MCF-7 and

Table 1. CMS model procedure

Days	Stress types and order
1	19:00 (the day before) wet bedding overnight; 10:00 restraint stress
2	15:00 cold; 17:00 light overnight
3	12:00 dark treatment, 3 hours; 15:00 forced swim
4	7:30 wet bedding, all day; without food and water, 24 hours
5	13:00 forced swim; 19:00 social isolation, overnight
6	14:00 cold; 15:00 dark treatment, 3 hours
7	19:00, wet bedding, cage tilt overnight
8	19:00 social isolation, without food and water, overnight
9	16:00 restraint stress; 19:00 light, overnight
10	9:00 forced swim; 10:00 restraint stress

MDA-MB-231 breast cancer cells and cancer progression in a mouse model. Our work emphasizes that the psychological problems of cancer patients may be relieved during the antitumor treatment process.

Materials and methods

Chronic mild stress (CMS) model

All animal procedures were performed according to protocols approved by the Institutional Animal Care and Use Committee of Shanghai Jiao Tong University School of Medicine. The CMS experiment was performed with 40 female C57BL/6 mice (14-16 g) obtained from the Laboratory Animal Center, Xinhua Hospital, Shanghai Jiaotong University School of Medicine. The CMS model was described previously [17]. Briefly, mice were kept on a 12-h light: 12-h dark cycle (light on at 7:00 a.m.) in at 25°C with water and food ad libitum. Mice were randomly assigned to the control or experimental group (n=20 per group). The experimental group was exposed to stressors, two per day, for 10 days (**Table 1**). Stressors included restraint stress (1 hour), cold (1 hour), forced swim (10 min), light/dark cycle disturbance (12 h), strobe light, social isolation (24 h), no/wet bedding (12 h), food and water deprivation (24 h), and cage tilt (12 h). Control group mice were not subjected to stress. Mice were weighed daily. Behavioral testing includes open field experiment (crossing score is the total numbers of grids mice crawl, rearing score is the times of raising forelimbs), forced swimming test, and sucrose preference experiment was used to confirm whether the animals had significant depressive symptoms after procedure of CMS. Serum was collected from all animals right after behavioral testing.

Electroencephalogram (EEG)

Mice were anesthetized with 5% isoflurane, and maintained at 1.5%. Anesthetized mice were implanted with electrodes. With the mouse on the stereotaxic instrument, a hole was drilled through the skull above the prefrontal cortex. The electrode was inserted into the subdural cortical material and fixed in place with dental cement. EEG data were recorded after surgery.

Enzyme-linked immunosorbent assay (ELISA)

Blood was kept at room temperature for 40 min to 1 h and then centrifuged (1500 g, 4°C, 10 min). To 300 μl of serum, an equal volume of hydrogen chloride was added, melted, and then centrifuged at low speed. Epinephrine was detected in the serum lysates using an ELISA kit from MEIMIAN according to manufacturer's instructions. Each sample was assayed in triplicate, and a mean OD value for each sample was calculated on the basis of the standard curve. Each serum sample was analyzed independently three times.

Cell culture

Human breast cancer cell lines (MCF-7 and MDA-MB-231) were purchased from the Cell Bank (Shanghai Institutes for Biological Sciences and Chinese Academy of Sciences). All cells were cultured in Dulbecco's Modified Eagle's medium (DMEM), supplemented with antibiotics, and 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific) at 37°C in a humidified atmosphere of 5% CO_2. Cells expressing green fluorescent protein were preserved in our laboratory.

Immunocytofluorescence

MCF-7 and MDA-MB-231 cells were put onto coverslips in 6-well plates at a density of 2×10^4 cells for 24 h. After washing with cold PBS, cells were fixed with 4% paraformaldehyde, permeabilized with 0.15% Triton X-100, and then

washed extensively with PBS. Cells were first incubated with primary antibodies against anti-β1-AR (dilution 1:100; cat. no. ab3442; Abcam) or anti-β2-AR (dilution 1:100; cat. no. ab61778; Abcam) at room temperature for 1 h. Finally, cells were incubated with secondary antibody (dilution 1:1000; cat. no. BD5003; Bioworld) for 1 h at room temperature in the dark. After washing with PBS, the cells were counterstained with 4,6-diamidino-2-phenylindole (DAPI) and visualized with a fluorescence microscope (Olympus).

Cell viability assay

Cell viability was assessed using CCK-8 assay (Life Technologies). The cells were plated into a 96-well plate (1×10^3 cells per well in 100 µl) with or without treatment and cultured at 37°Cwith 5% CO_2 for 24, 48, or 72 h. Following this treatment, 10 µl CCK-8 was added to each well, and, after incubating for 2 h, the optical density at 450 nm was quantified using the Multiskan FC enzyme immunoassay analyzer (Thermo Fisher Scientific, Inc.). This reading was divided by absorbance at 450 nm of untreated cells and multiplies by 100 to obtain the cell survival percentage. All experiments were carried out in triplicate.

Cell migration assay

A wound healing assay was performed to evaluate cell migration [18]. MCF-7 cells and MDA-MB-231 cells were seeded at a density of 2×10^4 cells per well into a well of a 6-well plate. Cells were pre-treated with or without propranolol for 45 min and then with or without epinephrine. After 48 h, cells were washed with PBS three times. Cells in the denuded zone of 10 random fields were quantified using Inverted fluorescence microscope. The migration indices were calculated as follows: $[(A_{t=0}h\text{-}A_{t=\Delta h})/A_{t=0h}] \times 100\%$, where, $A_{t=0h}$ is the area of the wound measured immediately after scratching, and $A_{t=}\Delta_h$ is the area of the wound at 48 h after scratching. When the samples were compared with the control cells, the differences were considered significant if $P<0.05$, $P<0.01$ and $P<0.001$.

Cell invasion assay

MCF-7 (3×10^3) or MDA-MB-231 (5×10^3) cells in 200 µl DMEM without fetal bovine serum were placed in the upper chamber of a transwell insert (24 well, 0.8-µm pore size; Corning Costar) coated with Matrigel (Sigma). The lower chambers were loaded with 10 µM epinephrine (Sigma) or 20 µM propranolol in 500 µl DMEM with 10% fetal bovine serum or culture medium only as a control. After incubation at 37°C for 24 h, cells were removed from the upper chamber using a cotton swab, and the cells on the lower membrane surface were fixed with 4% formalin, stained with 0.1% crystal violet for visualization, and counted under a microscope (Leica). Three experiments were performed independently.

Cell cycle assay

MCF-7 and MDA-MB-231 cells were either stimulated or not stimulated with 1 or 10 µM epinephrine combined with 0 µM propranolol, or 20 µM propranolol alone. After 24 h, cells trypsinized and centrifuged, washed twice with cold PBS, and fixed in cold 70% ethanol at 4°C overnight. Fixed cells were collected, washed twice with PBS, and suspended in PBS containing 10 µg/mL propidium iodide (Sigma) and 100 µg/mL RNase A, then incubated at 4°C for 30 min in the dark. Cell cycle distribution was determined using flow cytometry (Becton-Dickinson FACScan).

Protein extraction and western blot

Total protein was collected from 5×10^6 MCF-7 or MDA-MB-231 cells by lysis with 200 µl of ice-cold RIPA buffer with PMSF. The extracted protein concentration was determined using the BCA Protein Assay (cat. no. P0009; Beyotime Biotechnology). Approximately 30 µg protein was loaded in each well of a polyacrylamide-SDS gel. After electrophoresis, proteins were transferred onto PVDF membranes (Thermo Fisher). The membranes were initially blocked with 5% non-fat dry milk in Tris-buffered saline for 1 h at room temperature and then incubated with the primary antibody overnight at 4°C: anti-β1-AR (dilution 1:1000; cat. no. ab3442; Abcam), anti-β2-AR (dilution 1:1000; cat. no. ab61778; Abcam), p38MAPK (dilution 1:1000; cat. no. AF1111; Beyotime Biotechnology). This was followed by incubation with the rabbit secondary antibody (dilution 1:1000; cat. no. A0208; Beyotime Biotechnology) for 2 h. Immunoreactive bands were detected with Trident ECL (cat. no. P0018FM; Beyotime Biotechnology).

Xenograft tumor model

The xenograft model experiment was performed with 20 nude mice (14-16 g) purchased from Sino-British SIPPR/BK Lab Animal Co. Mice were housed 5 per cage and maintained on a 12-h light: 12-h dark cycle (lights on at 8.00 a.m.) 22 ± 2°C and with food and water ad libitum. All animal procedures complied with a protocol approved by the Institutional Animal Care and Use Committee of Shanghai Jiao Tong University School of Medicine. After 1 week of acclimation to the housing conditions, mice were injected subcutaneously with 1×10^7 MCF-7 cells suspended in 0.2 ml of PBS. Prior to implantation, we confirmed that tumor cells were negative for the presence of mycoplasma and mouse pathogens. Once the tumors were approximately 300 mm^3, mice were divided randomly into the following treatment groups (5 mice per group): a) PBS, b) epinephrine (10 mg/kg), c) propranolol (2 mg/kg), d) epinephrine (10 mg/kg) plus propranolol (2 mg/kg). All treatments were injected intraperitoneally in an absolute volume of 100 µl every other day for 10 days. Tumor growth was measured two times a week with a digital caliper 2BIOL (Besozzo). Xenograft volumes were calculated using the formula: volume=$(a \times b^2)/2$, where a is the largest superficial diameter and b is the smallest superficial diameter.

In vivo imaging technology

MCF-7 cells that stably express GFP were injected subcutaneously into mice. Once tumors reached 300 mm^3, mice were randomly divided into groups that were treated with PBS, epinephrine alone, propranolol alone, or the combination of epinephrine and propranolol. On the last day of animal experiment, mice were anaesthetized with isoflurane prior to imaging. Animals were then placed into the CCD camera system light-tight chamber (IVIS, Xenogen). The grayscale surface captured a reference image (digital photo) under low light.

Immunohistochemistry

The animals were sacrificed with narcotic overdose after last imaging. Tumors were excised and fixed in 4% paraformaldehyde for 24 h at 4°C and dehydrated in 30% sucrose in PBS solution for 24-48 h. Tumor sections were obtained by a cryostat (CM3050 S, Leica) at 40 µm, and every six sections were collected in one well. The DNA was denatured for 30 min at 37°C with 2 M HCl, neutralized by treatment for 10 min with 0.1 M borate buffer (pH 8.5), then washed with PBS three times for 10 min each wash. Sections were blocked by 5% goat serum in PBS solution containing 0.3% Triton X-100 to prevent non-specific binding. The sections were incubated with the primary antibodies: rat anti-PNCA (dilution 1:250; cat. no. ab29; Abcam), rabbit anti-Ki67 (dilution1:500; cat. no. ab92742; Abcam) overnight at 4°C in a humidified box. After washing with PBS three times (10 min each wash), sections were incubated with secondary antibodies (dilution 1:100; cat. no. ab15580; Abcam) for 2 h at room temperature and with DAB (cat. no. P0203; Beyotime Biotechnology) for 10 min, then counterstain with hematoxylin after 5 min washed running water. The numbers of PNCA- and Ki67-positive cells were counted from images obtained under a Leica confocal microscope.

Statistical analysis

The collected data were analyzed using IBM SPSS Statistics 22. Data are presented as means ± standard deviations (SD). Two groups were compared using the Student's *t*-test, and the data were analyzed using a one-way analysis of variance (ANOVA). $P<0.05$ was considered significant. All experiments were repeated at least 3 times, and representative data are shown.

Results

Epinephrine concentration correlates with symptoms of depression in a mouse model

To model the effects of depressive symptoms on tumor growth, we first established the CMS model and verified it (**Figure 1A-F**). Mice subjected to stress were compared to controls housed under standard conditions. Serum from mice subjected to CMS had significantly higher levels of epinephrine than controls (**Figure 1G**). There was a positive correlation between epinephrine level and degree of depression as suggested by open-filed tests, but in forced swimming test, there was no significant linear correlation (**Figure 1H-J**).

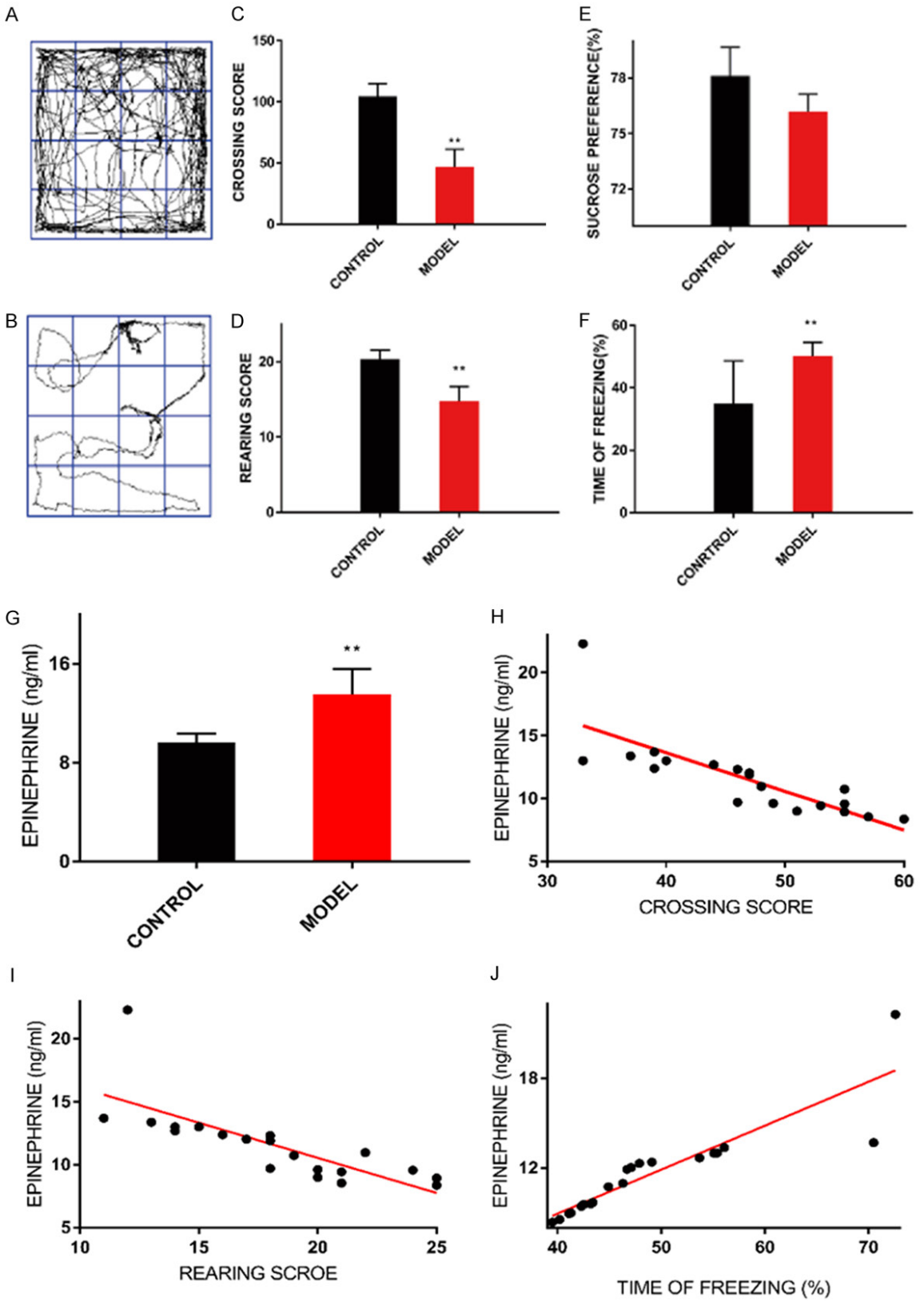

Figure 1. Epinephrine level in animals with depressive symptoms. (A) Typical depression mice field map. (B) Normal mice field map. (C) Crossing score of open field experiment. (D) Rearing score of open field experiment. (E) Sucrose preference experiment. (F) Forced swimming experiment results. (G) Epinephrine levels in control mice and mice subjected to CMS determined by ELISA. (H-J) Epinephrine levels in CMS mice plotted versus (H) open field score (crossing). (I) Open field test scores (rearing), and (J) Immobility time in the forced swimming test.

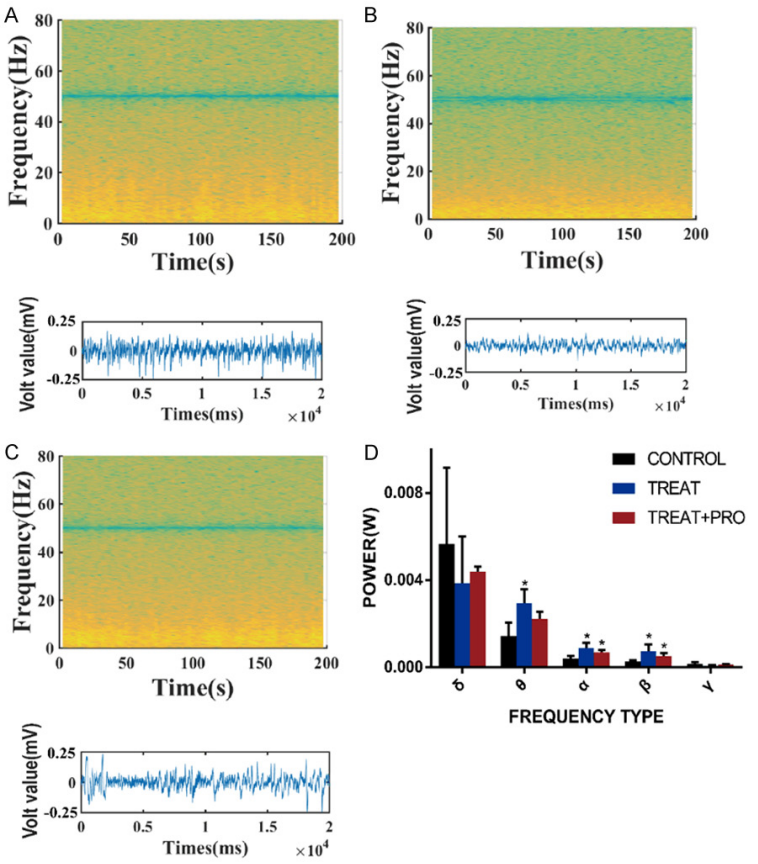

Figure 2. EEG indicates that propranolol partly alleviates EEG changes in depressed mice. (A-C) Pseudo-color and waveform of EEG, (A) control group, (B) CMS group and (C) PRO group. (D) EEG showed that depression induced changes in the mice brain waves that θ, α, β rhythm has increase in significance. Data are presented as means ± SD, n=3. *P<0.05, **P<0.01 relative to untreated control cells.

Depression may affect the brain electrical rhythm of animals, and β blockers can weaken this effect

Mice from both depressed and non-depressed groups were evaluated by electroencephalogram. Mice subjected to CMS had significantly increased intensities in brainwaves in θ, α, and β rhythm compared to non-stressed animals (**Figure 2D**). Treatment with propranolol reduced intensities at each of the three frequencies. This confirmed that the CMS model did depress the mice and that the β-AR blocker propranolol may reduce depression.

Abundant expression of beta receptor on breast cancer cell surface

In order to investigate whether epinephrine receptors are expressed in human breast cancer, we evaluated the expression of β-ARs in MCF-7 and MDA-MB-231 cells by western blot (**Figure 3A**) and immunofluorescence experiments (**Figure 3B**). Both experiments indicated that both β1-AR and β2-AR were expressed by MDA-MB-231 and MCF-7 cells.

CCK8 experiment provides the most suitable concentration of epinephrine in breast cancer cells

We explored the relationship between the effects of different concentrations of epinephrine and two kinds of breast cancer cells by a CCK8 experiment. We found that the optimal concentrations of MDA-MB-231 (**Figure 4A**). and MCF-7 (**Figure 4B**) breast cancer cell lines were 1 and 10 μM.

Epinephrine increases malignant properties of breast cancer cells in culture

In a wound healing assay, we observed that epinephrine stimulated cell migration and that this effect was abolished by propranolol (**Figure 5**). Using a Transwell assay we determined that the invasive abilities of both types of breast cancer cells were enhanced by epinephrine and that the enhancement was inhibited by the β-adrenergic receptor antagonist propranolol (**Figure 6**). Considered together, these results suggest that epinephrine promotes the malignancy of breast cancer.

Epinephrine promotes proliferation of MDA-MB-231 and MCF-7 cell lines

Furthermore, epinephrine caused increased accumulation of cells in S phase and decreased accumulation of cells in G phase. In cells treated with propranolol and epinephrine, the number of cells in S phase was reduced compared to those treated with epinephrine alone. This indicates that epinephrine promotes DNA synthesis in MDA-MB-231 (**Figure 7E**) and MCF-7 cell lines (**Figure 7J**).

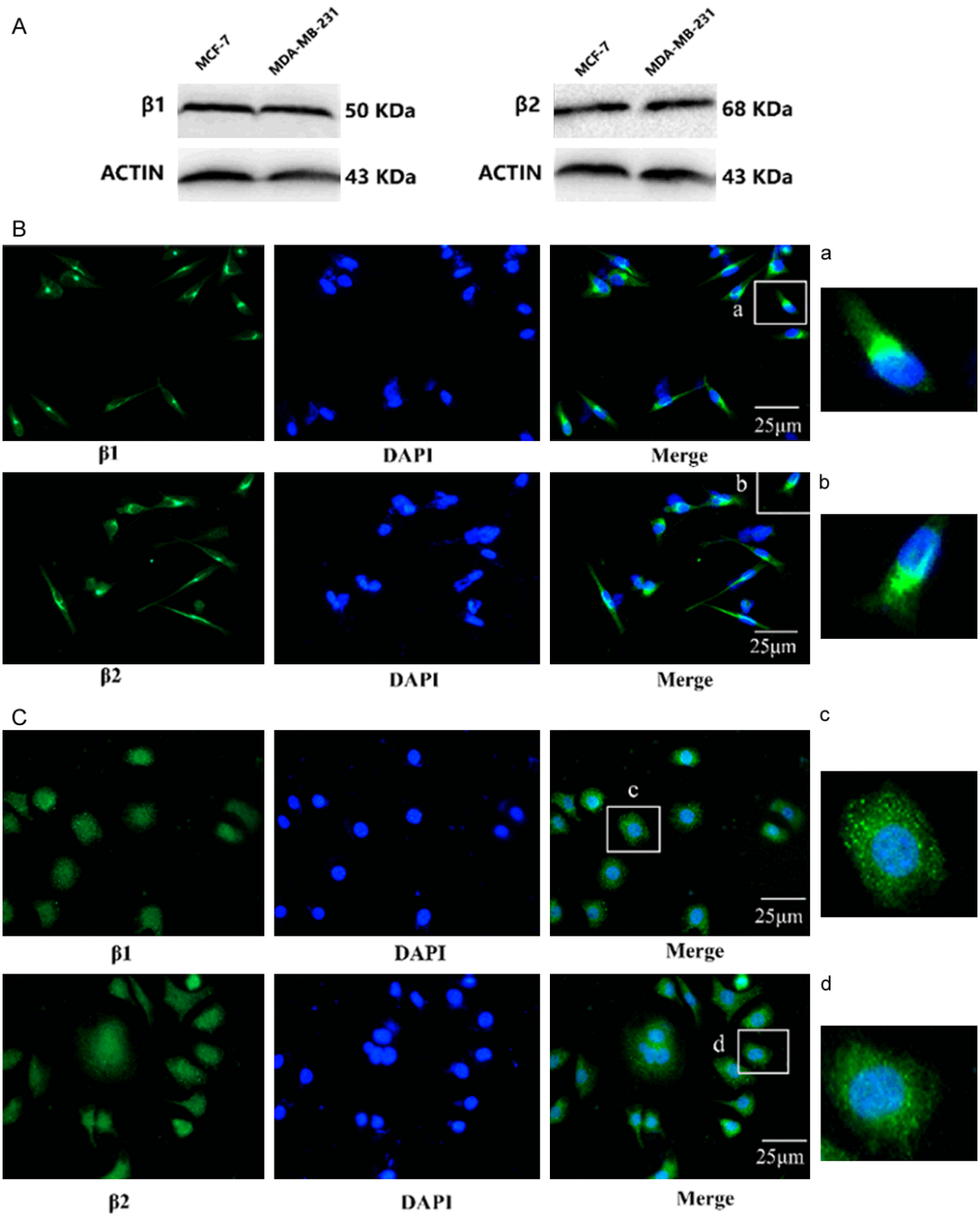

Figure 3. Expression of β-AR receptors in MCF-7 and MDA-MB-231 cell lines. (A) Expression of β1-AR and β2-AR in MCF-7 and MDA-MB-231 cells analyzed by western blot. (B, C) Immunofluorescence analysis of (B) MDA-MB-231 and (C) MCF-7 cells stained for β1-AR (upper images) and β2-AR (lower images); nuclei were stained with DAPI; (a-d) the expression of the beta adrenal receptor on the cell membrane.

Epinephrine influences MDA-MB-231 and MCF-7 cell lines by the p38 MAPK pathway

To determine the specific signaling pathway involved in the epinephrine-mediated enhancement of tumor growth, the activation states of key signaling proteins, including PI3K, AKT, mTOR (data not shown), and p38 MAPK, were measured in breast cancer cells treated with epinephrine in culture. Of the proteins evaluat-

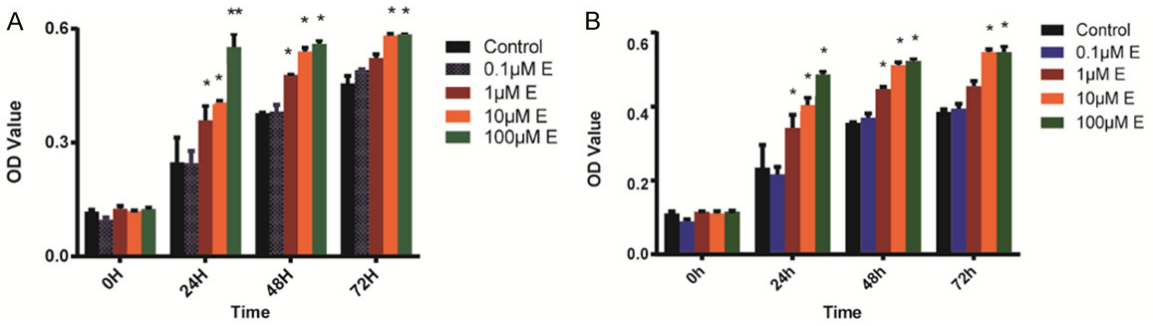

Figure 4. Epinephrine enhances cancer cell proliferation. (A, B) Proliferation of (A) MCF-7 and (B) MDA-MB-231 cells incubated with indicated concentrations of epinephrine was analyzed as a function of time using a CCK-8 assay. Data are presented as means ± SD, n=3. *P<0.05, **P<0.01 relative to untreated control cells.

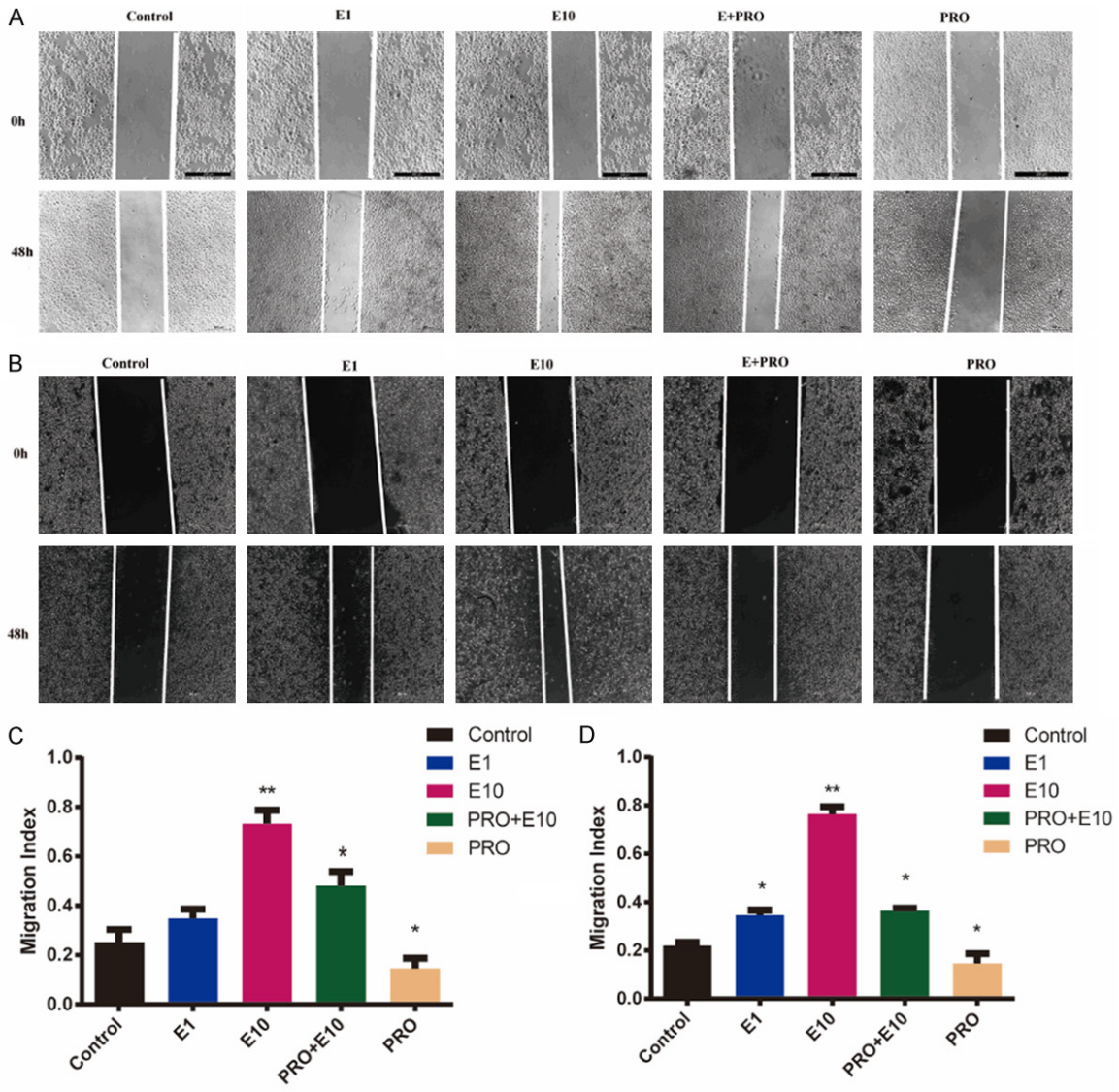

Figure 5. Epinephrine enhances migration of MCF-7 and MDA-MB-231 cells. Cell migration rates were quantitatively assessed by counting the number of cells in the exposed area 0 and 48 h after wound induction. (A, B) Images of (A) MCF-7 and (B) MDA-MB-231 cells treated with growth medium only (control), 1 μM epinephrine (E1), 10 μM epinephrine (E10), 20 μM propranolol plus 10 μM epinephrine (E10+PRO), or 20 μM propranolol (PRO). (C, D) Migration indices for (C) MCF-7 and (D) MDA-MB-231 cells treated as indicated. Data are means ± SD, n=3. *P<0.05, **P<0.01 relative to control.

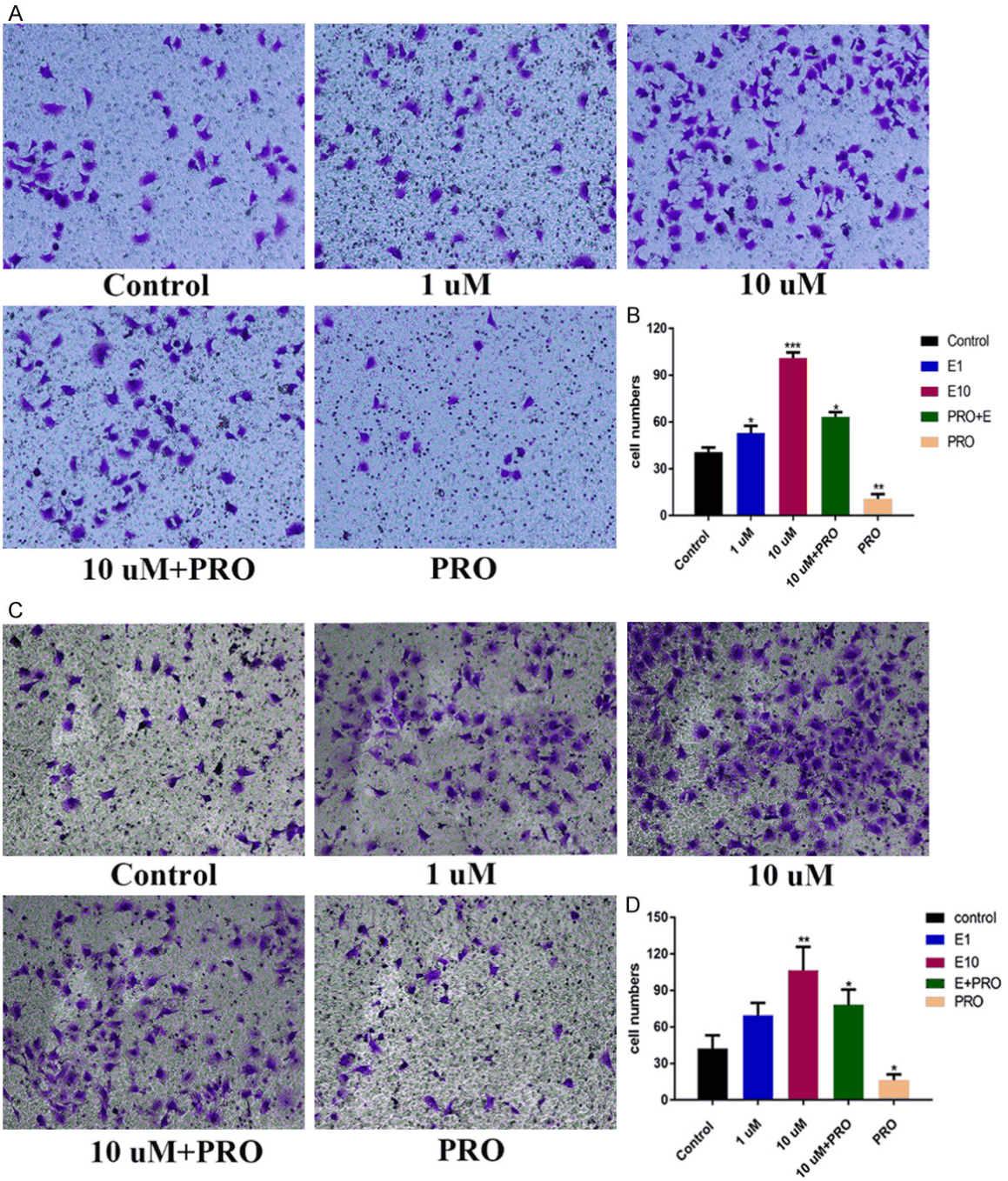

Figure 6. Epinephrine stimulates the invasion of breast cancer cells and is blocked by propranolol. A. MCF-7 cells were incubated in growth medium only (control), 1 μM epinephrine (E1), 10 μM epinephrine (E10), 20 μM propranolol plus 10 μM epinephrine (E10+PRO), or 20 μM propranolol (PRO). Images of cells that migrated through the membrane; cells were fixed and stained with crystal violet. B. Number of MCF-7 cells treated as indicated that migrated through the membrane. C. MDA-MB-231 cells were treated with growth medium only (control), E1, E10, E10+PRO, or PRO, and membranes were imaged. D. Number of MDA-MB-231 cells treated as indicated that migrated through the membrane. Data are means ± SD, n=3. *P<0.05, **P<0.01 relative to control.

ed, only p38 MAPK activation was altered. Treatment with epinephrine resulted in an increase in phosphorylation of p38 MAPK without altering p38 MAPK levels relative to control cells (**Figure 8A**, **8B**). Combination treatment with the β-AR antagonist propranolol complete-

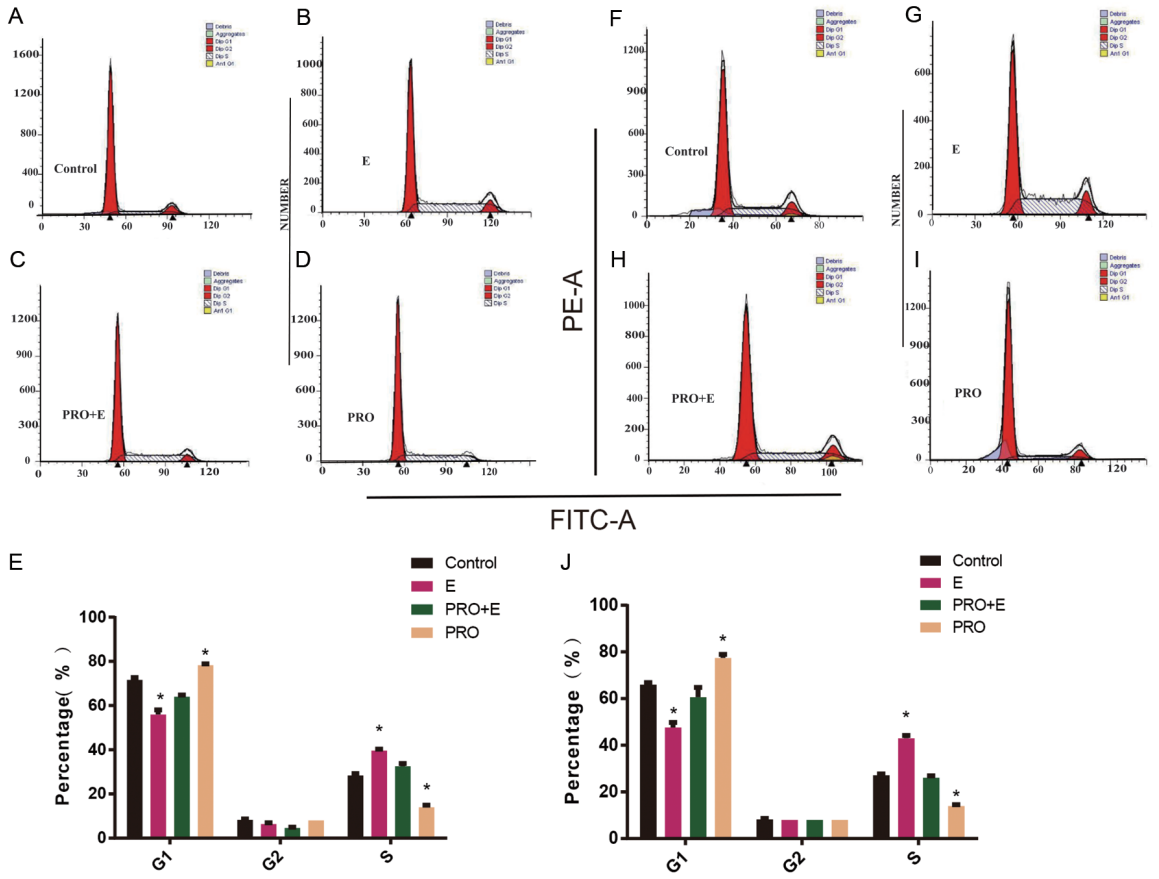

Figure 7. Epinephrine causes accumulation of breast cancer cells in the S phase. As the pictures show, both (A-D) MCF-7 and (F-I) MDA-MB-231 analysis of cell cycle distribution. E increased the S-phase and decreased the G-phase, which was reversed by pre-treating with PRO in (E) MCF-7 and (J) MDA-MB-231. The photograph shows the results determined by flow cytometry. Data are means ± SD, n=3. *P<0.05 relative to control.

ly blocked epinephrine-induced p38 MAPK activation, which indicated that p38 MAPK mediates the effect of epinephrine.

Epinephrine promotes tumor growth in vivo and propranolol inhibits this effect

We reasoned that we might see more definitive effects if mice were treated with epinephrine. Mice were MCF-7 cells that stably express GFP were injected subcutaneously. Once tumors reached 300 mm^3, mice were randomly divided into groups that were treated with PBS, epinephrine alone, propranolol alone, or the combination of epinephrine and propranolol. The tumor volume increased with time, the E group grew faster than the other groups, and the PRO group was significantly slower than the control group (**Figure 9A**). At 21 days after injection, the amount of tumor luminous flux was significantly higher in epinephrine treated mice than in controls, and this effect was relieved by combined treatment with propranolol. However, we had not observed tumor cells invasion of adjacent tissues and distant sites (**Figure 9B**). After sacrifice, tumors were excised and weighed. Tumors from mice treated with epinephrine were heaviest; tumors from mice treated with propranolol were lightest (**Figure 9C**). Immunohistochemistry of tumor sections revealed a significant increase in expression of Ki-67 and PNCA in tumors cells from epinephrine-treated mice relative to controls that was antagonized by propranolol in the mice treated with the combination of drugs (**Figure 9D**). Thus, increased levels of epinephrine, a hormone associated with depression, promoted tumor growth, and the β-adrenergic receptor antagonist could abolish this effect.

Discussion

Previous analyses of patients with different types of cancer have shown that chronic stress,

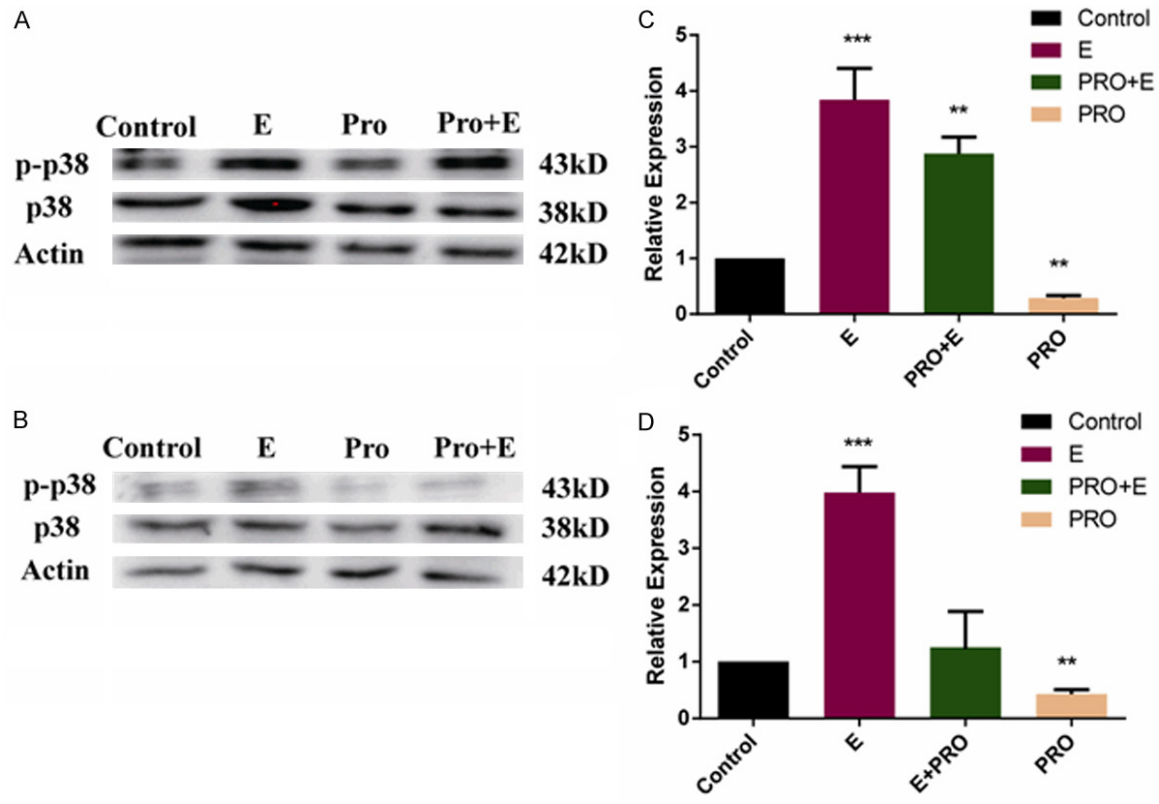

Figure 8. Epinephrine triggers proliferation of breast cancer by enhancing p38 MAPK signaling. (A, B) Western blot analysis of p38 MAPK in (A) MDA-MB-231 and (B) MCF-7 cells after treatment with medium (control), 10 μM epinephrine (E), 20 μM propranolol (PRO), or 10 μM epinephrine plus 20 μM propranolol (E+PRO) for 24 h. Actin was used as a loading control. (C, D) Quantification of p38 phosphorylation was determined as an increase in the ratio of phos-P38MAPK to total P38 MAPK. (C) MDA-MB-231, (D) MCF-7 Data are means ± SD, n=3. **P<0.01, ***P<0.001 relative to control.

especially depression, can accelerate tumor development [19-21], but the mechanisms of promotion of cancer progression in depression is unknown. It has been reported that norepinephrine [22], epinephrine and dopamine [23] play an important role in depression, and drugs that regulate epinephrine, norepinephrine and dopamine are used to intervene in depressive symptoms [23]. In this study, we first established a chronic depression model in mice and measured the levels of epinephrine and norepinephrine in the blood of depressed mice. We found that the levels of epinephrine and norepinephrine (Data do not show that experiments are under way) in depressed mice were significantly higher than those in the control group, and EEG studies also found that changes of rhythm in mice after depression could be partially reversed by propranolol, an epinephrine inhibitor. Therefore, epinephrine as a key factor has been used to study the relationship between epinephrine and breast cancer progression.

MCF-7, estrogen receptor positive cells, and MDA-MB-231, estrogen receptor negative cells, were the cells used in this study. We chose these two cells because the expression of estrogen receptor is an important feature of breast cancer [24]. In cell experiments, we first examined the expression of β-adrenergic receptors in two kinds of cells and found that these two receptors were abundantly expressed on the cell membrane, which laid a foundation for us to explore the biologic effects of epinephrine on breast cancer cells. Although there has been agreement that the epinephrine is a factor in cancer growth, conflicting results have been reported. For example, Dethlefsen et al. reported that epinephrine could inhibit proliferation of breast cancer [25], whereas Reeder et al. reported that epinephrine increased invasiveness of breast cancer [26]. Here we explored the concentration of epinephrine in breast cells and evaluated the effects of epinephrine on proliferation, migration, and invasion of two breast cancer cell lines.

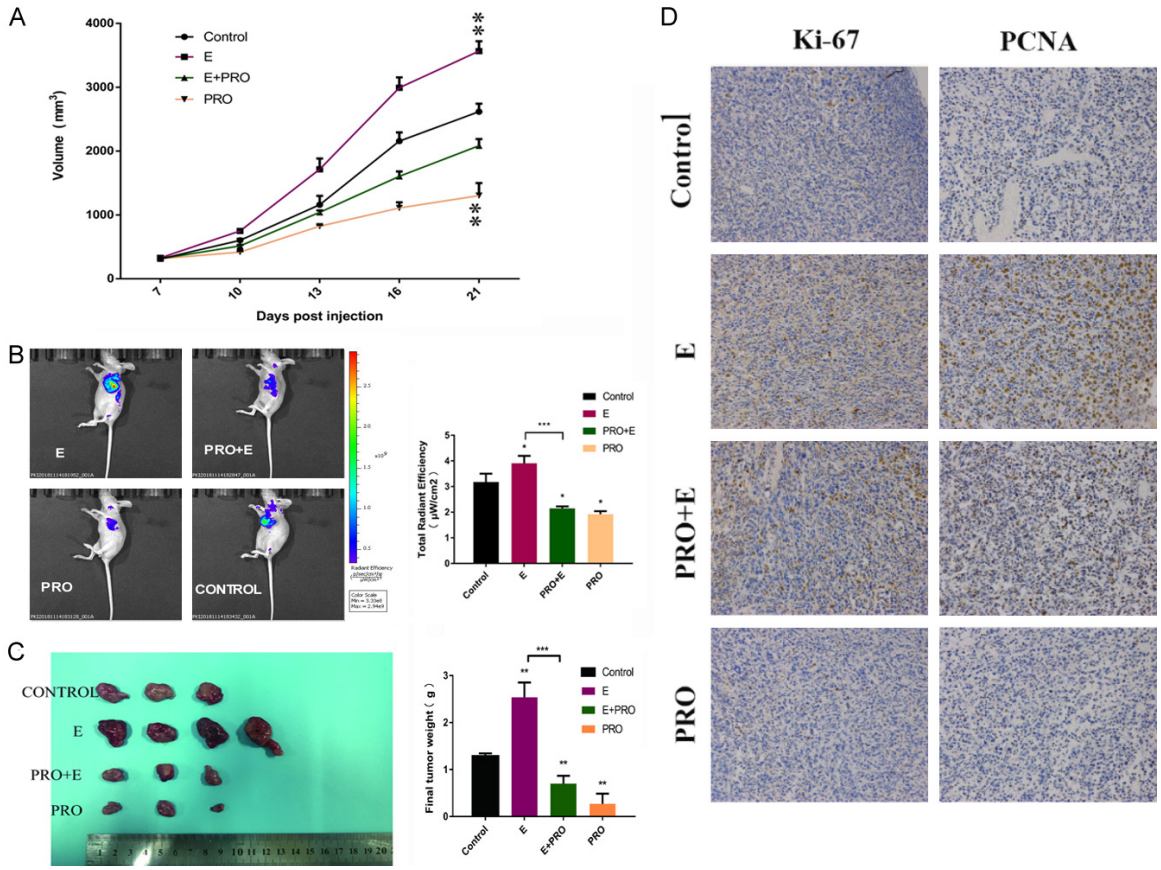

Figure 9. Epinephrine enhances tumor growth *in vivo*. (A) The tumor volume E Group grew faster than the other groups, and the PRO group was significantly slower than the control group. (B) Total luminous flux quantified by bioluminescence imaging in tumor-bearing mice treated with PBS (control), epinephrine alone (E), The combination of epinephrine and propranolol (PRO+E), or propranolol alone (PRO) with time. Images of representative mice and quantification of luminescence on day 21. (C) Photographs of representative breast tumors from each group and quantification of tumor weight. (D) Immunohistochemical analysis of Ki-67 and PNCA in representative tumor sections from each group. Data are means ± SD, n=3. *P<0.05, **P<0.01, ***P<0.001 relative to control.

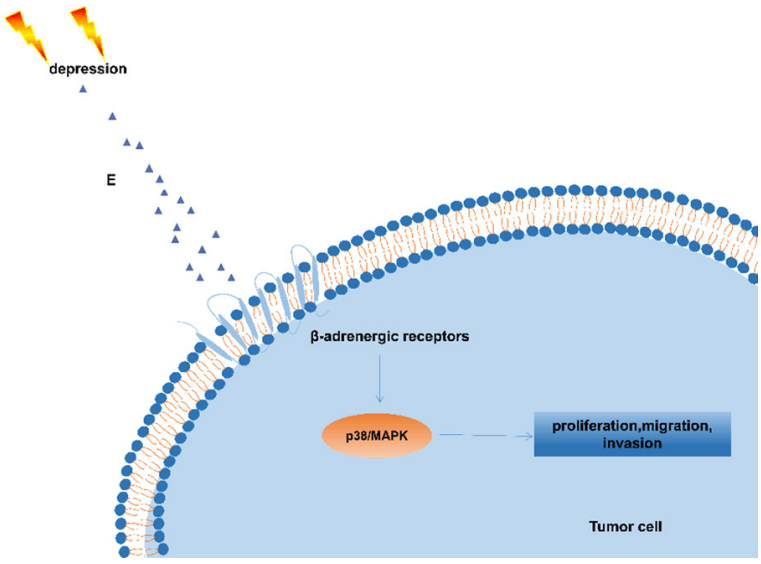

Figure 10. Depression increases levels of epinephrine in serum. Epinephrine activates β2-AR, which results in activation of the p38 MAPK signal pathway, enhancing proliferation, migration, and invasion of tumor.

We found that epinephrine did increase the proliferation, invasion, and metastasis of breast cancer cells.

p38 MAPK and PI3K/AKT signaling pathways are common pathways related to invasion and metastasis of breast cancer [27, 28]. We found that epi-

nephrine has no significant effect on PI3K/AKT pathway in breast cancer cells, but can significantly regulate the p38 pathway. The effects of p38 MAPK signaling pathways depend on the type of cancer. Downregulation of the p38 MAPK pathway significantly decreases liver cancer cell proliferation [29], but the activation of p38 MAPK signaling suppresses non-small lung cancer development [30]. Here we found that phosphorylation of p38 MAPK increased significantly in breast cancer cells after epinephrine treatment, but it did not alter levels of p38 protein expression. Propranolol, a β-adrenergic receptor blocker, significantly decreased protein expression. These results indicated that the p38 MAPK pathway plays a key role in influencing breast cancer cell proliferation when β-adrenergic receptors are activated and suggested that epinephrine might increase malignancy of breast cancer through the p38 MAPK signaling pathway.

The nude mice tumorigenesis experiment and in vivo imaging experiment also found that epinephrine can promote the growth of tumors, while injecting epinephrine plus propranolol into nude mice did not cause significant growth of tumors. Propranolol alone can even delay the growth of tumors, which may be related to the neutralization of endogenous adrenaline in nude mice by propranolol. However, partly since the malignancy of breast cancer is weaker than other malignant tumors, and the immune system and microenvironment inside the body, we have not observed distant metastasis of tumors in animal in vivo imaging.

In conclusion, our study has found that epinephrine, as a significant regulatory factor in the blood of cancer patients, can promote the proliferation, invasion and migration of cancer cells through the p38 MAPK pathway (**Figure 10**). This provides guidance for our clinical work: The treatment of breast cancer should not only consider the cancer itself, but also the psychological state of the patients. We advocate timely and targeted psychological interventions for breast cancer patients with depressive symptoms as a complement to anti-cancer treatments. This will improve quality of life for breast cancer patients and may also improve prognosis. This "whole patient" strategy for breast cancer treatment will have value.

Conclusion

Taken together, the present study demonstrates that epinephrine enhances proliferation, invasiveness, and migration of human breast cancer cells by activation of the p38 MAPK signaling pathway. Serum epinephrine can be used as an indicator of the degree of depression in breast cancer patients. Physicians should consider the psychological states of their breast cancer patients to improve the quality of life and prognosis.

Acknowledgements

This research was supported by the National Nature Science Foundation of China (81572859) and it was funded by the Shanghai Commission of Health and Family Planning (20184Y0016, 201640201, 201840003, 201740027) and the Shanghai Chongming Science and Technology Committee (CKY201803).

Disclosure of conflict of interest

None.

Address correspondence to: Gang Ding, Shanghai International Medical Center, Shanghai 201318, China. E-mail: ddinggang@hotmail.com; Feng Jiang, Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, Chongming Branch, Shanghai 202150, China. E-mail: jf365321@163.com

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