P2X7 receptor stimulates breast cancer cell invasion and migration via the AKT pathway

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Abstract. Purinergic signaling has been implicated in the regulation of many cellular processes. A high concentration of ATP has been observed in the tumor microenvironment, suggesting a possible role of extracellular ATP in tumor progression. The P2X7 receptor, which belongs to the ligand-gated ion channel receptor family, is involved in tumor development and metastasis. In the present study, we found that extracellular ATP stimulated the invasion and migration of human T47D breast cancer cells, in a dose-dependent manner. BzATP (ATP analogue), but not ADP, also promoted invasion and migration. We further found that the P2X7 receptor was highly expressed in the T47D cells. After knockdown of the P2X7 receptor, ATP-stimulated invasion and migration were markedly inhibited. Moreover, activation of the P2X7 receptor by ATP downregulated the protein level of E-cadherin and upregulated the production of MMP-13. In addition, ATP time-dependently induced the activation of AKT via the P2X7 receptor, and the AKT pathway was required for the ATP-mediated invasion and migration. Taken together, our results revealed that activation of the P2X7 receptor by ATP promotes breast cancer cell invasion and migration, possibly via activation of the AKT pathway and regulation of E-cadherin and MMP-13 expression. Therefore, the P2X7 receptor may be a useful therapeutic target for the treatment of breast cancer.

Introduction

Purinergic signaling acts as an important pathway in the regulation of cell growth, differentiation and development (1). Extracellular ATP, which is widely distributed in the tumor microenvironment, has been reported to be involved in the progression of cancer (2). ATP acts through P2 receptors, which are further categorized as P2X and P2Y receptors. P2Y receptors belong to the G-protein coupled receptors, while P2X receptors belong to the ligand-gated ion channel receptors. To date, seven P2X receptor subtypes (P2X1-7) and eight P2Y receptor subtypes (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13 and P2Y14) have been cloned in human cells (3). As one subtype of the P2X receptors, the P2X7 receptor has been found to be highly expressed in many types of cancers, such as acute myelogenous leukemia (AML), acute lymphoblastic leukemia (ALL), chronic myelogenous leukemia (CML) and thyroid papillary cancer (4,5). However, the level of the P2X7 receptor is low in tissues of complex hyperplasia with atypia or in endometrial adenocarcinoma and uterine epithelial cancer (6,7).

Breast cancer is one of the most common cancers among women worldwide. Advancements in treatment including surgery, chemotherapy and radiotherapy have increased the overall survival rate of breast cancer patients (8). However, invasion and metastasis remain the major reasons for breast cancer-related mortality (9). One study reported that P2X7 receptor activation participated in the SK3 channel- and cystein cathepsin-dependent cancer cell invasiveness in MDA-MB-435s breast cancer cells (10), but the role of the P2X7 receptor in breast cancer cell invasion and metastasis requires further clarification.

In the present study, we identified that P2X7 receptor activation via extracellular ATP promoted the invasion and migration of T47D breast cancer cells. We also elucidated the function of the AKT pathway in the P2X7-mediated cell invasion and migration.

Materials and methods

Reagents. ATP, BzATP, ADP, P2X inhibitor APPDS and AKT inhibitor LY294002 were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Antibodies to E-cadherin and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies to phospho-AKT and AKT were obtained from Cell Signaling Technology (Danvers, MA, USA).

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Cell culture. Human T47D breast cancer cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and were maintained in RPMI-1640 medium, supplemented with 10% fetal bovine serum (FBS), 100 mg/ml streptomycin, 100 U/ml penicillin and 2 mM L-glutamine. Cells were grown at 37˚C in a humidified atmosphere of 95% air and 5% CO_2.

Transwell invasion assay. A 24-well cell culture Transwell chamber (Corning Costar, San Diego, CA, USA) was used to analyze the cell invasion capacity. The filters of the upper inserts were coated with Matrigel before being used. The upper inserts were seeded with $1x10^5$ cells/200 μl in RPMI-1640 medium and the lower inserts were filled with RPMI-1640 medium, supplemented with 20% FBS. After stimulation with different nucleotides (ATP, BzATP or ADP), the cells that invaded through the Matrigel and filters were fixed with methanol, and the nuclei were labeled with 4',6-diamidino-2-phenylindole (DAPI). Nuclei were counted using immunofluorescence microscopy (Nikon, Tokyo, Japan) at x200 magnification.

Transwell migration assay. Cell migration capacity was also determined using 24-well cell culture Transwell chambers (Corning Costar). Briefly, the upper inserts were seeded with $5x10^5$ cells/200 μl in RPMI-1640 medium, and the lower inserts were filled with RPMI-1640 medium supplemented with 20% FBS as a chemoattractant. The cells were stimulated with or without the different nucleotides (ATP, BzATP or ADP). After an 18-h incubation at 37˚C, the migrated cells were fixed with methanol, and the nuclei were labeled with DAPI. Finally, the nuclei were counted in seven random fields using immunofluorescence microscopy (Nikon) at x200 magnification.

RNA extraction, reverse transcription and real-time PCR. Total RNA was isolated from the T47D cells by TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Then 2 μg of RNA was reverse-transcribed into cDNA using the cDNA synthesis kit (Tiangen Biotech Co., Ltd., Beijing, China). Real-time PCR was performed with a SYBR-Green PCR kit (Tiangen Biotech) and the primers are listed in Table I. The fold-change in the expression of each objective gene relative to β-actin was calculated based on the $2^{-\Delta\Delta Ct}$ method.

Small interfering RNA transfection. A P2X7 siRNA (siP2X7) was purchased from Shanghai Genechem Co., Ltd. (Shanghai, China), with the sequence of 5'-CCGAGAAACAGGCGA UAAU-3'. A scramble sequence not targeting any known gene was used as a control siRNA (siCtrl). T47D cells were plated in 24-well plates at the density of $1x10^4$ cells/ml. Six hours later, the cells were transfected with siP2X7 or siCtrl using Lipofectamine 2000 (Invitrogen). After 36 h of transfection, real-time PCR was performed to assess the knockdown efficiency.

Western blot analysis. The cells were stimulated with or without the different nucleotides (ATP, BzATP or ADP) for various times, and the inhibitors were applied 30 min before ATP stimulation when the inhibitors were used. The cells were lysed in ice-cold RIPA buffer containing protease and phosphatase inhibitors (Applygen Technologies Inc., Beijing, China). Protein concentrations were determined with the BCA protein assay kit (Applygen Technologies). Then fifty micrograms of proteins were separated on 10% SDS gel electrophoresis and transferred to a PVDF membrane. After blocking with 5% BSA in buffered saline, the membrane was further probed with primary antibodies overnight at 4˚C, and then incubated with the secondary antibodies for 1 h at room temperature. The immunoreactive bands were detected using ECL reagents (Applygen Technologies).

Table I. Real-time PCR primers.

Gene	Sequence (5'-3')
P2X1	GGCTGACTACGTCTTCCCAG GCGCAGTAGCCTTGAGTCT
P2X2	AGCTGGGCTTTATCGTGGAGA TTGGGGTTGCACTCCGATG
P2X3	AGTCGGTGGTTGTGAAGAGC AGCCTTCTCGTGCAAGAAAAC
P2X4	CTACCAGGAAACTGACTCCGT GGTATCACATAATCCGCCACAT
P2X5	CTGTCGCTGTTCGACTACAAG CCCATACGACCAGGTACGC
P2X6	GAACCCCAGTTTTCCATCATCA GGCGTCACAAGGAAGTTGGT
P2X7	TATGAGACGAACAAAGTCACTCG GCAAAGCAAACGTAGGAAAAGAT
MMP-13	ACTGAGAGGCTCCGAGAAATG GAACCCCGCATCTTGGCTT
β-actin	AGCGCGGCTACAGCTTCA CGTAGCACAGCTTCTCCTTAAT

ELISA assay. After stimulation with or without ATP, the supernatant was collected and centrifuged at 10,000 x g for 15 min at 4˚C. The MMP-13 ELISA kit was purchased from Invitrogen (Carlsbad, CA, USA) and used to measure the protein level of MMP-13, according to the manufacturer's instructions.

Analysis of data. Experiments were repeated at least three times. Data are expressed as the means ± SD, and were analyzed using the Student's t-test. Statistical significance is indicated when $P<0.05$.

Results

Extracellular ATP promotes the invasion and migration of breast cancer cells. To determine the effect of ATP on the invasion and migration of breast cancer cells, we performed Transwell invasion and migration assays in the T47D cells. The results showed that ATP produced a concentration-dependent (100-300 μM) increase in the invasion and migration capacities of the T47D cells, with a maximal effect

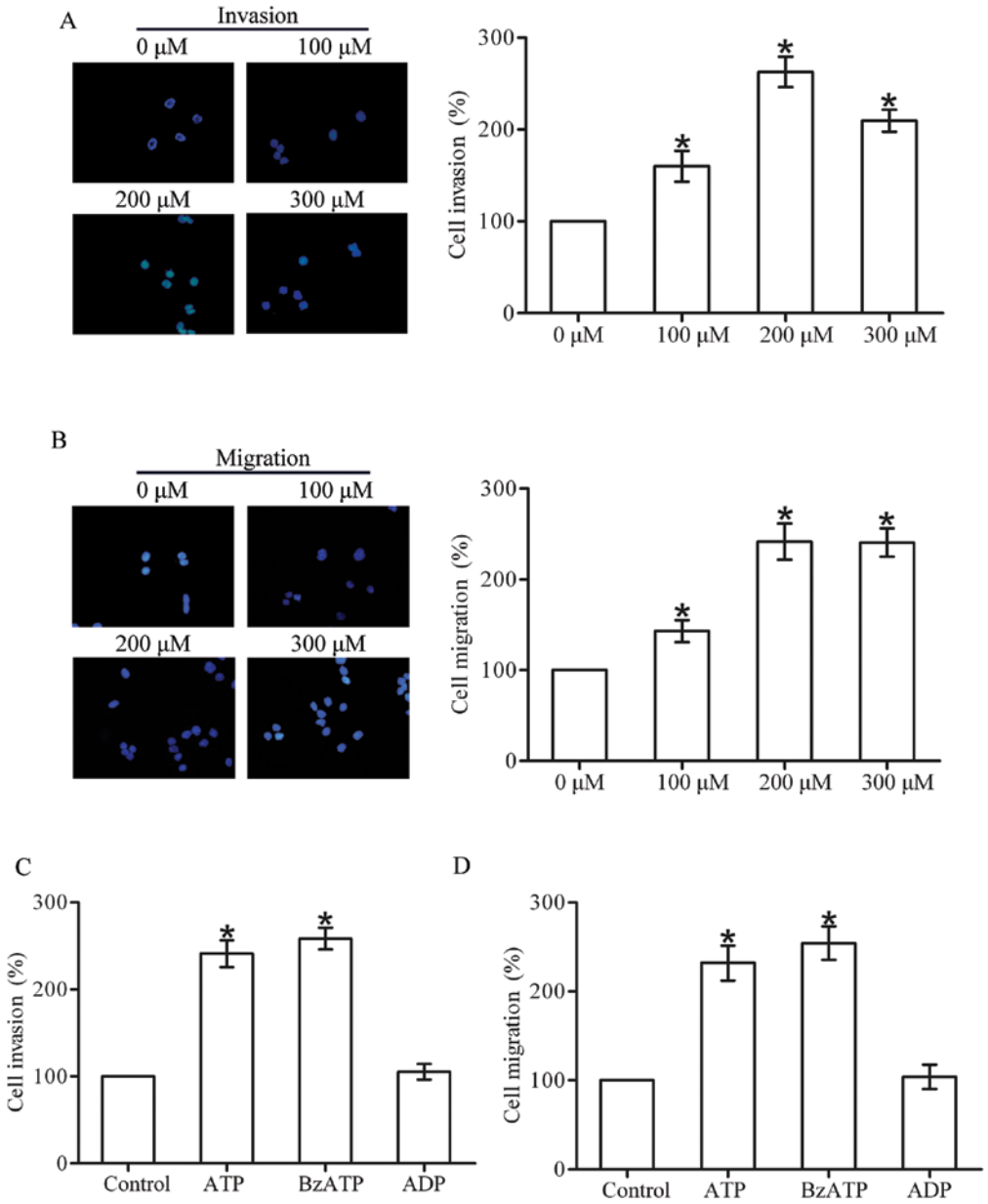

Figure 1. Effect of purinergic nucleotides on breast cancer cell invasion and migration. Human breast cancer T47D cells were stimulated with different concentrations of ATP. (A) Effect of ATP on the invasion of T47D cells after an 18-h incubation with ATP. (B) Effect of ATP on the migration of T47D cells after an 18-h incubation with ATP. (C) Invasion of T47D cells was observed after an 18-h incubation with ATP (200 μM), BzATP (200 μM) or ADP (200 μM). (D) Migration of T47D cells was observed after an 18-h incubation with ATP (200 μM), BzATP (200 μM) or ADP (200 μM); *P<0.05, compared with the controls.

occurring at 200 μM (Fig. 1A and B). Therefore, subsequent experiments were carried out using 200 μM. Furthermore, the ATP analogue BzATP also promoted the invasion and migration of the T47D cells. However, ADP had little effect on the invasion and migration (Fig. 1C and D). Together, these data suggest that extracellular ATP promotes the invasion and migration of breast cancer cells.

Effect of P2X7 receptor activation on the invasion and migration of breast cancer cells. Next, we found that PPADS (100 μM), a non-selective P2X receptor antagonist, attenuated the ATP-induced invasion and migration of the T47D cells (Fig. 2A and B). Seven P2X receptor subtypes (P2X1-7) have been cloned in human cells, and the P2X7 receptor has been reported to play an important role in tumor progression (11). Using real-time PCR, we found that the P2X7 receptor was significantly expressed in the T47D cells (Fig. 2C). Thus, we silenced the expression of P2X7 receptor in the T47D cells by transfection of siRNA (Fig. 2D), and then investigated the effect of P2X7 knockdown on cell invasion and migration. We found that knockdown of the P2X7 receptor markedly inhibited the invasion and migration of the T47D cells promoted by ATP stimulation (Fig. 2E and F), indicating the involvement of the P2X7 receptor in breast cancer cell invasion and migration.

Activation of the P2X7 receptor affects the levels of E-cadherin and MMP-13. E-cadherin is essential for tumor invasion and metastasis (12). Using western blot analysis, we found that ATP and its analogue BzATP downregulated the protein level

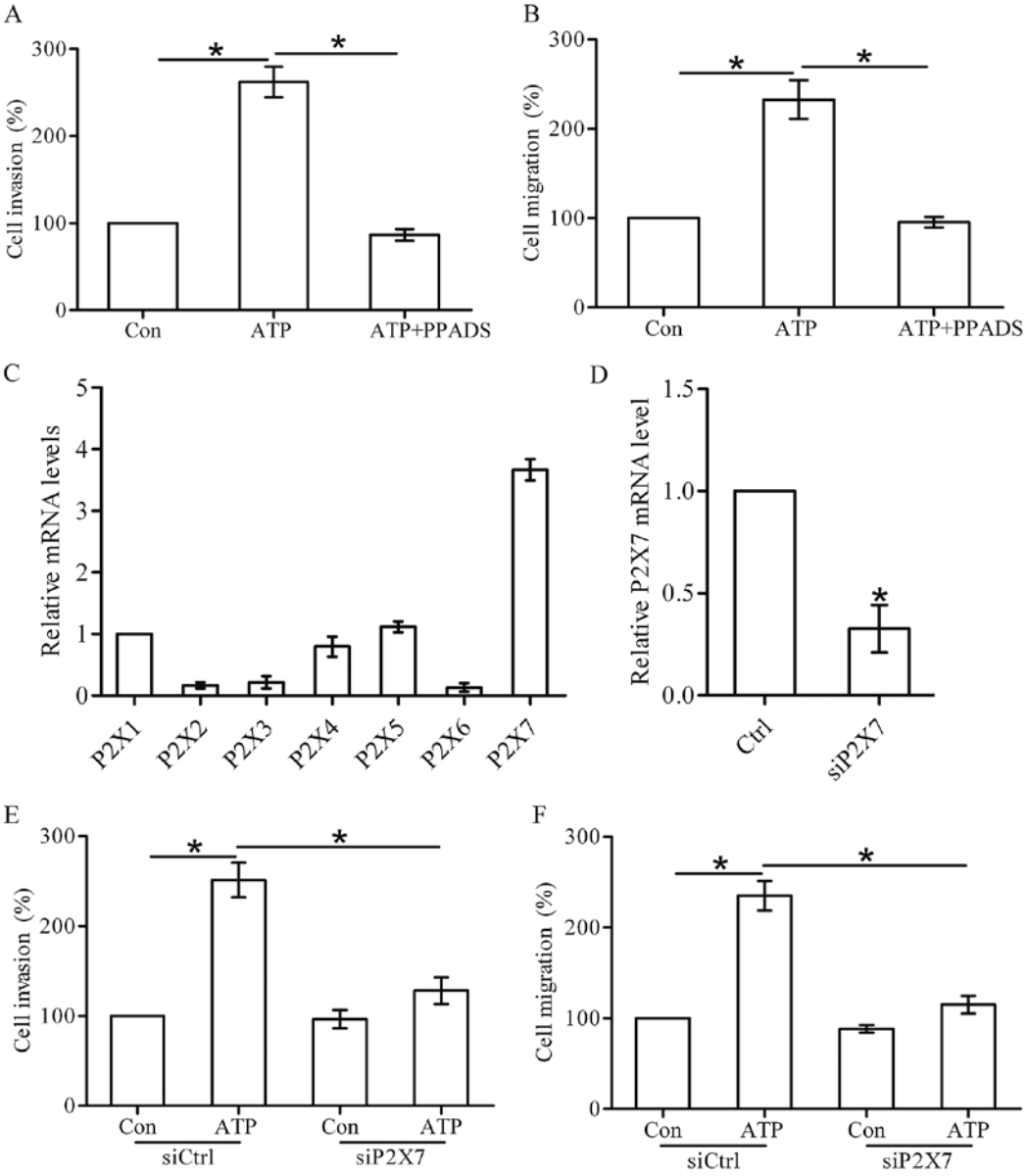

Figure 2. Involvement of the P2X7 receptor in ATP-mediated breast cancer cell invasion and migration. (A and B) T47D cells were pretreated with PPADS (P2X7 inhibitor, 100 μM) for 30 min, and then stimulated with or without ATP. Cell invasion and migration abilities were determined by Transwell invasion and migration assays. (C) The mRNA expression level of P2X receptor subtypes (P2X1-7) were detected by real-time PCR. (D) T47D cells were transfected with control siRNA (siCtrl) or P2X7 siRNA (siP2X7), and knockdown efficiency was examined by real-time PCR. (E) Effect of P2X7 receptor knockdown on ATP-induced invasion of T47D cells. (F) Effect of P2X7 receptor knockdown on ATP-induced migration of T47D cells; *P<0.05.

of E-cadherin in the T47D cells, whereas ADP did not affect the protein level of E-cadherin (Fig. 3A). MMP-13 plays an important role in tumor invasion. In the present study, real-time PCR and ELISA assay showed that ATP and its analogue BzATP increased the expression and secretion of MMP-13 in the T47D cells. However, ADP did not affect the production of MMP-13 (Fig. 3B and C).

Extracellular ATP regulates the expression of E-cadherin and MMP-13 via the P2X7 receptor. As shown in Fig. 4A, after stimulation of ATP, the expression of E-cdherin was decreased in the control siRNA (siCtrl) cells. However, the expression of E-cadherin did not show any change in the P2X7 siRNA (siP2X7) cells stimulated with ATP. Furthermore, ATP increased the expression and secretion of MMP-13 in the siCtrl cells, whereas ATP stimulation had little effect on MMP-13 production in the siP2X7 cells (Fig. 4B and C). These findings imply that activation of the P2X7 receptor by ATP decreases the expression level of E-cadherin and MMP-13 in breast cancer cells.

Activation of the P2X7 receptor by ATP induces the AKT pathway in breast cancer cells. AKT, a pivotal kinase in the cell signaling pathway, participates in tumor metastasis and development (13). Therefore, we examined whether ATP could stimulate the activation of the AKT pathway in breast cancer cells. As shown in Fig. 5A, ATP (200 μM) time-dependently stimulated a marked increase in the level of phosphorylated AKT in the T47D cells, and peak activation occurred at 30 min. Moreover, western blot analysis showed that the ATP-induced

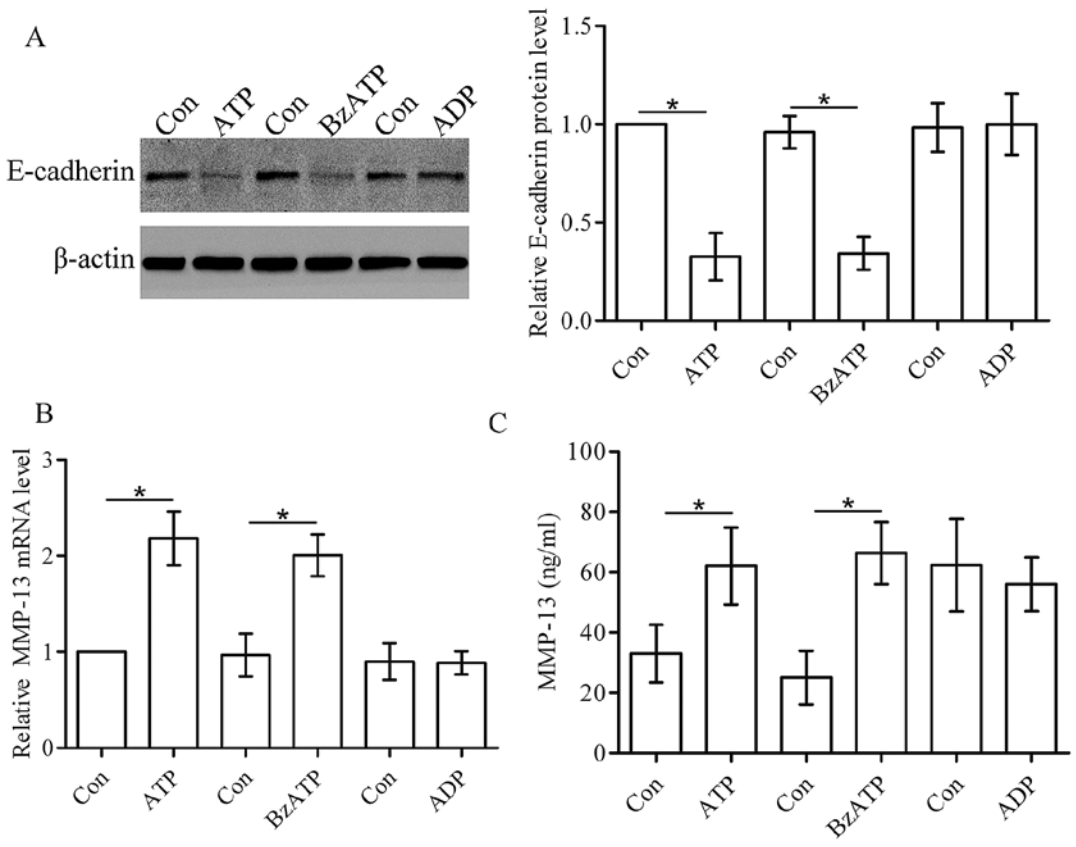

Figure 3. P2X7 receptor activation participates in the regulation of E-cadherin and MMP-13 expression in breast cancer cells. (A) T47D cells were incubated with ATP (200 μM), BzATP (200 μM) or ADP (200 μM) for 18 h, and the protein level of E-cadherin was detected by western blot analysis. (B) T47D cells were incubated with ATP (200 μM), BzATP (200 μM) or ADP (200 μM) for 18 h, and the mRNA level of MMP-13 was detected by real-time PCR. (C) T47D cells were incubated with ATP (200 μM), BzATP (200 μM) or ADP (200 μM) for 18 h, and the secretion of MMP-13 was detected by ELISA assay; *P<0.05.

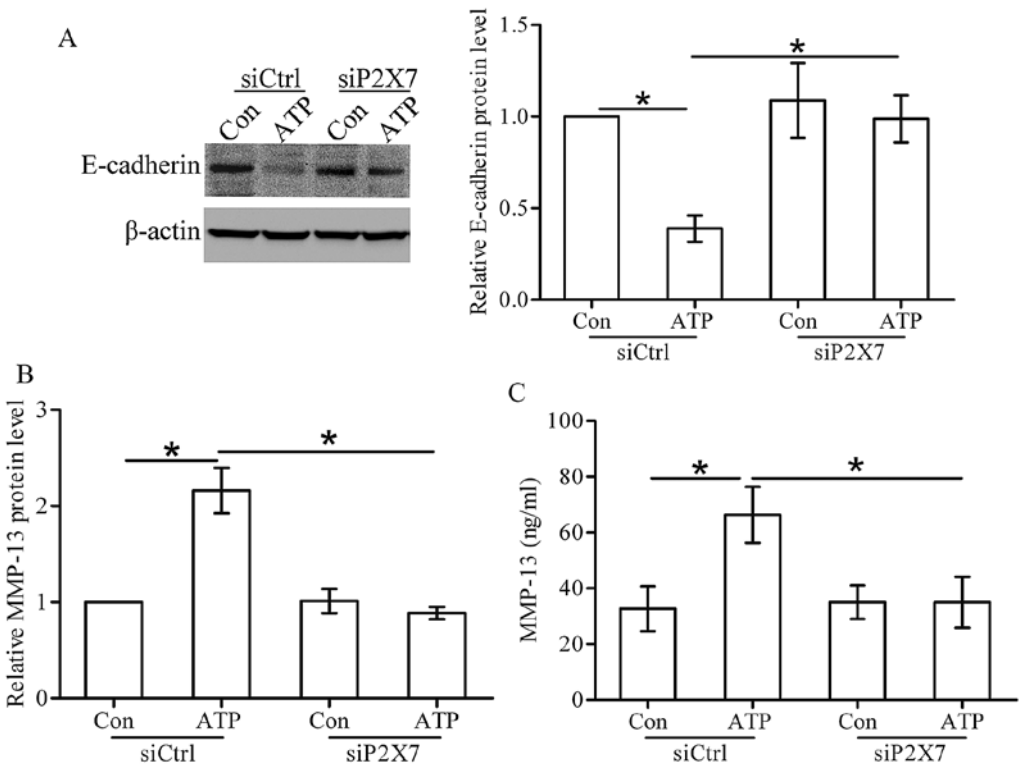

Figure 4. Effect of P2X7 receptor knockdown on E-cadherin and MMP-13 expression. (A) Western blot analysis showing the protein level of E-cadherin upon ATP stimulation for 18 h in the siCtrl or siP2X7 cells. (B) Real-time PCR and (C) ELISA assay showing the expression and secretion of MMP-13 upon ATP stimulation for 18 h in the siCtrl or siP2X7 cells; *P<0.05.

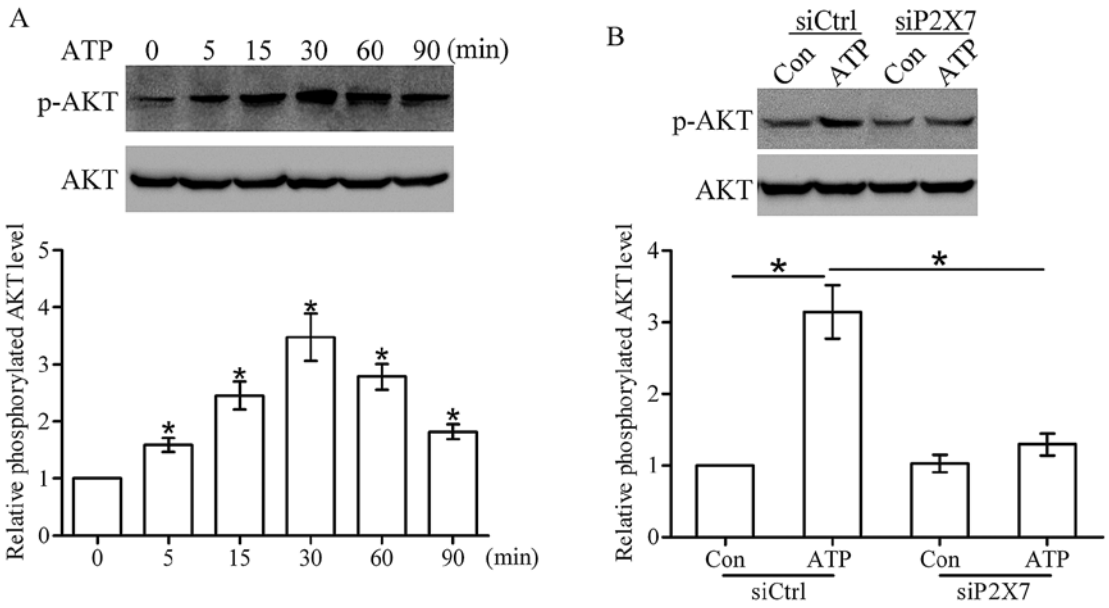

Figure 5. ATP activates the AKT pathway through the P2X7 receptor. (A) T47D cells were stimulated with 200 μM ATP for 5, 15, 30, 60 or 90 min, and the level of phosphorylated AKT was detected by western blot analysis. (B) Western blot analysis showing the level of phosphorylated AKT upon ATP stimulation for 30 min in the siCtrl or siP2X7 cells; *P<0.05.

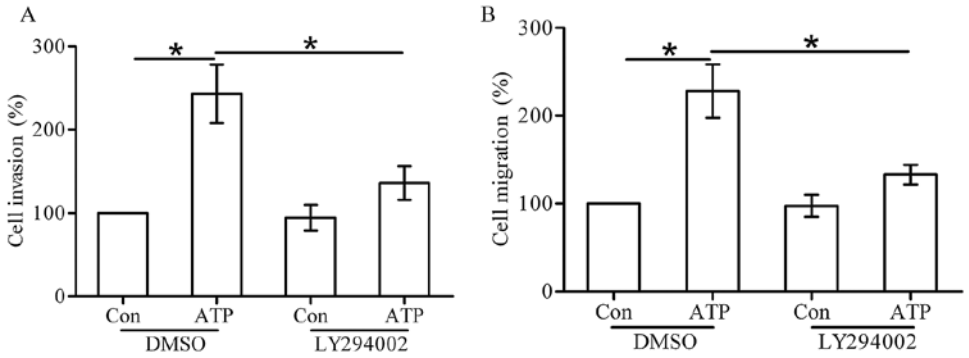

Figure 6. Role of the AKT pathway in ATP-induced breast cancer cell invasion and migration. T47D cells were pretreated with LY294002 (AKT inhibitor, 10 mM). (A and B) To assess the invasion and migration abilities, T47D cells were stimulated with or without ATP, and then subjected to Transwell invasion and migration assays; *P<0.05.

activation of AKT was greatly inhibited after knockdown of the P2X7 receptor (Fig. 5B), suggesting that the P2X7 receptor contributes to the ATP-induced activation of the AKT pathway in breast cancer cells.

Effect of the AKT pathway on P2X7-mediated invasion and migration. To determine whether the P2X7 receptor-stimulated cell invasion and migration are mediated through the AKT pathway, T47D cells were pretreated with 10 mM LY294002 for 30 min before ATP stimulation. Transwell invasion and migration assays showed that LY294002 inhibited the cell invasion and migration induced by ATP (Fig. 6A and B). These data confirm that the AKT pathway is required for the invasion and migration induced by ATP.

Effect of the AKT pathway on P2X7-mediated expression changes of E-cadherin and MMP-13. Western blot analysis showed that ATP stimulation decreased the protein level of E-cadherin in the DMSO-treated cells, whereas the effect of ATP on E-cadherin expression was attenuated in the LY294002-treated cells (Fig. 7A). Furthermore, real-time PCR and ELISA assay showed that ATP stimulation increased the expression and secretion of MMP-13 in the DMSO-treated cells, while in the LY294002-treated cells, this effect was significantly attenuated (Fig. 7B and C).

Discussion

Many reports have proved the important functions of purinergic signaling in tumor progression. In the present study, we examined the capability of P2X7 receptor activation to induce breast cancer cell invasion and migration. We found that ATP and its analogue BzATP stimulated the invasion and migration of human breast cancer T47D cells. P2X receptor inhibitor PPADS suppressed the ATP-mediated cell invasion and migration. Furthermore, knockdown of the P2X7 receptor inhibited the invasion and migration stimulated by ATP. Altogether, our data suggest that activation of the P2X7

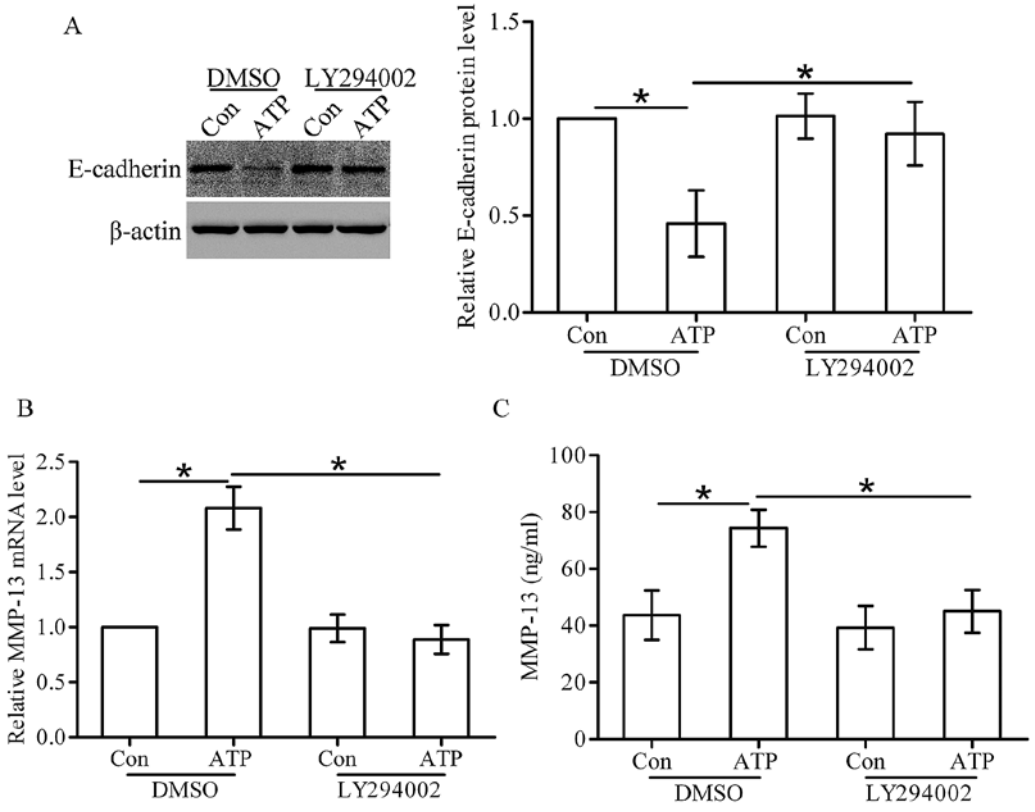

Figure 7. Role of the AKT pathway in the ATP-mediated expression changes of E-cadherin and MMP-13 in breast cancer cells. T47D cells were pretreated with LY294002 (AKT inhibitor, 10 mM). (A) To assess the E-cadherin protein level, T47D cells were stimulated with or without ATP for 18 h, and then subjected to western blot analysis. (B) To assess the MMP-13 mRNA level, T47D cells were stimulated with or without ATP for 18 h, and then subjected to real-time PCR. (C) To assess the MMP-13 protein level, T47D cells were stimulated with or without ATP for 18 h, and then subjected to ELISA assay; $^{*}P<0.05$.

receptor by ATP contributes to the invasion and migration of breast cancer cells.

Previously, studies have found that extracellular ATP stimulation leads to a decrease in the growth of tumor cells (14). Further studies showed that ATP participates in tumor motility, invasion and metastasis (15,16). In the present study, we found that ATP dose-dependently increased the invasion and migration of T47D cells. We further found that BzATP also stimulated cell invasion and migration, whereas ADP stimulation did not affect the invasion and migration of T47D cells, confirming the involvement of ATP in regulating breast cancer cell invasion and migration. ATP functions via P2 receptors, which are divided into P2X and P2Y receptors. In the present study, we found that the P2X receptor inhibitor PPADS could greatly suppress the ATP-induced cell invasion and migration, suggesting that P2X receptors may participate in the invasion and migration of breast cancer cells. There are seven P2X receptor subtypes (P2X1-7) that have been cloned in human cells. Using real-time PCR, we found that the P2X7 receptor was significantly expressed in the T47D cells. Studies have reported that expression of the P2X7 receptor is elevated in thyroid papillary cancer, and is closely associated with poor prognostic factors and lymph node metastasis (4,17,18). It was reported that activation of the P2X7 receptor increased the growth of B16 melanoma cells *in vitro* and *in vivo* (19,20) and influenced the motile activity of lung cancer cells (21). Jelassi *et al* (10) found that P2X7 receptor activation enhanced SK3 channel- and cystein cathepsin-dependent cancer cell invasiveness of MDA-MB-435s breast cancer cells. In the present study, we found that the knockdown of the P2X7 receptor inhibited the ATP-mediated invasion and migration of T47D cells, further confirming that P2X7 receptor activation promotes breast cancer cell invasion and migration.

Epithelial-mesenchymal transition (EMT) is essential for the invasion and metastasis of breast cancer cells (22). Davis *et al* (23) showed that ATP stimulated the expression of vimentin in MDA-MB-468 breast cancer cells, and Li *et al* (15) further found that ATP increased the protein levels of E-cadherin and Snail in prostate cancer cells, suggesting that ATP may affect the EMT process in tumor cells. E-cadherin, which is an important EMT-related marker in tumors, regulates cell-cell adhesion and is often weakly expressed in tumor cells (24). In the present study, we found that ATP and its analogue BzATP downregulated the expression of E-cadherin, but ADP had little effect on the expression of E-cadherin in breast cancer cells. Using siRNA technology, we confirmed that ATP stimulation decreased the protein level of E-cadherin via the P2X7 receptor.

A member of the metalloproteinases (MMPs), MMP-13 plays a key role in regulating tumor invasion and metastasis. Many studies have reported that MMP-13 contributes to the bone metastasis of breast cancer (25,26). It was reported that extracellular ATP stimulated MMP-13 mRNA expression in DU-145 prostate cancer cells (27). However, there is no report concerning the effect of the P2X7 receptor on MMP-13 expression. Here, we found that activation of the P2X7 receptor by

ATP promoted the expression and secretion of MMP-13 in breast cancer cells.

Many intracellular signaling pathways can be activated by the P2X7 receptor. Studies have confirmed that activation of the P2X7 receptor enhanced the proliferation of ovarian carcinoma cells via the AKT and ERK1/2 pathways (28), and mediated tumor cell death via PI3K/AKT and AMPK-PRAS40-mTOR signaling pathways (29). In the present study, we found that activation of the P2X7 receptor by ATP time-dependently induced the AKT pathway in the T47D cells. It is well known that the AKT pathway plays a vital role in tumor progression (30). The present study showed that blocking of the AKT pathway attenuated the effect of ATP on the invasion and migration in T47D cells. We further identified that inhibition of the AKT pathway suppressed the changes in P2X7-mediated E-cadherin and MMP-13 expression. These data indicate that activation of the P2X7 receptor stimulates breast cancer cell invasion and migration via the AKT pathway.

In conclusion, the present study demonstrated that ATP stimulation promotes the invasion and migration of breast cancer cells via activation of the P2X7 receptor. The function of the P2X7 receptor may be triggered by activation of the AKT pathway and subsequent regulation of E-cadherin and MMP-13 expression. Thus, the P2X7 receptor could act as a new target for the anticancer therapy of breast cancer.

Acknowledgements

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