$P2Y_2$ nucleotide receptor-mediated extracellular signal-regulated kinases and protein kinase C activation induces the invasion of highly metastatic breast cancer cells

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Abstract. Tumor metastasis is considered the main cause of mortality in cancer patients, thus it is important to investigate the differences between high- and low-metastatic cancer cells. Our previous study showed that the highly metastatic breast cancer cell line MDA-MB-231 released higher levels of ATP and exhibited higher $P2Y_2R$ activity compared with the low-metastatic breast cancer cell line MCF-7. In addition, $P2Y_2R$ activation by ATP released from MDA-MB-231 cells induced hypoxia-inducible factor-1α expression, lysyl oxidase secretion and collagen crosslinking, generating a receptive microenvironment for pre-metastatic niche formation. Thus, in the present study, we investigated which $P2Y_2R$-related signaling pathways are involved in the invasion of breast cancer cells. The highly metastatic breast cancer cells MDA-MB-231 and SK-BR-3 showed higher invasion than MCF-7 and T47D cells at a basal level, which was abolished through $P2Y_2R$ knockdown or in the presence of apyrase, an enzyme that hydrolyzes extracellular nucleotides. MDA-MB-231 cells also showed high levels of mesenchymal markers, such as Snail, Vimentin and N-cadherin, but not the epithelial marker E-cadherin and this expression was inhibited through ATP degradation or $P2Y_2R$ knockdown. Moreover, SK-BR-3 and MDA-MB231 cells exhibited higher ERK and PKC phosphorylation levels than T47D and MCF-7 cells and upregulated phospho-ERK and -PKC levels in MDA-MB-231 cells were significantly downregulated by apyrase or $P2Y_2R$ knockdown. Specific inhibitors of ERK, PKC and PLC markedly reduced the invasion and levels of mesenchymal marker expression in MDA-MB-231 cells. These results suggest that over-activated ERK and PKC pathways are involved in the $P2Y_2R$-mediated invasion of breast cancer cells.

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Abbreviations: ATP, adenosine triphosphate; CTRL, control; DAPI, 4',6-diamino-2-phenylindole; EC, endothelial cell; ECM, extracellular matrix; ECL, enhanced chemiluminescence; EMT, epithelial-mesenchymal transition; ER, estrogen receptor; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; HER2, human epidermal growth factor receptor-2; MAPK, mitogen activated protein kinase; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; PKC, protein kinase C; PLC, phospholipase C; PR, progesterone receptor; $P2Y_2R$, $P2Y_2$ receptor; RT-PCR, reverse transcription-polymerase chain reaction; siRNA, small interfering RNA; TBS-T, Tris-buffered saline/Tween-20; TNBC, triple-negative breast cancers

Key words: adenosine triphosphate, epithelial-mesenchymal transition, extracellular signal-regulated kinase, invasion, $P2Y_2$ receptor, protein kinase C

Introduction

Breast cancer is a common malignancy in females worldwide and is a heterogeneous disease that encompasses several distinct entities with different biological characteristics and clinical behaviors. Currently, breast cancer patients are managed according to different treatment approaches based on various clinical parameters in conjunction with the assessment of the status of sex steroid receptor (estrogen and progesterone receptors). Approximately 70 to 80% of primary breast cancers are positive for estrogen receptor (ER) and/or progesterone receptor (PR), such as MCF-7 breast cancer cells and ER^+ breast cancers typically have a better prognosis and are often responsive to antiestrogen therapy; however, ER-independent breast cancer cells, such as MDA-MB-231, are more aggressive, possess high potential to metastasize and are unresponsive to antiestrogens (1). Thus, it is important to investigate the differences between high- and low-metastatic cancer cells.

In a previous study, we showed that the highly metastatic breast cancer cell line MDA-MB-231, released significantly more ATP than the less metastatic breast cancer cell line MCF-7 or normal epithelial or endothelial cells (ECs) under both normoxic and hypoxic conditions (2,3). In addition, MDA-MB-231 cells showed higher $P2Y_2$ purinergic receptor ($P2Y_2R$) activity and increased invasion into the extracellular matrix (ECM) compared with MCF-7 cells (2). $P2Y_2R$ is a G protein-coupled purinergic receptor equally activated by both extracellular ATP and UTP (4). Many studies have shown

that extracellular purines accumulate in the tumor microenvironment and directly affect cancer progression through purinergic receptors. The activation of $P2Y_2Rs$ also supports the progression of each step of metastasis, including angiogenesis, intravasation and invasion and tumor growth (5-7). Thus, it is important to determine which $P2Y_2R$-related signaling pathway is involved in the invasion of breast cancer cells.

Gq-coupled $P2Y_2R$ activates several intracellular signal transduction pathways, resulting in intracellular calcium mobilization and phospholipase C (PLC) and protein kinase C (PKC) activation. Through the extracellularly oriented RGD domain, $P2Y_2R$ interacts with $\alpha_V\beta_{3/5}$ integrins to regulate the activities of Rho and ROCK, which regulate cell movement. Src-homology-3 binding domains (PXXP) within the C terminus of $P2Y_2R$ bind Src to enable ATP or UTP to transactivate growth factor receptors and downstream mitogen-activated protein kinases (MAPKs). Accordingly, in the present study, we investigated whether $P2Y_2R$ activation mediates breast cancer cell invasion through PKC and extracellular signal-regulated kinase (ERK) signaling pathways.

Materials and methods

Materials. RPMI-1640 medium and fetal bovine serum (FBS) were purchased from HyClone (South Logan, UT, USA). Antibiotics (penicillin/streptomycin), glutamine and collagenase were purchased from Gibco-BRL (Rockville, MD, USA). Anti-Snail, anti-N-cadherin, anti-β-catenin and anti-E-cadherin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Control siRNA or $P2Y_2R$ siRNA was obtained from Bioneer (Daejeon, Korea). Cell culture inserts (8 μm) and the Basement Membrane Matrix (Matrigel) were obtained from BD Bioscience (San Jose, CA, USA). Enhanced chemiluminescence (ECL) western blotting detection reagent was purchased from Amersham (Buckinghamshire, UK). All other chemicals, including adenosine triphosphate (ATP), uridine 5'-triphosphate (UTP) and anti-β-actin antibody, were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture. The human breast cancer cell lines MCF-7, MDA-MB-231, SK-BR-3 and T47D were obtained from the Korea Cell Line Bank (Seoul, Korea). The cells were grown in RPMI-1640 supplemented with 10% FBS, 100 IU/ml penicillin and 10 μg/ml streptomycin.

Gene silencing with small interfering RNA (siRNA). Gene silencing experiments were performed with three independent $P2Y_2R$ siRNAs. The cells were transfected with 100 nM control or $P2Y_2R$ siRNA in serum-containing medium using Turbofect® (Thermo Scientific, Rockford, IL, USA). The gene silencing efficiency was determined by reverse transcription-polymerase chain reaction (RT-PCR) and western blot analysis.

RT-PCR. RT-PCR was performed using the TOPscript One-step RT PCR DryMix (Enzynomics, Daejeon, Korea) according to the manufacturer's instructions. The following primer sets were used: $hP2Y_2R$, 5'-GTG CTC TAC TTC CTG GCT-3' and 5'-CTG AAG TGT TCT GCT CCT AC-3'; hGAPDH, 5'-TCA ACA GCG ACA CCC ACT CC-3' and 5'-TGA GGT CCA CCA CCC TGT TG-3'. Thirty cycles of amplification were performed under the following conditions: melting at 95˚C for 30 sec, annealing at 56˚C for 30 sec and extension at 72˚C for 30 sec.

Measurement of intracellular calcium ion concentration ($[Ca^{2+}]_i$). The $[Ca^{2+}]_i$ concentration was measured as previously described, with minor modifications (2). Briefly, the cells were stained with 5 μM fluo-3-AM and washed with physiological solution (125 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM HEPES, 5 mM glucose and 1 mM $CaCl_2$). Subsequently, the cells were treated with ATP and the fluorescent images were scanned using a confocal microscope (IX70 Fluoview, Olympus; excitation wavelength: 488 nm, emission wavelength: 530 nm). The changes in $[Ca^{2+}]_i$ were calculated as $(F_{max}-F_0)/F_0$ (F, fluorescence intensity; F_0, basal fluorescence intensity before treatment; F_{max}, maximum level of fluorescence intensity, which occurred after the addition of ATP).

Extracellular ATP release measurements. The cells were incubated for 15 min at 37˚C with HEPES buffer (pH 7.4) containing AOPCP, a selective inhibitor of ecto-5'-nucleotidase. The cells were treated with or without TNF-α for an additional 5 min. The supernatants were collected at the indicated time-points and ATP release was measured using the Enliten ATP Assay system (Promega, Madison, WI, USA). ATP levels were calculated based on an ATP standard curve.

Western blot analysis. The cells were lysed using Pro-PREP protein extraction solution (iNtRON Biotechnology, Seoul, Korea). Subsequently, the lysate was centrifuged at 13,000 rpm for 15 min at 4˚C and the supernatants were collected for determination of the protein concentration using the Bradford method. Aliquots of 40 μg of protein were subjected to 7.5-12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis for 2 h at 100 V. The separated proteins were transferred onto Hybond-P^+ polyvinylidene difluoride membranes (Amersham). The membranes were blocked with 5% nonfat milk in Tris-buffered saline containing 0.05% Tween-20 (TBS-T) for 2 h at room temperature, followed by incubation with the indicated primary antibodies. The bound antibodies were detected using horseradish peroxidase-conjugated secondary antibodies and ECL western blotting detection reagent (Bionote, Gyeonggi-do, Korea).

Matrigel invasion assay. The upper chambers of the inserts were coated with 100 μl of Matrigel (1 mg/ml, BD Bioscience). Control or $P2Y_2R$ siRNA-transfected breast cancer cells ($2x10^5$ cells/insert) were added to the upper chambers in serum-free media and 500 μl of RPMI media with or without apyrase was added to the lower chambers. The cells were incubated for 16 h to facilitate invasion, and subsequently the cells on the lower part of the insert membranes were stained with 4',6-diamidino-2-phenylindole (DAPI) and counted in a 500x500 μm field under an Olympus microscope (CKX41) equipped with a camera (Nikon, DS-U3). Three independent experiments were performed in triplicate.

Data analysis. Image Master® VDS (Pharmacia Biotech Inc., San Francisco, CA, USA) was used for scanning densitometry.

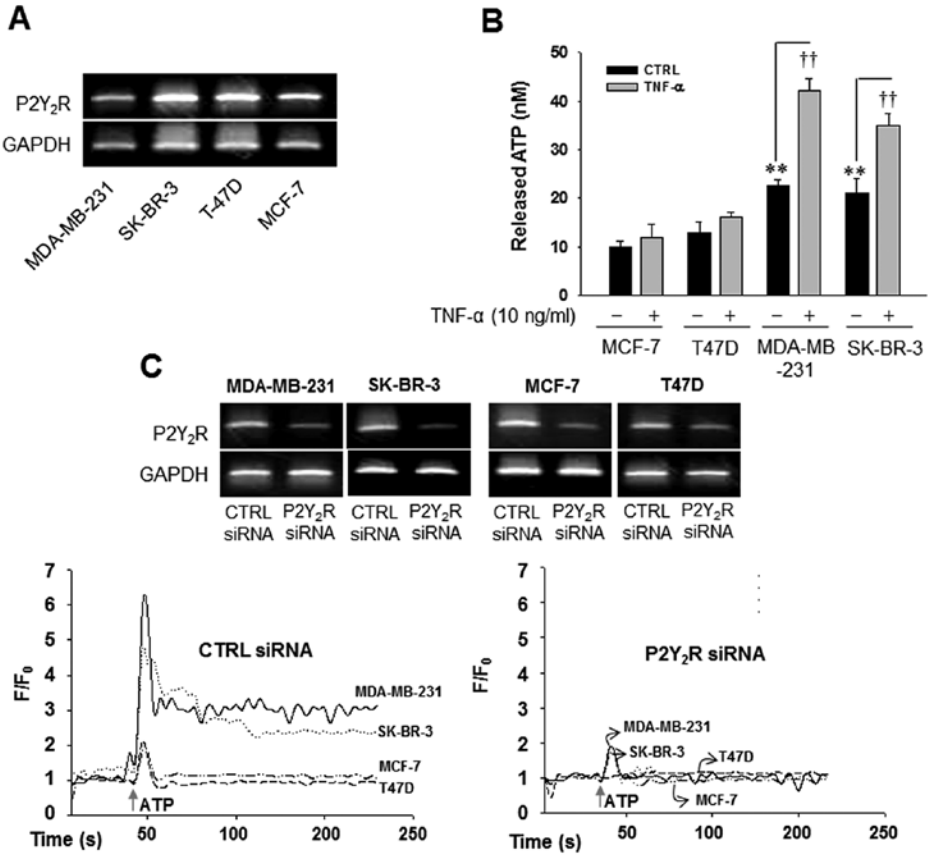

Figure 1. Highly metastatic breast cancer cells MDA-MB-231 and SK-BR-3 show much higher ATP release and $P2Y_2R$ activity than less metastatic breast cancer cells MCF-7 and T47. (A) $P2Y_2R$ expression level was determined by RT-PCR in several breast cancer cell types exhibiting different metastatic properties, including MDA-MB-231, SK-BR-3, MCF-7 and T47D cells. The results were confirmed by repeated experiments. (B) The amount of ATP released into the extracellular medium was measured as described in the Methods. Data represent mean values ± SEM of three independent experiments (significant compared with the control of MCF-7 cells, $^{**}P<0.01$; compared with the control of MDA-MB-231 or SK-BR-3 cells, $^{††}P<0.01$. (C) Intracellular Ca^{2+} levels were determined in MDA-MB-231, SK-BR-3, MCF-7 and T47D cells to measure the $P2Y_2R$ activity. CTRL siRNA- or P2Y2R siRNA-transfected breast cancer cells were stained with 5 μM fluo-3-AM and subsequently treated with ATP and fluorescent images were scanned as described in the Methods. The results were confirmed by at least two independent experiments. The arrows indicate the points at which ATP (10 μM) was added. The net change in Ca^{2+} levels was normalized to $(F_{max}-F_0)/F_0$. CTRL, control.

All results are representative of three independent experiments performed in triplicate. Significant differences within experiments were evaluated using one-way analysis of variance and the Scheffe *post-hoc* test. $P<0.05$ was considered statistically significant.

Results

Highly metastatic breast cancer cells MDA-MB-231 and SK-BR-3 show much higher ATP release and $P2Y_2R$ activity than less metastatic breast cancer cells MCF-7 and T47D. In a previous study, the highly metastatic breast cancer cell line MDA-MB-231 released ATP at a much higher level than the less metastatic breast cancer cell line MCF-7, although $P2Y_2R$ expression was not different between the two cell types (2). Thus, we confirmed the levels of ATP released into the extracellular medium in several breast cancer cells with different metastatic properties. Fig. 1 showed that there were no significant differences between $P2Y_2R$ mRNA expression in highly metastatic breast cancer cells (MDA-MB-231 and SK-BR-3) and low metastatic breast cancer cells (MCF-7 and T47D) (Fig. 1A); however, the highly metastatic breast cancer cells MDA-MB-231 and SK-BR-3 released markedly more ATP in comparison to the low metastatic breast cancer cells MCF-7 and T47D, In addition, TNF-α, an essential factor in tumor progression and metastasis (8,9), significantly enhanced the release of ATP in MDA-MB-231 and SK-BR-3 (Fig. 1B). Next, we compared $P2Y_2R$ activity between the highly metastatic breast cancer cells MDA-MB-231 and SK-BR-3 and the low metastatic breast cancer cells MCF-7 and T47D. As shown in Fig. 1C, ATP (10 μM) a $P2Y_2R$ agonist elicited immediate and rapid augmentation in $[Ca^{2+}]_i$ in MDA-MB-231 and SK-BR-3, which were significantly reduced in $P2Y_2R$ knocked down MDA-MB-231 and SK-BR-3. As expected, the transient elevation of $[Ca^{2+}]_i$ levels in MCF-7 and T47D were much lower than MDA-MB-231 and SK-BR-3, suggesting higher $P2Y_2R$ activity in response to nucleotides in MDA-MB-231 and SK-BR-3.

$P2Y_2R$ activation by ATP released from highly metastatic breast cancer cells increases invasion of breast cancer cells. Next, to investigate whether nucleotides released from highly metastatic breast cancer cells could increase the invasion of these cells, we performed Matrigel invasion assays. Control siRNA- or $P2Y_2R$ siRNA-transfected breast cancer cells were seeded on the Matrigel-coated insert wells in serum-free

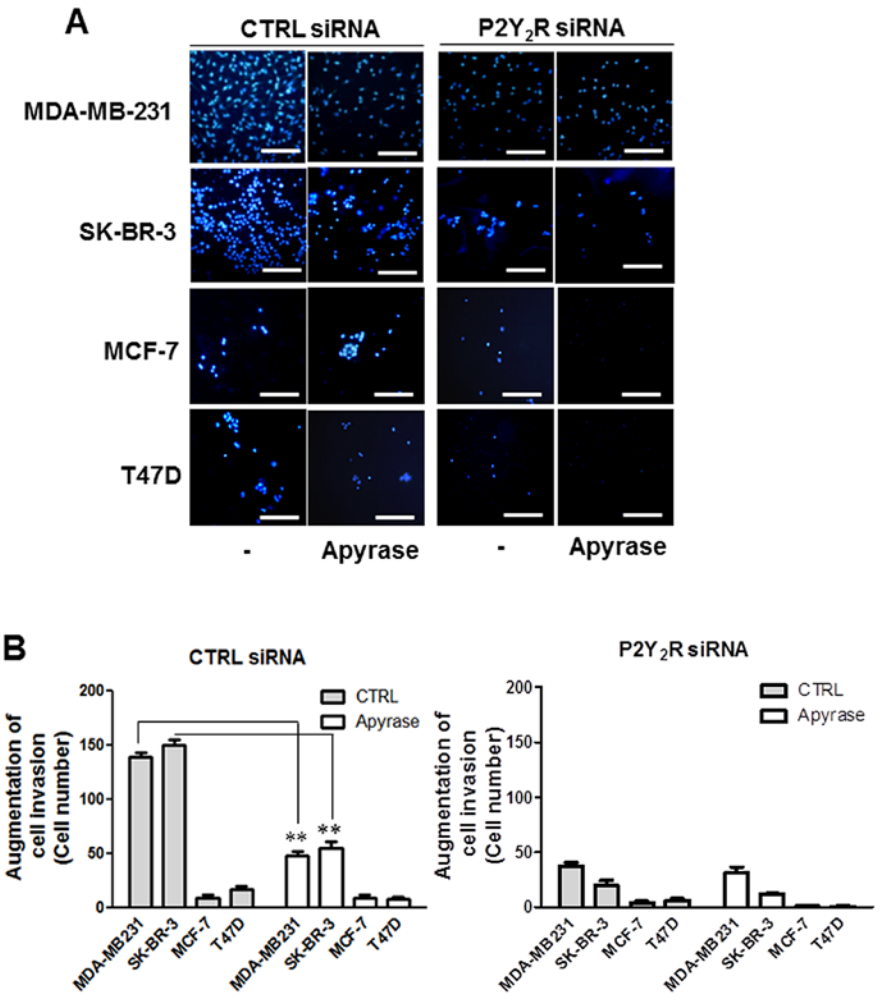

Figure 2. $P2Y_2R$ activation by ATP released from highly metastatic breast cancer cells increases invasion of breast cancer cells. CTRL or $P2Y_2R$ siRNA-transfected breast cancer cells (highly metastatic breast cancer cells, MDA-MB0231 and SK-BR-3; low metastatic breast cancer cells, MCF-7 and T47D) were seeded onto the upper chambers which were coated with Matrigel and 500 μl of RPMI media with or without apyrase was added to the lower chambers as described in the Methods. After incubation for 16 h, the invaded breast cancer cells across the insert membranes were stained with DAPI, counted under a light microscope (A) and quantified (B). The data represent the means ± SEM of three independent experiments (significant compared with the control, $^{**}P<0.01$; scale bar: 100 μm). CTRL, control.

media and RPMI media with or without apyrase was added to the lower chambers. After 16 h-incubation, MDA-MB-231 and SK-BR-3 showed a higher invasion than MCF-7 and T47D in basal level, which was abolished in the presence of apyrase. In addition, the induced invasion of MDA-MB-231 and SK-BR-3 was significantly reduced in $P2Y_2R$ siRNA-transfected MDA-MB-231 and SK-BR-3. These results suggest that ATP released from highly metastatic breast cancer cells increases invasion of breast cancer cells through $P2Y_2R$ activation (Fig. 2).

ATP released from highly metastatic breast cancer cells induces the expression of mesenchymal markers, Snail, Vimentin and N-cadherin, but not the epithelial marker E-cadherin, through $P2Y_2R$ activation in MDA-MB-231 cells. Next, we assessed whether $P2Y_2R$ activation by ATP released from the highly metastatic breast cancer cells affects the expression of epithelial-mesenchymal transition (EMT)-related proteins, including the mesenchymal markers Snail, Vimentin and N-cadherin and the epithelial marker E-cadherin. The levels of the mesenchymal markers Snail, Vimentin and N-cadherin were highly induced at a basal level in MDA-MB-231 cells, and this effect was significantly reduced in the presence of apyrase or after $P2Y_2R$ knockdown. In contrast, the epithelial marker E-cadherin was not detected at the basal level and ATP degradation using apyrase or siRNA-mediated $P2Y_2R$ knockdown induced E-cadherin levels (Fig. 3), thereby implicating the release of ATP from MDA-MB-231 cells in EMT through $P2Y_2R$ activation.

ERK and PKC pathways are over-activated in highly metastatic breast cancer cells. As shown in Fig. 2 and 3, ATP-mediated $P2Y_2R$ activation increased invasion and EMT-related protein expression in highly metastatic breast cancer cells. Thus, we examined which $P2Y_2R$-related signaling pathway is involved in these responses. Preliminary data suggested that ERK/MAPK and PKC pathways were activated in MDA-MB-231 cells compared with MCF-7 cells (data not shown). Thus, we further examined the levels of phospho-ERK and phospho-PKC in SK-BR-3 and T47D cells. The results shown in Fig. 4A indicated that SK-BR-3 and MDA-MB231 cells exhibited upregulated ERK and PKC phos-

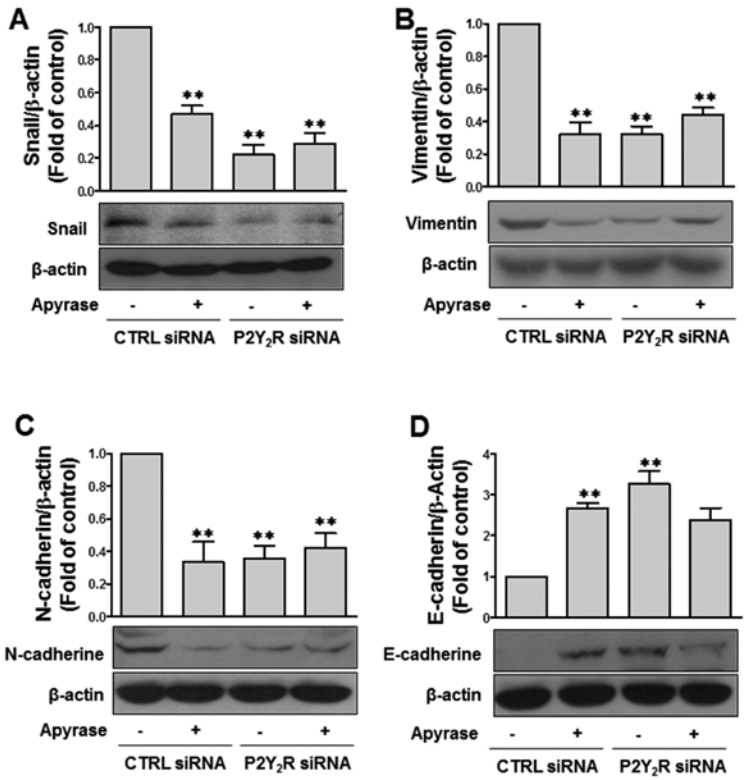

Figure 3. ATP released from highly metastatic breast cancer cells MDA-MB-231 induces the expression of mesenchymal markers Snail, Vimentin and N-cadherin, but not the epithelial marker E-cadherin, through $P2Y_2R$ activation. CTRL or $P2Y_2R$ siRNA-transfected MDA-MB-231 cells were treated with or without apyrase for 24 h and subsequently the cell lysates were extracted using lysis buffer. The levels of the epithelial marker E-cadherin (D) and the mesenchymal markers Snail (A), Vimentin (B) and N-cadherin (C) were determined by western blot analysis. The data are presented as the means ± SEM of three independent experiments (significant compared with the control, $^{**}P<0.01$). CTRL, control.

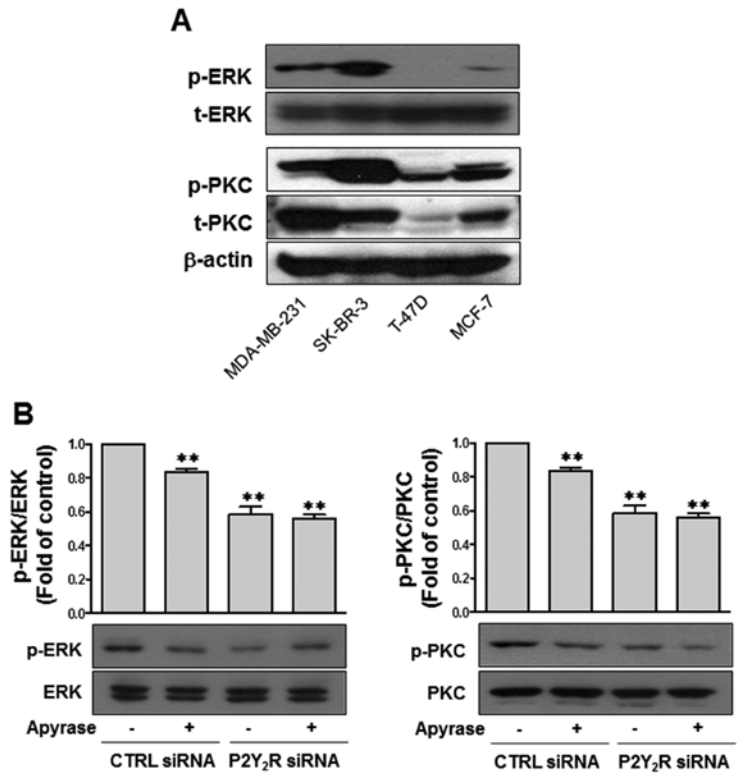

Figure 4. ERK and PKC are over-activated in highly metastatic breast cancer cells (MDA-MB-231 and SK-BR-3) compared with low metastatic breast cancer cells (MCF-7 and T47D). (A) Cell lysates were obtained from MDA-MB0231, SK-BR-3, MCF-7 and T47D cells and phospho-ERK and phospho-PKC levels were determined by western blot analysis. (B) CTRL or $P2Y_2R$ siRNA-transfected MDA-MB-231 cells were treated with or without apyrase for 24 h and phospho-ERK and phospho-PKC levels were determined from cell lysates by western blot analysis. The data are presented as the means ± SEM of three independent experiments (significant compared with the control, $^{**}P<0.01$). CTRL, control.

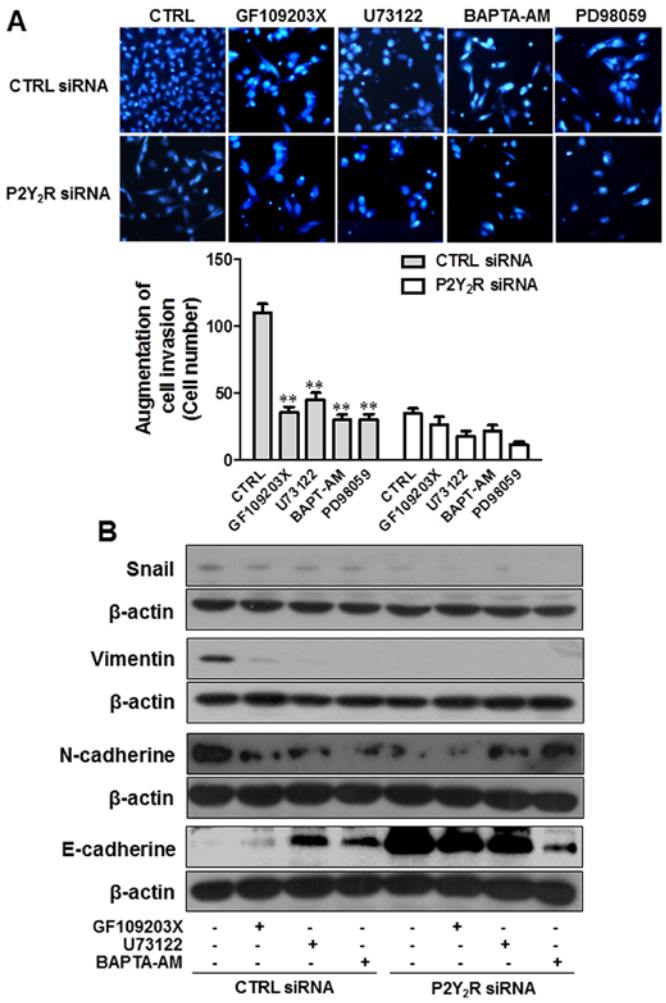

Figure 5. ERK and PKC pathways are involved in the P2Y$_2$R-mediated breast cancer cell invasion and EMT-related protein expression. (A) CTRL or P2Y$_2$R siRNA-transfected breast cancer cells were seeded onto the upper chambers which were coated with Matrigel and subsequently treated with the PKC inhibitor GF109203X (1 μM), the PLC inhibitor U73122 (1 μM), the intracellular Ca^{2+} chelator BAPTA-AM (1 μM) or the ERK inhibitor PD98059 (1 μM) for 16 h. The invaded breast cancer cells in the insert membranes were stained with DAPI and counted as described in Fig. 2. The data are presented as the means ± SEM of three independent experiments (significant compared with the control, **P<0.01. (B) CTRL or P2Y$_2$R siRNA-transfected breast cancer cells were treated with various inhibitors for 24 h as described in Fig. 5A. The levels of the EMT-related proteins E-cadherin, N-cadherin, Vimentin and Snail were determined by western blot analysis. The results were confirmed by repeated experiments. CTRL, control.

phorylation levels at the basal level; however, phospho-ERK and phospho-PKC were not detectable or significantly low in T47D and MCF-7 cells. As shown in Fig. 4B, the upregulated ERK and PKC phosphorylation levels in MDA-MB-231 cells were significantly downregulated after treatment with apyrase or transfection with P2Y$_2$R siRNA. These data suggest that over-activated ERK and PKC pathways are associated with ATP-mediated P2Y$_2$R activation in highly metastatic breast cancer cells, such as MDA-MB-231.

ERK and PKC pathways are involved in the P2Y$_2$R-mediated breast cancer cell invasion and EMT-related protein expression. To confirm the involvement of ERK and PKC pathways in the enhancement of P2Y$_2$R-mediated invasion and EMT-related protein expression, MDA-MB-231 cells were treated with specific inhibitors of ERK, PKC and PLC. As expected, treatment with specific inhibitors (PD98059, an ERK inhibitor; GF109203X, a PKC inhibitor; U73122, a PLC inhibitor) markedly reduced the invasion of MDA-MB-231. The intracellular Ca^{2+} chelator BAPTA-AM also inhibited the invasion of MDA-MB-231 cells (Fig. 5A). Induced levels of Snail, Vimentin and N-cadherin expression at the basal level were reduced by treatment with GF109203X, U73122 and BAPTA-AM, but E-cadherin expression was induced by these inhibitors and these responses were a P2Y$_2$R-dependent (Fig. 5B). These results suggest that ATP released from highly metastatic breast cancer cells activates the P2Y$_2$R pathway, which mediates ERK and PKC-PLC activation, resulting in the invasion and EMT of highly metastatic breast cancer cells.

Discussion

In metastasis, cancer cells spread from the site of origin to adjacent sites and this process is responsible for the majority of cancer-related mortalities, including breast cancer (10-12). Therefore, many studies have focused on elucidating the molecular mechanisms of metastasis. It has been suggested that the tumor microenvironment affects tumor progression and the formation of metastases. Recent studies have highlighted

the role and accumulation of nucleotides, such as ATP, within the tumor microenvironment, which directly affects cancer progression through purinergic receptors. Among the receptors engaged by extracellular ATP (P2 receptors), the $P2Y_2R$ is most consistently expressed (or overexpressed) on tumor cells. The activation of $P2Y_2Rs$ supports the progression of each step of metastasis, including angiogenesis, intravasation and invasion and tumor growth (5-7). In a previous study, we reported that MDA-MB-231, a highly metastatic breast cancer cell line, released higher levels of ATP and showed higher $P2Y_2R$ activity compared with the low-metastatic breast cancer cell line MCF-7 and the ATP-mediated activation of $P2Y_2R$ plays an important role in cancer metastasis through the modulation of crosstalk between cancer cells and ECs (2). In addition, we showed that $P2Y_2R$ activation by ATP released from highly metastatic breast cancer cells induces HIF-1α expression, lysyl oxidase secretion and collagen crosslinking, generating a receptive microenvironment for pre-metastatic niche formation (3). In the present study, we also confirmed that the highly metastatic breast cancer cell lines SK-BR-3 and MDA-MB-231 released markedly higher levels of ATP and showed higher $P2Y_2R$ activity compared with the low metastatic breast cancer cell lines MCF-7 and T47D. Furthermore, we observed that ERK and PKC pathways are activated in highly metastatic breast cancer cells and ATP-mediated $P2Y_2R$ activation induces EMT invasion through ERK and PKC pathways.

Breast cancer is a heterogeneous disease, classified into luminal A (ER^+ or PR^+, $HER2^-$), luminal B (ER^+ or PR^+, $HER2^+$), HER2-positve (ER^- and PR^-, $HER2^+$) and triple-negative (ER^- and PR^-, $HER2^-$) subtypes. Generally, triple-negative breast cancers (TNBCs) are known to be an aggressive group of breast cancers with higher rates of relapse compared with ER/PR^- and HER2-positive breast cancers, despite optimal locoregional and systemic therapies. However, molecular analyses, including microarray, DNA copy-number variation and DNA sequencing, have shown significant biological diversity within this subgroup (13-15). Moreover, it has been reported that ER-independent (ER^-) breast cancers are more aggressive, possess high metastatic potential and are unresponsive to antiestrogens (1,16). Indeed, MDA-MB-231 cells present as TNBCs with highly metastatic characteristics. Although SK-BR-3 cells are not TNBCs, these metastatic breast cancer cells are ER-negative cells ($ER^-/PR^-/HER2^+$) and both SK-BR-3 and MDA-MB-231 cells exhibit increased invasiveness.

Tumor metastasis is responsible for most cancer deaths. Signal transduction in the microenvironment around the primary tumor locus may trigger tumor metastasis, particularly at the migration stage. Sustained MAPK signaling involved in uncontrolled tumor cell migration relies on crosstalk between integrin, receptor tyrosine kinase and PKC. In a previous study, we reported that the conditions of the tumor microenvironment, specifically the high level of ATP released from cancer cells, induced tumor metastasis through $P2Y_2R$ activation (2). Gq-coupled $P2Y_2R$ activation by ATP or UTP results in intracellular calcium mobilization and PKC and phosphatidylinositol 3-kinase (PI3K) activation. In addition, src-homology-3 binding domains (PXXP) within the C-terminus of $P2Y_2R$ bind Src to enable ATP or UTP to transactivate growth factor receptors and downstream MAP kinases (17), suggesting that $P2Y_2R$-mediated MAPK and PKC pathways are involved in the induction of breast cancer cell invasion. Accordingly, we determined the levels of phospho-MAPK and phospho-PKC in the highly metastatic breast cancer cell line MDA-MB-231. SK-BR-3 and MDA-MB-231 showed activated ERK and PKC levels, and upregulated ERK and PKC phosphorylation levels in MDA-MB-231 cells were significantly downregulated by treatment with apyrase or through $P2Y_2R$ knockdown. Therefore, we suggest that $P2Y_2R$ activation by ATP released from highly metastatic breast cancer cells increases metastasis through ERK and PKC pathways. These results are consistent with reports that ERK regulates migration in several cell types (18,19) and MAPK and PKC signaling pathways are involved in the regulation of MMP-9 transcription, closely associated with the MMP-9 activity in numerous cancer cells (20,21).

During EMT, epithelial tumor cells lose polarized organization and cell-cell junctions. Thus, the cells undergo changes in shape and cytoskeletal organization and acquire mesenchymal characteristics important for metastasis (22,23). The loss of E-cadherin expression in epithelial tumors has been associated with a more invasive phenotype and metastasis (24). N-cadherin promotes cell motility and migration, effects opposite to that of E-cadherin (24). PI3K/Akt activation results in the phosphorylation of GSK-3β (inactivation of GSK-3β), which in turn increases Snail and β-catenin protein levels, ultimately resulting in the suppression of E-cadherin transcription and induction of N-cadherin expression. In the present study, we observed that the $P2Y_2R$-mediated activation of ERK and PKC pathways induced invasion and metastasis through the modulation of the EMT process. According to Martiáñez *et al* (25), $P2Y_2R$ activation by UTP induced N-cadherin expression via downstream pathways, such as ROCK, PLC, Ca^{2+} and PKC and MAPKs, including ERK in Schwann cells, a type of peripheral myelinating glial cell. N-cadherin expression through $P2Y_2R$ activation could also involve JNK, P38 and ERK pathways in MDA-MB-231 cells. However, in the present study, we focused on identifying the different signaling pathways involved in high and low-metastatic cancer cells, and it was shown that ERK/MAPK and PKC are over-activated in the MDA-MB-231 cells compared with MCF-7 cells. Thus, it is proposed that ATP released from highly metastatic breast cancer cells and the subsequent $P2Y_2R$ activation by ATP mediate ERK and PKC-PLC activation, resulting in invasion and EMT of highly metastatic breast cancer cells.

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