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ATP-P2Y2-β-catenin axis promotes cell invasion in breast cancer cells

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Key words

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Extracellular adenosine 5′-triphosphate (ATP), secreted by living cancer cells or released by necrotic tumor cells, plays an important role in tumor invasion and metastasis. Our previous study demonstrated that ATP treatment *in vitro* could promote invasion in human prostate cancer cells via P2Y2, a preferred receptor for ATP, by enhancing EMT process. However, the pro-invasion mechanisms of ATP and P2Y2 are still poorly studied in breast cancer. In this study, we found that P2Y2 was highly expressed in breast cancer cells and associated with human breast cancer metastasis. ATP could promote the *in vitro* invasion of breast cancer cells and enhance the expression of β-catenin as well as its downstream target genes CD44, c-Myc and cyclin D1, while P2Y2 knockdown attenuated above ATP-driven events *in vitro* and *in vivo*. Furthermore, iCRT14, a β-catenin/TCF complex inhibitor, could also suppress ATP-driven migration and invasion *in vitro*. These results suggest that ATP promoted breast cancer cell invasion via P2Y2-β-catenin axis. Thus blockade of the ATP-P2Y2-β-catenin axis could suppress the invasive and metastatic potential of breast cancer cells and may serve as potential targets for therapeutic interventions of breast cancer.

Breast cancer is the most common malignancy in women worldwide, and tumor invasion and metastasis are the major detrimental events that threaten patients' life. Regarding tumor invasion, tumor cells themselves can obtain invasive capacities through epithelial-mesenchymal transition (EMT)[1,2] and acquisition of stem-like properties and so on.[3,4] In addition, interactions between tumor cells and their adjacent microenvironment also play pivotal roles in tumor invasion and metastasis.[5,6] Our laboratory has been focusing on an important tumor microenvironment molecule, adenosine 5′-triphosphate (ATP).

Adenosine 5′-triphosphate, acting as an intracellular source of energy and an extracellular signaling molecule, is released extracellularly in physiological conditions like neurotransmission as well as in pathological situations such as tissue damage and tumor necrosis.[7] ATP at tumor microenvironment is in hundreds micromolar range, but it is generally undetectable in normal tissues,[8] suggesting that ATP may play an important role in host-tumor interaction. ATP initiates signaling pathways through the activation of transmembrane receptors, P2 receptors. There are two subfamilies of P2 receptors, including seven subtypes of P2X ion channel receptors (P2X1 to P2X7) and eight subtypes of G protein-coupled receptors (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11 to P2Y14).[9,10]

Our previous research demonstrated that ATP could promote invasiveness of human prostate cancer cells through P2Y receptors.[11] Subsequently, we revealed that P2Y2, a preferred receptor for ATP, stimulated the invasion of prostate cancer cells by enhancing EMT process.[12] Chadet and colleagues found that P2Y2 receptor increases MCF-7 breast cancer cell migration by activating MEK-ERK1/2 signaling pathway.[13] Using xenograft tumor model, Jin H and colleagues found that the highly metastatic breast cancer cells MDA-MB-231 released higher levels of ATP and showed a higher P2Y2 activity compared with the lowly metastatic breast cancer cells MCF-7. They also demonstrated that ATP stimulated the adhesion and migration of MDA-MB-231 cells to endothelial cells (ECs) through the expression of vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 in MDA-MB-231 or ECs. Their results suggest that P2Y2 promotes cancer metastasis through the modulation of crosstalk between cancer cells and ECs.[14] However, the pro-invasion mechanisms of ATP and P2Y2 are still poorly investigated. In this study, we demonstrated for the first time that ATP-P2Y2-β-catenin axis promoted breast cancer cell invasion.

Materials and Methods

Antibodies and chemicals. ATP and iCRT-14 were purchased from Sigma (St Louis, MO, USA). ATP was dissolved in sterile normal saline and used at a concentration of 100 μM. The iCRT-14 was dissolved in DMSO and used at a concentration of 25 μM. The antibodies used in this study are as follows: anti-β actin (TA-09, ZSGB-BIO, Beijing, China), anti-P2Y2

(H-70; SC-20124, Santa Cruz, CA, USA), anti-β-catenin (61053, BD Biosciences, San Jose, CA, USA), anti-Myc (K422; BS2462, Bioword Technology, St Louis, MN, USA), anti-Axin2 (E2A6978, Enogene, Nanjing, China), anti-CD44 (ZM-0537, ZSGB-BIO, Beijing, China), Ki-67 (GM001, Gene Tech, Shanghai, China).

Cell lines and culture conditions. Human breast cancer cell lines (MCF-7, MDA-MB-231) and the immortalized human normal breast epithelial cell line MCF-10A were purchased from American Type Culture Collection (ATCC) and preserved in our laboratory. These cell lines were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS (GIBCO, Grand Island, NY, USA) at 37°C with 5% CO_2 in a humid atmosphere.

P2Y2 gene silencing. P2Y2 gene was silenced with small interfering RNA (siRNA) or small hairpin RNA (shRNA). Two P2Y2 siRNA oligonucleotides (siRNA1 and siRNA2) were purchased from Genepharma (Shanghai, China) and the sequences are as follows: P2Y2 siRNA1, 5′-GUGCUAACAGUUGCCUUGA-3′ and P2Y2 siRNA2, 5′-GCCCAAGAGAUGAACAUCU-3′. A scramble siRNA oligonucleotide was used as negative control siRNA (designated as NC). Cells were transfected with siRNAs using Lipofectamine RNAiMAX Reagent (Invitrogen). An oligonucleotide labeled with fluorescence was applied to directly observe the efficiency of siRNA transfection.

P2Y2 shRNA plasmid as well as a negative control shRNA (NC) was constructed in our lab as described by Li WH *et al.*[12] By using Lipofectamine 2000 (Invitrogen), we transfected MDA-MB-231 cells with P2Y2 shRNA or control shRNA, and selected stable clones using G418 (GIBCO).

***In vitro* cellular invasion and migration assays.** The cell invasion assays were carried out as described by Li WH *et al.*[12,15] Briefly, each polycarbonate filter in 24-well Transwell chamber (Costar, San Diego, CA, USA) was coated with matrigel (BD Biosciences) and incubated at 37°C for 30 min. Then, the NIH3T3-conditioned medium was filled into lower chamber, and cells were placed in the upper chamber. Cells were allowed to invade for a period of time at 37°C with or without ATP treatment. Subsequently, the membranes were fixed with 4% formaldehyde, and the invaded cells were stained with crystal violet and counted at 200 magnification under the microscope.

Cell migration assays were carried out in 24-well Transwell chambers. A 6.5-mm polycarbonate filter (8-μm pore size) separated the upper and lower compartments of the chamber. Similarly, NIH3T3-conditioned medium (600 μL) was filled into lower chamber while cells were placed in the upper chamber. The cells were allowed to migrate for a period of time at 37°C with or without ATP treatment. Subsequently, the migrated cells were stained and evaluated as described above.

MTT assay. Cell proliferation was assayed using MTT reagent (Leagene, Beijing, China) according to the manufacturer's protocol. Briefly, cells were seeded in 96-well plates at a density of 1×10^3 cells/well and four wells were assigned to each experimental treatment. After 1–6 days of culture, 20 μL of MTT was added to each well and incubated for 4 h at 37°C. Then, the reaction was stopped by removal of MTT and 150 μL of DMSO was added into each well to dissolve the formazan crystals. Absorbance at 490 nm was measured by using a Microplate reader (Bio-Rad, Hercules, CA, USA). All measurements were done in triplicate.

Quantitative real-time PCR. Total RNA was extracted from cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Before reverse transcription, DNase (Promega) was utilized to get rid of genomic DNA within the total RNA. Then, 2 μg of total RNA was reverse transcribed using M-MLV reverse transcriptase (Promega, Madison, Wisconsin, USA) in accordance with the manufacturer's instructions. Quantitative real-time PCR was performed on ABI StepOne Real-Time PCR System (Life Technologies, Carlsbad, CA, USA) using SYBR Green Real-time PCR Master Mix (TOYOBO, Japan). The gene-specific primer sequences are listed in Table S1. The mRNA relative expression level was evaluated by using $2^{-\Delta\Delta Ct}$ method.

Western blotting analyses. Cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed in cell lysis buffer, which contained both protease inhibitors and phosphatase inhibitors. Protein concentration was determined using a BCA protein assay kit. Western blotting was carried out using standard protocol. Immunoreactive bands were visualized by using enhanced chemiluminescence reagent (Applygen Technologies Inc, Beijing, China), and quantified by densitometry using Quantity One software (Bio-Rad).

Differentially expressed gene profiles. MCF-7 cells were cultured in medium with ATP (100 μM) or without ATP for 24 h. Total RNA was isolated from cells using Trizol (Life Technologies), and cDNA microarray was carried out on Affymetrix Human Genome U133 Plus 2.0 Array (Affymetrix, CA, USA) following standard protocols. This cDNA array represents over 47 000 transcripts and 38 500 clear human genes. Genes with differential expressions between ATP treated cells and the control of untreated cells were identified as those with at least a 1.5-fold change and $P < 0.01$. Subsequently, gene ontology and pathway analysis were further performed on these differentially expressed genes by Gene Cluster and TreeView software.

Immunofluorescence assay. Cells were grown on coverslips and fixed in 4% paraformaldehyde at room temperature for 10 min. After PBS washing, the cells were blocked with 10% goat serum at 37°C for 30 min, and incubated at 4°C with anti-β-catenin overnight, and then probed with a tetramethyl rhodamine isothiocyanate (TRITC)-conjugated secondary antibody (Sigma) at 37°C for 2 h. Subsequently, cells were stained with DAPI and observed under a fluorescence microscope.

TOP-Flash/FOP-Flash reporter assay. After seeded into 24-well plates one day before transfection, MCF-7 cells were transfected with Super 8 × TOP-Flash/FOP-Flash (100 ng) plasmid containing 1 ng of pRL using Lipofectamine 2000. Twenty-four hours later, cells were treated with or without ATP. The activities of both firefly and Renilla luciferase reporters were examined using a Dual Luciferase Assay Kit (Promega) in accordance with the manufacturer's instruction. The transcriptional activity of TOP-Flash reporter is presented as the relative ratio of firefly luciferase activity to Renilla luciferase activity.

Xenograft tumorigenesis assays. Female NOD/SCID nude mice of 6–8 weeks were bred in specific pathogen-free conditions at the Center of Experimental Animals (Peking University, Beijing, China). All the mice were handled in accordance with the Guidelines of Animal Experiments by Peking University and National Institutes of Health. Experimental procedures for using laboratory animals were approved by the Institutional Animal Care and Use Committee of Peking University (no. LA2011-72). MDA-MB-231 stable cell clones, which expressed P2Y2 shRNA (shRNA1 and shRNA2) or a scramble shRNA (NC), were suspended in PBS and 4×10^6 cells were injected directly into mammary fat pads of the mice ($n = 6$ for each group), respectively. The primary tumor was monitored weekly. Seven weeks after injection, all the animals were killed and dissected. The xenograft tumors were measured in volume. Partial primary tumors and mice organs including

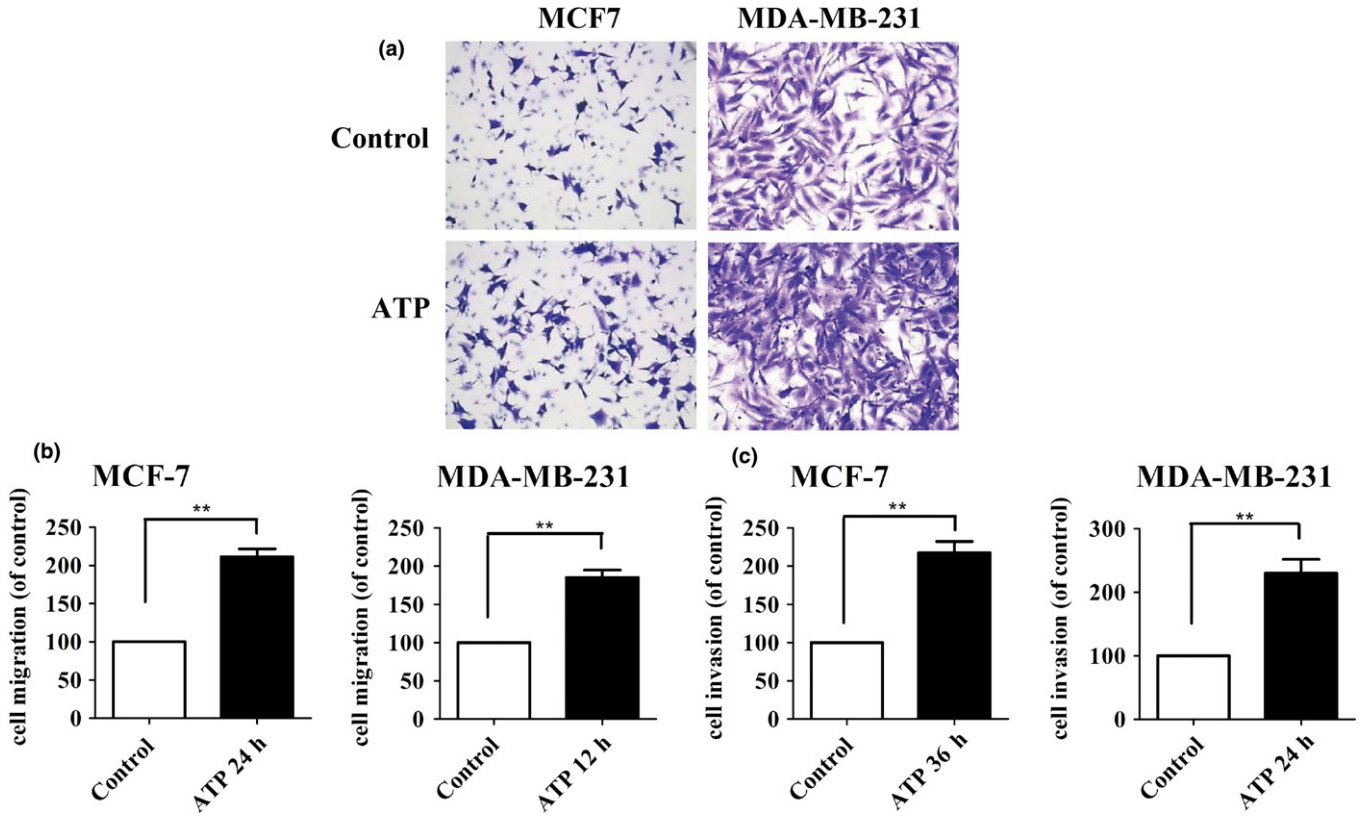

Fig. 1. Boyden chamber assay shows that ATP treatment (100 μM) promotes breast cancer cells' migration (a, b) and invasion (c) in MCF-7 and MDA-MA-231 cells. *$P < 0.05$, **$P < 0.01$.

lungs, livers and kidneys were fixed in neutral paraformaldehyde, embedded in paraffin and sectioned into 4 μm-thick slices. Tumor tissue slices were used for histological and immunohistochemical stainings. Slices from organs were examined for micrometastasis. Partial fresh primary tumors were used for RNA or protein extraction.

HE staining and Immunohistochemical staining. For histological examination, 4 μm sections were stained with hematoxylin and eosin (HE) using standard protocol. Immunohistochemical staining was performed using a standard procedure. Briefly, 4 μm sections were incubated with Ki-67 or CD44 primary antibody, then with anti rabbit/mouse HRP polymer, and visualized with DAB. Ki-67 and CD44 positive rate on each section were assessed by counting at least 500 cells under a light microscope.

Statistical analyses. All experiments in this study were repeated at least three times unless stated otherwise. Results were generally presented as mean ± SD (standard deviation) and illustrated in the histogram. Student's *t*-test was used for comparing the difference between two groups, and one-way ANOVA for comparing three or more groups. Relationships between P2Y2 expression level and clinicopathological parameters of breast cancers were evaluated using χ^2 test. Statistical analysis was performed using SPSS 17.0 software (Chicago, IL, USA). Significance was set at $P \leq 0.05$.

Results

ATP promotes migration and invasion of breast cancer cells. To investigate the effect of ATP on the migration and invasion of breast cancer cells, we performed Boyden Chamber assay in MCF-7 and MDA-MB-231 cells. The number of migrating cells after 100 μM ATP treatment was 2.11- and 1.85-fold of the control cells in MCF-7 and MDA-MB-231, respectively, and the number of invading cells after 100 μM ATP treatment was 2.17- and 2.30-fold of the control cells in MCF-7 and MDA-MB-231 respectively (Fig. 1). To exclude the possibility that the data of invasion and migration assays might be influenced by ATP's effect on cellular proliferation, we performed MTT assay. We found that ATP inhibited the proliferation of MCF-7 and MDA-MB-231 cells (Fig. S1). These results suggest that ATP can enhance the migration and invasion of breast cancer cells.

P2Y2 receptor is highly expressed in breast cancer and associated with human tumor metastasis. In an attempt to identify the candidate P2Y subtypes involved in breast carcinoma invasion, we examined P2Y subtypes in breast cancer cell lines. Using qRT-PCR, we demonstrated that MCF-7 and MDA-MB-231 breast cancer cell lines predominantly expressed P2Y2 mRNA and P2Y6 mRNA (Fig. 2a). By using Western blotting, we found that P2Y2 protein was highly expressed in these breast cancer cell lines compared with MCF-10A mammary epithelial cells (Fig. 2b).

Then, we investigated the expression of P2Y2 in human breast cancer tissues using the Cancer Genome Atlas (TCGA) data by using the online database (https://www.oncomine.org/resource/) and Ma Breast data.[16] We found that P2Y2 expression level was higher in breast cancer tissues than in normal tissues (TCGA data; Fig. 2c,d). Next, we examined the association of P2Y2 expression with clinical parameters based on 1,173 cases of breast cancer data from TCGA, which is provided on the website of Cancer Genomics Brower of California Santa Cruz (UCSC; https://genome-cancer.ucsc.edu/), and found that P2Y2 high expression was associated with lymph node metastasis ($P = 0.037$; Fig. 2e). These clinical data

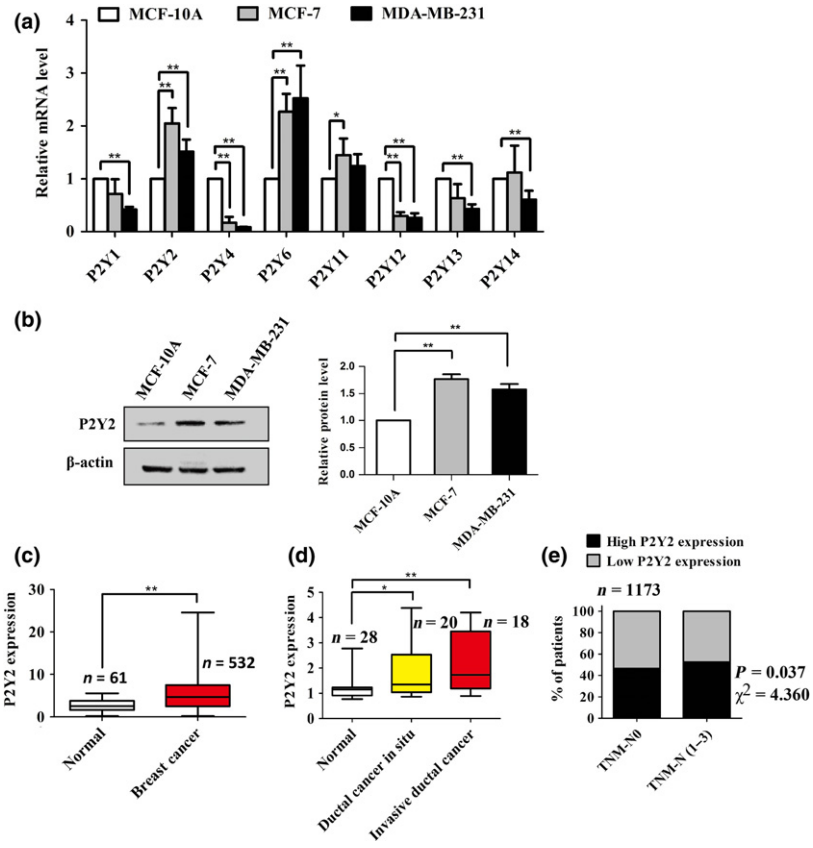

Fig. 2. P2Y2 is highly expressed in breast cancer and associated with tumor invasion and metastasis. (a) The mRNA expressions of P2Y2 receptor subtypes were examined by real-time PCR in MCF-10A, MCF-7 and MDA-MB-231. (b) The protein expression of P2Y2 was examined by western blotting in breast cancer cells and immortal normal mammary cells MCF-10A. (c) Analysis of TCGA datasets showed a significant difference of P2Y2 mRNA expression between breast cancers and normal mammary tissues. (d) Analysis of Ma breast datasets showed a significant difference of P2Y2 mRNA expression between ductal cancer *in situ*, invasive ductal cancer and normal mammary tissues. (e) P2Y2 mRNA expressions in TCGA datasets were associated with lymph node metastasis. We analyzed 1173 cases of breast cancers with Tumor-Node-Metastasis (TNM) staging data, which include the size and extension of the primary tumor, its lymphatic involvement, and the presence of distant metastases to classify the progression of cancer. TNM-N0 represents no lymph node metastasis, and TNM-N1-3 refers to different extent of lymph node metastasis. $*P < 0.05$, $**P < 0.01$.

indicate that P2Y2 expression is associated with breast cancer metastasis.

Since ATP is the preferred ligand for P2Y2 receptor, P2Y2 may be an important molecule for the ATP-mediated invasion. Therefore, we concentrated our research on P2Y2 receptor.

P2Y2 receptor is involved in ATP-driven migration and invasion of breast cancer cells. In an attempt to uncover the effect of P2Y2 receptor in ATP-mediated migration and invasion, two different siRNA targeting P2Y2 were designed to knockdown or silence P2Y2 in breast cancer cells. Both P2Y2 siRNA effectively knocked down P2Y2 expression in MCF-7 and MDA-MB-231 cells (Fig. 3a). After knocking down of P2Y2, the ATP-mediated migration and invasion were significantly inhibited (Fig. 3b,c), suggesting that P2Y2 plays an important role in these process.

β-catenin is the downstream signaling of ATP. To identify the candidate downstream genes of ATP stimulation, we performed cDNA microarray in MCF7 cells, and found 333 genes to be up-regulated (Fold Change ≥ 1.5) and 670 genes to be down-regulated (Fold Change ≤ 0.67) after ATP treatment. Among those genes, 25 genes related with cancer were examined in MCF-7 and MDA-MB-231 cells by using quantitative real-time PCR, and the heat maps were shown in Figure 4(a). The real-time PCR analysis and cDNA microarray data consistently demonstrated that expressions of β-catenin and its downstream target gene CD44 were significantly increased in MCF-7 and MDA-MB-231 cells after treatment with 100 μM ATP for 24 h (Fig. 4a,b). Immunofluorescence assay demonstrated that ATP treatment upregulated β-catenin expression in both cytoplasm and nucleus, indicating that ATP not only stimulates β-catenin expression but also accelerates its translocation from cytoplasm to nucleus in breast cancer cells (Fig. 4c). Then, we further investigated whether ATP could activate β-catenin. A key step in β-catenin activation is the complex formation between β-catenin and T-cell factor (TCF)/lymphoid-enhancing factor (LEF) family of transcription factors to cause target gene expression, such as CD44, MYC, cyclin D1 and Axin2.[17] In the present study, western blotting showed that the total β-catenin was slightly increased in MCF-7 and MDA-MB-231 cells after ATP treatment (Fig. 4d,e). Moreover, ATP could increase the activity of TOPFLASH luciferase reporter (Fig. 4f), which has binding sites for Wnt transcription factor TCF and is expressed when β-catenin is active. ATP could also promote the expressions of β-catenin's target genes c-Myc and Axin2 (Fig. 4g). These results indicate that β-catenin signaling is activated after ATP treatment.

β-catenin is involved in ATP-driven migration and invasion. To investigate whether β-catenin is involved in ATP-mediated migration and invasion, we used β-catenin/TCF complex inhibitor iCRT14 to block β-catenin/TCF transcription. Quantitative RT-PCR showed that iCRT14 could inhibit the expression of β-catenin/TCF target genes c-Myc, cyclin D1 and CD44 in MCF-7 (Fig. 5a) and MDA-MB-231 cells (Fig. 5b). Boyden Chamber assay demonstrated that iCRT14 could attenuate ATP-driven migration (Fig. 5c) and invasion (Fig. 5d) in breast cancer cells.

P2Y2 receptor is involved in the activation of β-catenin signaling. To reveal whether P2Y2 is essential in the ATP-mediated activation of β-catenin, we established two stable P2Y2-knockdown clones in MDA-MB-231 cells (designated as P2Y2 shRNA1 and P2Y2 shRNA2) by using P2Y2-shRNA constructs, and one negative control-clone (transfected with a control scramble shRNA and designated as NC; Fig. 6a). In P2Y2 knockdown clones, the ATP-mediated migration and invasion

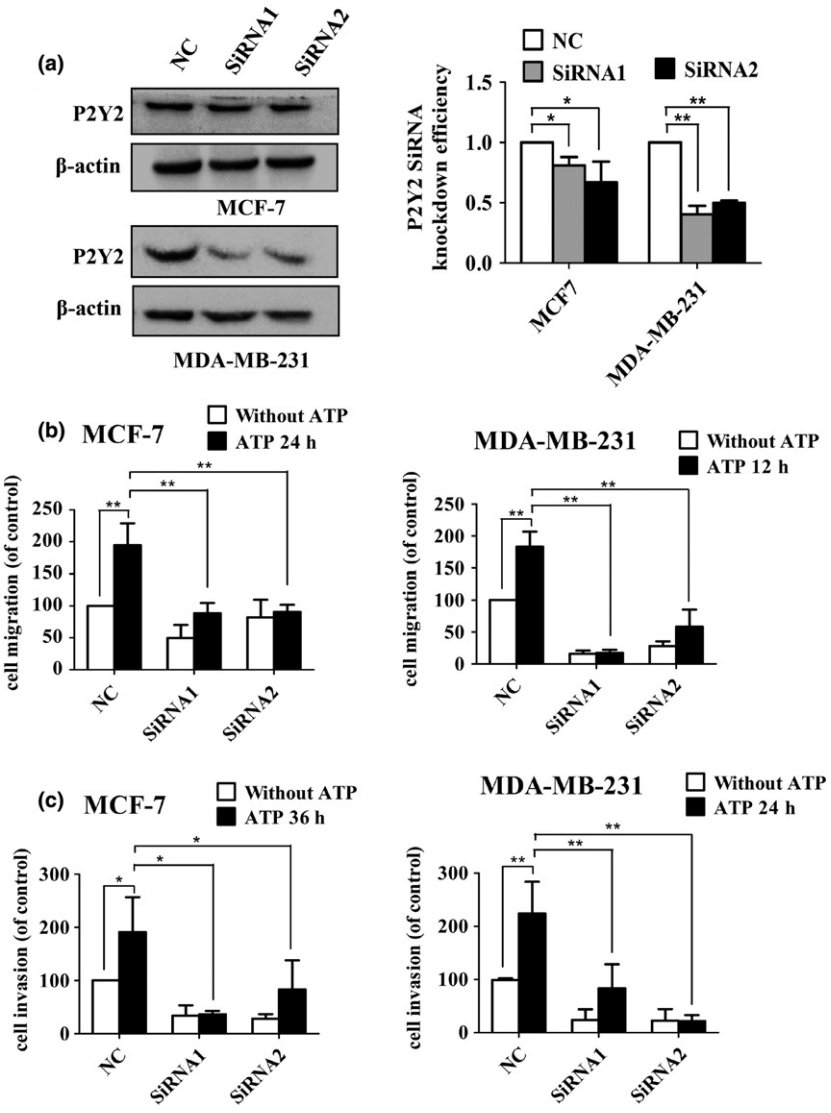

Fig. 3. P2Y2 knockdown inhibits ATP-mediated migration and invasion *in vitro*. (a) Cells were transfected with P2Y2-siRNAs (siRNA1, siRNA2) or a negative control siRNA (NC). Knockdown efficiency was evaluated by western blotting. (b) Effects of P2Y2 silencing on ATP-mediated migration. MCF-7 and MDA-MB-231 cells were incubated with ATP for 24 h and 12 h respectively. (c) Effects of P2Y2 silencing on ATP-mediated invasion. MCF-7 and MDA-MB-231 cells were incubated with ATP for 36 h and 24 h respectively. $^{*}P < 0.05$, $^{**}P < 0.01$.

were significantly inhibited (Fig. S2). Moreover, we found that P2Y2 knockdown attenuated ATP-driven expression of β-catenin (Fig. 6b), and inhibited the expressions of β-catenin's target genes c-Myc, cyclin D1 and CD44 in MCF-7 (Fig. 6c) and MDA-MB-231 cells (Fig. 6d). These results indicate that P2Y2 is involved in the activation of β-catenin.

P2Y2 receptor is required for *in vivo* invasion and metastasis of breast cancer. Finally, we investigated the effect of P2Y2 receptor on *in vivo* invasion and metastasis by using NOD/SCID mice. Two stable MDA-MB-231 P2Y2-shRNA and one NC clones were injected directly into mammary fat pads of NOD/SCID mice. Tumor volumes were measured every 7 days for 48 days. All but two of the xenograft tumors injected with P2Y2-shRNA clones were much smaller than those injected with NC clone (Fig. 7a,b). Notably, regardless of their sizes, none of the xenograft tumors in the mice injected with P2Y2-shRNA clones showed invasion and metastasis (Fig. 7c). However, 66.7% (4/6 N) of mice in control group exhibited lung metastasis and 83.3% (5/6N) of mice in control group exhibited liver metastasis (Fig. 7d). Then, we examined the mRNA expression levels of β-catenin signaling related molecules in xenograft tumors. The results showed that expressions of β-catenin, c-Myc, cyclin D1 and CD44 were remarkably decreased in P2Y2-knockdown tumors (Fig. 7e). Immunohistochemical staining demonstrated that CD44 and Ki-67 positive cells were much fewer in P2Y2-shRNA cells than in control cells ($P < 0.001$; Fig. 7f). The positive rates of Ki-67 in NC, shRNA1, shRNA2 clone were $(44.34 \pm 8.08)\%$, $(11.43 \pm 6.73)\%$ and $(12.52 \pm 9.13)\%$ respectively. The positive rates of CD44 in NC, shRNA1 and shRNA2 clones were $(82.30 \pm 8.22)\%$, $(8.90 \pm 2.55)\%$ and $(10.50 \pm 2.15)\%$, respectively.

Discussion

Adenosine 5′-triphosphate can be secreted by living cancer cells or released by necrotic tumor cells,[18,19] therefore, ATP can reach much high concentrations in tumor microenvironment compared with that in healthy tissues where it is normally undetectable.[8] Some studies demonstrated that ATP stimulated the growth of several human cancer cells, such as Caco-2 colon cancer cells,[20] OVCAR-3 ovarian cancer cells [21] and A549 human lung cancer cells.[22] However, some other studies showed that extracellular ATP inhibited the proliferation of Caco-2[23] and glioblastoma cells.[24] Apart from influencing cellular proliferation, extracellular ATP was demonstrated by our group to promote invasion of prostate

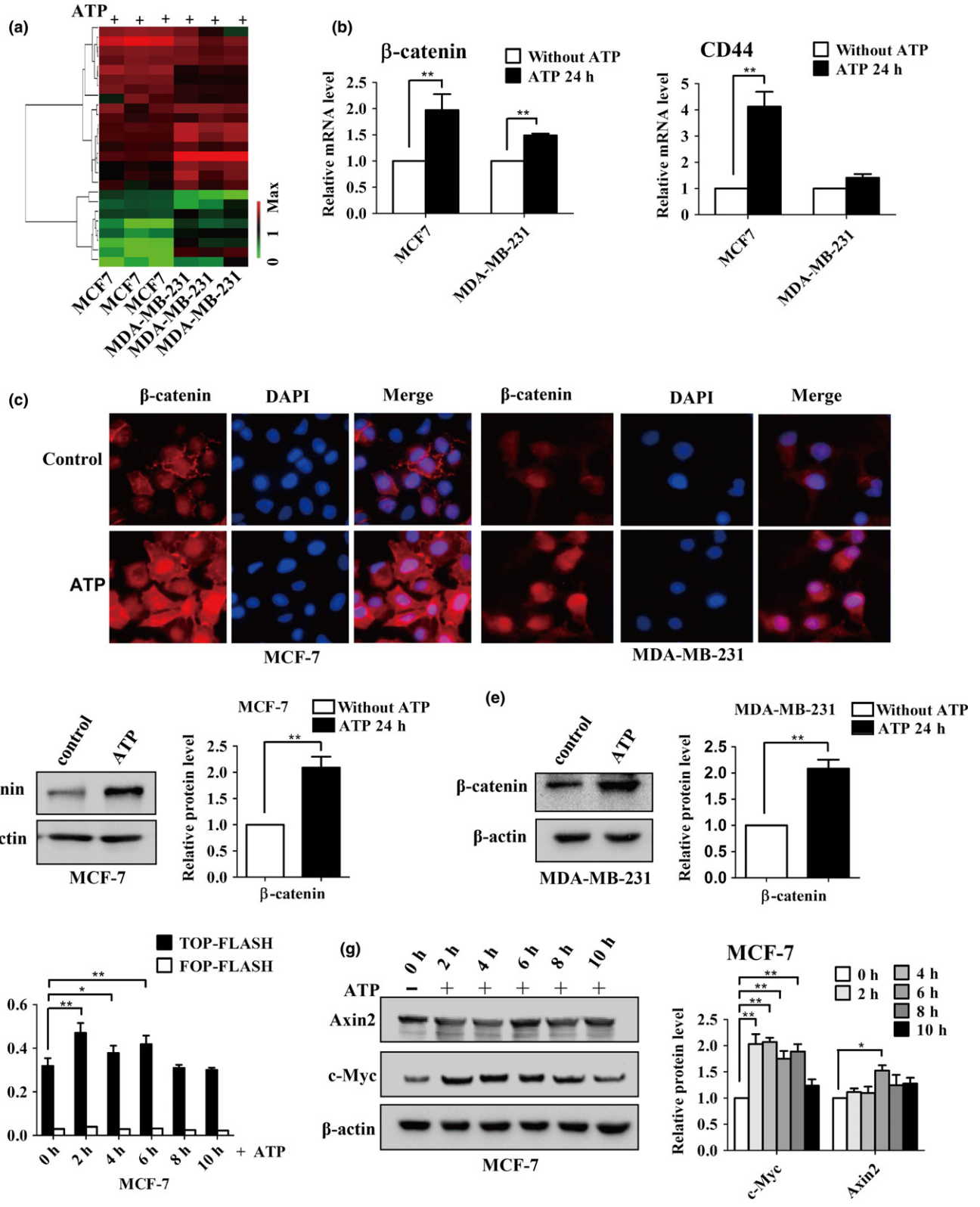

Fig 4. ATP activates β-catenin. (a) Heat maps, generated from quantitative RT-PCR data, demonstrated genes with differential expressions between ATP treatment and the control of untreated cells. (b) Quantitative RT-PCR showed that ATP could up-regulate β-catenin and CD44 mRNA. (c) Immunofluorescence showed that ATP could stimulate the expression of β-catenin in both the cellular cytoplasm and nucleus. (d, e) Western blotting demonstrated that ATP could upregulate β-catenin protein expression. (f) TOPFLASH luciferase reporter assay indicated that ATP could activate β-catenin/TCF-mediated transcription. MCF-7 cells were transfected with the indicated plasmids and treated with or without ATP, then luciferase activity was tested. (g) Western blotting showed that protein expressions of β-catenin/TCF target genes, c-Myc and Axin2, were increased after ATP treatment. $*P < 0.05$, $**P < 0.01$.

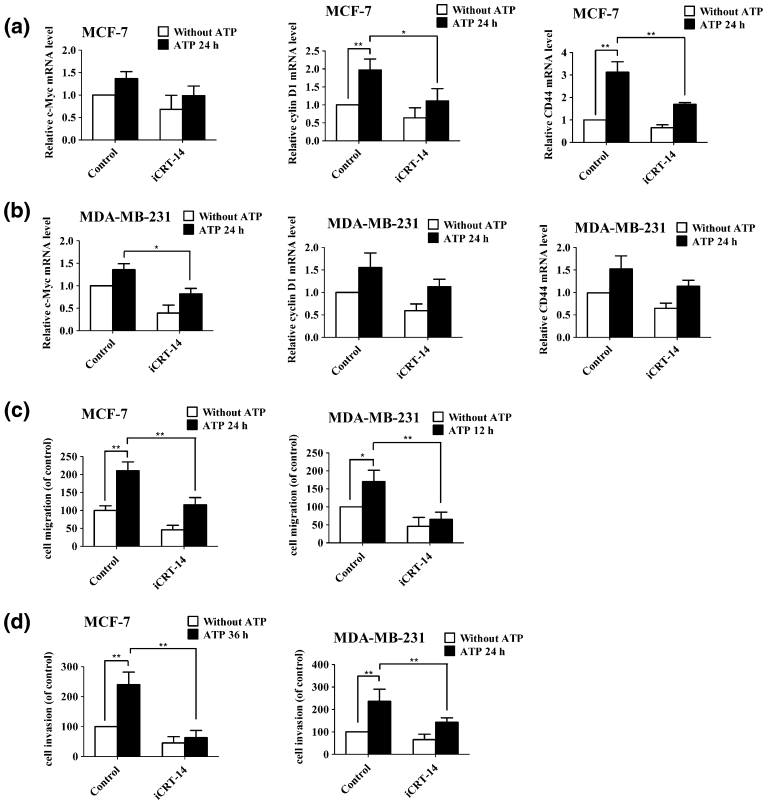

Fig. 5. β-catenin/TCF complex inhibitor iCRT14 attenuated ATP-driven *in vitro* migration and invasion. Quantitative RT-PCR results showed that iCRT14 could inhibit the expression of β-catenin/TCF target genes c-myc, cyclin D1 and CD44 in MCF-7 (a) and MDA-MB-231 cells (b). Inhibitor iCRT-14 attenuated ATP-mediated migration (c) and invasion (d) in MCF-7 and MDA-MB-231 cells. $*P < 0.05$, $**P < 0.01$.

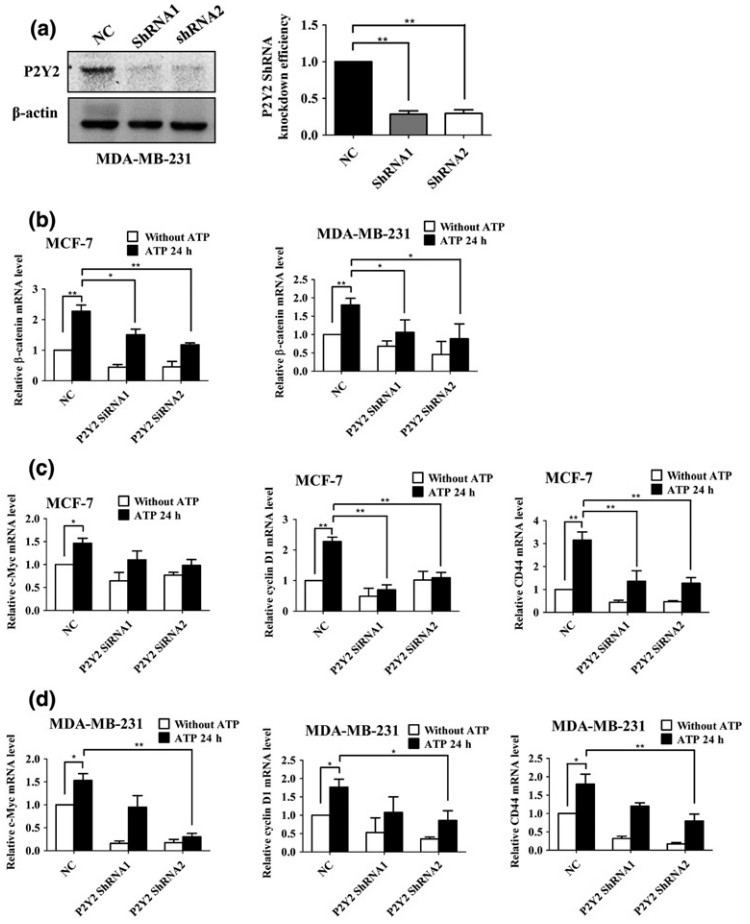

Fig. 6. P2Y2 is essential in activation of β-catenin. (a) MDA-MB-231 cells were transfected with P2Y2-shRNA (shRNA1, shRNA2) or a negative control shRNA (NC). Knockdown efficiency was evaluated by western blotting. (b) qRT-PCR detected the expressions of β-catenin in P2Y2-knockdown MCF-7 and MDA-MB-231 cells. (c, d) qRT-PCR detected the expressions of β-catenin/TCF target genes c-Myc, cyclin D1 and CD44 in P2Y2-knockdown MCF-7 and MDA-MB-231 cells. $*P < 0.05$, $**P < 0.01$.

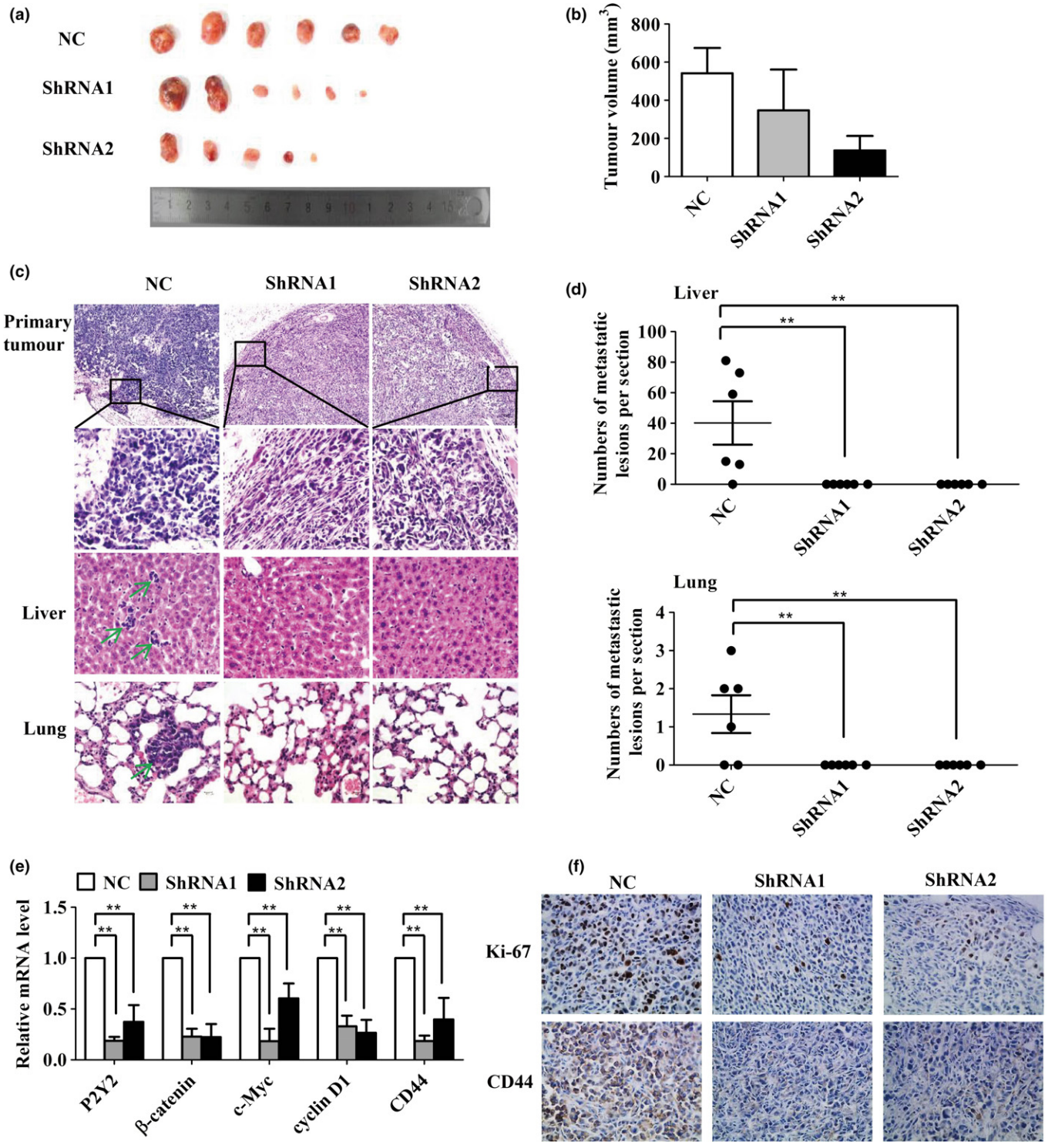

Fig. 7. Knockdown of P2Y2 inhibited *in vivo* invasion and metastasis of breast cancer cells. (a, b) MDA-MB-231-P2Y2 shRNA (shRNA1, shRNA2) or scramble shRNA (NC) were injected directly into mammary fat pads of the mice (n = 6 for each group). Tumor volumes on the 48th days were measured and shown by a histogram. (c) Representative images of xenograft tumor, liver and lung sections (HE staining). Metastatic lesions were indicated by green arrows. (d) The number of micrometastatic lesions per section in the lung and liver of tumor-bearing mice. (e) The mRNA expressions of P2Y2, β-catenin, c-Myc, cyclin D1 and CD44 in xenograft tumors were detected by qRT-PCR. (f) Representative photograph of Ki-67 and CD44 immunohistochemical staining in xenograft tumors. $^{**}P < 0.01$.

cancer cells by activating Rho GTPase and upregulating MMPs.[25] Chadet S and colleagues found that ATP enhanced the migration of human MCF-7 breast cancer cells, but it exhibited minor or no effect on tumor cellular proliferation.[13] In this study, we found that ATP inhibited the proliferation of MCF-7 and MDA-MB-231 cells, but promoted tumor cells' migration and invasion. The reported opposing results might be due to the use of different concentration or time scales of ATP treatment, or different cancer cell lines, or due to the activation of different subtypes of receptors. The migration

and invasion capacities of cancer cells determine the development of metastases, which is the major cause of cancer patients' death. Therefore, the focus of this study is to reveal the mechanism underlying ATP-mediated invasion.

Adenosine 5′-triphosphate exerts its various biological effects via P2 receptors. The study of P2 receptors in cancer biology is a promising research field. P2Y2 have drawn much attention from some researchers. P2Y2 were found to be highly expressed in squamous cell carcinomas and basal cell carcinomas of skin, and stimulate proliferation of A431 cells, a human cutaneous squamous cell carcinoma cell line.[26] It has been shown that P2Y2 has pro-proliferative effect on MCF-7 breast cancer cells,[27] but anti-proliferative and apoptotic effects on colorectal and esophageal cancer cells.[28–30] P2Y2 has also been demonstrated to support several steps of metastasis, including angiogenesis, intravasation and invasion.[31] Jin H and colleagues showed that activation of the P2Y2 by ATP enhanced MDA-MB-231 cell invasion through interaction with endothelial cells.[14] Joo YN investigated the effect of P2Y2 on breast cancer cell metastasis to distant sites. They found that P2Y2 played an important role in the formation of pre-metastatic niche, specifically in the recruitment of hematopoietic bone marrow-derived cells to distant crosslinked collagen through activation of the hypoxia inducible factor-1α (HIF-1α) and lysyl oxidase (LOX).[32] Chadet S and colleagues demonstrated that P2Y2 enhanced breast cancer cell migration through MEK-ERK1/2-dependent signalling pathway.[13] In this study, we showed that the human breast cancer cells MCF-7 and MDA-MB-231 expressed high levels of P2Y2 mRNA. By using P2Y2-knockdown *in vitro* system and *in vivo* mice model injected with P2Y2-knockdown cells, we revealed that P2Y2 promoted breast cancer cellular migration, invasion and metastasis via upregulating β-catenin.

β-catenin serves a dual function, namely in cadherin-mediated cell adhesion and in canonical Wnt signaling. Wnt/β-catenin signaling governs many biological processes such as regulation of stem cell self-renewal, cell proliferation, differentiation and apoptosis.[33] Apart from Wnt pathway, several non-Wnt factors, such as vascular endothelial growth factor (VEGF), epidermal growth factor (EGF) and fibroblast growth factors (FGFs), have been demonstrated to induce the expression or stabilization of β-catenin protein, and/or to activate β-catenin.[34–36] The main manifestation of β-catenin activation is the formation of a complex between β-catenin and T-cell factor (TCF)/Lymphoid-enhancing factor (LEF) family of transcription factors to cause target gene expression, such as MYC, cyclin D1 and CD44.[37–39] In the present study, we showed that ATP enhanced the expression of β-catenin as well as its target gene c-Myc, cyclin D1 and CD44 in breast cancer cells MCF-7 and MDA-MB-231. We demonstrated that P2Y2-knockdown decreased ATP-enhanced expression of β-catenin as well as its target gene CD44, cyclin D1 and c-Myc, and also attenuated ATP-driven migration and invasion, indicating that ATP-P2Y2-β-catenin axis promote the invasion of breast cancer cells. Apart from ATP and P2Y2, β-catenin, CD44, cyclin D1 and c-Myc all have been reported to promote cancer invasion or metastasis,[40–42] which supports the above notion.

Because purinergic receptors are widely distributed and usually more than one subtype can be found on a cell,[7,43] the mechanism of ATP-driven tumor invasion would be very complicated. Chadet S showed that MCF-7 cells contain all P2Y receptor subtypes and both P2Y2 and P2Y4 could promote cells invasion.[13] Our previous study demonstrated that human prostate cancer cells contained all P2 receptor subtypes and both P2Y2 and P2X7 could promote tumor cellular invasion.[12,15] In this study, we showed that both MCF-7 and MDA-MB-231 cells expressed all P2Y receptor subtypes, especially high levels of P2Y6, P2Y11 in addition to P2Y2. Whether ATP exerts its role on invasion via P2Y6 or P2Y11 and the specific action mechanism are still unclear. Further studies are warranted.

In summary, our results demonstrated that ATP promoted invasion and metastasis of breast cancer via P2Y2-β-catenin axis. Thus blockage of the P2Y2-β-catenin axis could suppress the metastatic potential of breast cancer cells and may serve as potential targets for therapeutic interventions of breast cancer.

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Conflict of Interest

The authors declare no conflict of interests.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. MTT assay shows that ATP (100 μM) inhibits breast cancer cells' proliferation in MCF-7 (a) and MDA-MA-231 cells (b). vs the control group, ** indicates $P < 0.01$.

Fig. S2. Effects of P2Y2 knockdown on ATP-mediated migration (a) and invasion (b). ** indicates $P < 0.01$.

Table S1. Real-time PCR primers.