$P2Y_2R$-mediated transactivation of VEGFR2 through Src phosphorylation is associated with ESM-1 overexpression in radiotherapy-resistant-triple negative breast cancer cells

HANA JIN[1], YOUNG SHIN KO[1,2], SEUNG PIL YUN[1,2], SANG WON PARK[1,2] and HYE JUNG KIM[1,2]

[1]Department of Pharmacology, College of Medicine, Institute of Health Sciences;
[2]Department of Convergence Medical Science, Gyeongsang National University,
Jinju, Gyeongsangnam-do 52727, Republic of Korea

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Abstract. We previously reported that radiotherapy-resistant (RT-R) triple negative breast cancer (TNBC) cells upregulate the expression of endothelial-specific molecule-1 (ESM-1) compared with TNBC cells. In addition, ESM-1 is involved in an increased proliferation and invasion of RT-R-TNBC cells compared with TNBC cells. It was further identified that, in RT-R-TNBC cells, $P2Y_2$ purinergic receptor ($P2Y_2R$)-mediated activation of p21-activated kinase 1 (PAK1), protein kinase C (PKC), c-Jun N-terminal kinase (JNK) and p38 MAPKs is related to ESM-1 expression via forkhead box O1 (FoxO1) regulation. Notably, it has been reported that $P2Y_2R$ mediates the transactivation of vascular epithelial growth factor receptor 2 (VEGFR2), and VEGFR2 is known to be involved in ESM-1 expression. Therefore, in the present study, the involvement of VEGFR2 in the $P2Y_2R$-mediated ESM-1 upregulation in RT-R-TNBC cells and the relationship between $P2Y_2R$ and VEGFR2 activation was further examined. Western blotting and reverse transcription-PCR were used to monitor the expression of ESM-1, and the results demonstrated that extracellular ATP treatment regulated the expression of ESM-1 in a $P2Y_2R$-dependent manner in RT-R-MDA-MB-231 cells. In addition, extracellular ATP activated Src and VEGFR2 after 5 min of incubation, which was abolished by knockdown of $P2Y_2R$ expression. VEGFR2 activation in response to ATP was also decreased by inhibiting Src activity, suggesting that ATP-activated $P2Y_2R$ regulates VEGFR2 phosphorylation via Src activation. Furthermore, ATP-induced ESM-1 expression was decreased by transfection with VEGFR2 small interfering RNA (siRNA). ESM-1-related signaling molecules, PAK1, PKC, JNK and p38 MAPKs, and the transcriptional regulator, FoxO1, which were activated by ATP, were also decreased following transfection with VEGFR2 siRNA. These results suggest that $P2Y_2R$-mediated transactivation of VEGFR2 through Src phosphorylation is associated with ESM-1 overexpression in RT-R-TNBC cells.

Correspondence to: Dr Hye Jung Kim, Department of Pharmacology, College of Medicine, Institute of Health Sciences, Gyeongsang National University, 816 Beongil 15 Jinjudaero, Jinju, Gyeongsangnam-do 52727, Republic of Korea
E-mail: hyejungkim@gnu.ac.kr

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Introduction

Endothelial-specific molecule-1 (ESM-1) is a dermatan sulfate proteoglycan expressed and secreted generally from endothelial cells and renal epithelium, and is known to play a significant role in the inflammatory response (1). It has also been reported that ESM-1 is expressed not only in endothelial cells but also in various types of cancer, such as lung, bladder, kidney, liver, brain and colorectal cancer, and is involved in tumor progression, metastasis and angiogenesis (2-5). In addition, Rocha *et al* (6) reported that ESM-1 promotes the proliferation and survival of cancer cells and regulates vascular epithelial growth factor (VEGF) signaling, which induces angiogenesis of endothelial cells in the tumor microenvironment (TME), suggesting that ESM-1 may be considered as a new tumor biomarker and a target for cancer therapy.

Cancer cells create the TME through various interactions that stimulate surrounding stromal cells, leading to the infiltration of immune cells and induction of angiogenesis by secreting various cytokines and chemokines, such as TNF, interleukin-1, VEGF, CCL2, CXCL-8 and CXCL12 (7). Moreover, cancer cells and stromal cells in the TME release high concentrations of nucleotides through various stimuli, including hypoxia (8,9). Among the secreted nucleotides, ATP has been identified as a regulator of diverse signaling transduction pathways and has been reported to activate several purinergic receptors, a family of cell transmembrane receptor (10). In particular, the $P2Y_2$ purinergic receptor ($P2Y_2R$) is expressed in most tissues and cells, including breast cancer cells (11,12). Our previous study determined that $P2Y_2R$, when activated by extracellular ATP, promotes the proliferation and metastasis of triple negative breast cancer (TNBC) cells and is involved in breast tumor

growth *in vivo* (13). TNBC is a type of invasive breast cancer that accounts for 10-15% of all breast cancer cases. Compared with other types of breast cancer, TNBC has a higher mortality rate, higher recurrence frequency and poor prognosis. The risk of early distant recurrence within 5 years after diagnosis is ~3 times higher than that of non-TNBC (14). In addition, TNBC is complex to treat due to the lack of estrogen receptor, progesterone receptor and human epidermal growth factor receptor 2 (15). As hormone therapy is unsuitable, patients with TNBC are generally treated with chemotherapy (CT) and radiotherapy (RT) before or after surgery to diminish tumor mass or eliminate any remaining cancer cells. Nevertheless, the main limitation of RT for the treatment of TNBC is the potential of some TNBC cells to develop mechanisms involved in RT resistance during treatment. The acquirement of RT resistance causes a higher local recurrence rate and mortality in patients with TNBC (16). Therefore, to overcome the therapy limitation of acquiring RT resistance (RT-R), we established RT-R-TNBC cells in a previous study and have been investigating the differences between RT-R-TNBCs and TNBCs (17). In the previous study, it was demonstrated that ESM-1 is the most upregulated gene in the RT-R-TNBC cells compared with the parental TNBC cells, and it was determined that the ESM-1 overexpressed in RT-R-TNBC cells plays essential roles in tumor progression, including tumor growth and metastasis (18). Moreover, it has been determined that ESM-1 expression is upregulated through the $P2Y_2R$-p21-activated kinase 1 (PAK1)-FoxO1 signaling pathway in RT-R-TNBC cells (19).

Notably, it has also been reported that activation of $P2Y_2R$ in vascular endothelial cells can transactivate and interact with vascular epithelial growth factor receptor 2 (VEGFR2), which regulates downstream signaling pathways RhoA and Rac1, and the expression of various molecules, including vascular cell adhesion molecule-1 (20,21). Moreover, Abid *et al* (22) demonstrated that VEGFR2 activation regulates ESM-1 expression in endothelial cells. Therefore, it can be hypothesized that $P2Y_2R$ may be involved in activating VEGFR2 and its signaling pathways in breast cancer cells, particularly in terms of ESM-1 overexpression. However, this hypothesis still needs to be clarified and, to the best of our knowledge, no related reports exist. Therefore, the aim of the present study was to investigate whether ATP-activated $P2Y_2R$ regulates ESM-1 expression through the transactivation of VEGFR2, and to identify signaling molecules and pathways linking $P2Y_2R$, VEGFR and ESM-1 expression in RT-R-MDA-MB-231 cells.

Materials and methods

Establishment of RT-R-MDA-MB-231 cells. The human breast cancer cell line, MDA-MB-231, was purchased from the Korean Cell Line Bank (Korean Cell Line Research Foundation), and RT-R-MDA-MB-231 cells were established by repeatedly irradiating MDA-MB-231 cells 25 times with 2 Gy X-ray (to accomplish a total of 50 Gy), as described previously (20). RT-R-MDA-MB-231 cells were cultured in RPMI-1640 medium (cat. no. SH30027.01; Cytiva) supplemented with 10% FBS (cat. no. F0900; GenDEPOT) and 1% penicillin and streptomycin (cat. no. SV30010; Cytiva). The cells were maintained at 37˚C in humidified air containing 5% CO_2 and used for five cell passages.

Reagents and cell treatment. ATP (cat. no. A2383) was purchased from Sigma-Aldrich (Merck KGaA), and Src family kinase inhibitor, PP2 (cat. no. S7008), was obtained from Selleck Chemicals. Cells were treated with ATP (100 μM) in serum-free media in a time-dependent manner at 37˚C to determine the length of time required to express the maximum level of ESM-1, phosphorylated (phosphor)-Src or phosphor-VEGFR2. To detect ESM-1 mRNA and protein levels, cells were treated with ATP from 1 to 48 h (1, 3, 6, 12, 24 and 48 h), and the mRNA and protein levels were determined by reverse transcription (RT)-PCR and western blot analysis, respectively. To detect phosphor-Src and phosphor-VEGFR2, cells were treated with ATP from 5 to 240 min (5, 10, 30, 60, 120 and 240 min), and the levels of phosphor-Src or phosphor-VEGFR2 were determined by western blot analysis. Following this, the level of ESM-1 mRNA and protein in further experiments was determined after 24 h and the level of phosphor-Src and phosphor-VEGFR2 was determined after 5 min of ATP treatment. The treatment time of ATP for Forkhead box O1 (FoxO1) and other phosphorylated proteins such as phosphor-protein kinase C (PKC), phosphor-PAK1, phosphor-c-Jun N-terminal kinase (JNK) and phosphor-p38 were based on our previous study (19). The level of FoxO1 was determined after 8 h, phosphor-PKC, phosphor-PAK1 and phosphor-JNK after 5 min, and phosphor-p38 after 60 min of ATP treatment. PP2 was pretreated at 37˚C for 1 h before the ATP treatment when required.

Small interfering RNA (siRNA) transfection. RT-R-MDA-MB-231 cells were transfected with 100 nM negative control siRNA (control siRNA), $P2Y_2R$ siRNA or VEGFR2 siRNA (Bioneer Corporation) (Table I) using Lipofectamine 3000 (cat. no. L300015; Thermo Fisher Scientific, Inc.) in serum-free medium for 6 h at 37˚C. Then, the medium containing the siRNA mixture was replaced with complete medium. After additional incubation for 18 h at 37˚C, the transfected cells were used for the indicated experiments.

Total RNA extraction and reverse transcription (RT)-PCR. The 100 μM ATP-treated or siRNA-transfected cells were harvested, and total RNA was extracted using TRIzol reagent (cat. no. 15596018; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. RT-PCR was performed using TOPscript One-step RT-PCR Drymix (cat. no. RT421; Enzynomics Co., Ltd.) according to the manufacturer's protocol. Primer sets are listed in Table II. After denaturation at 95˚C for 10 min, a total of 27 amplification cycles were performed under the following conditions: Denaturation at 95˚C for 30 sec, annealing at 57.5˚C for 30 sec and extension at 72˚C for 1 min. GAPDH was used as the internal control for normalization. From each reaction mix, 10 μl was subjected to 1% agarose gel electrophoresis and stained with ethidium bromide solution diluted in 0.5X Tris-Acetate-EDTA buffer. Each band was quantified by scanning densitometry using Image Master® VDS (GE Healthcare) and Image J software version 1.48 (National Institutes of Health) and normalized with GAPDH.

Protein extraction from whole cell lysate or nuclear/cytosolic fractions and western blot analysis. After the treatment with ATP as aforementioned, cells were harvested and lysed with radioimmunoprecipitation assay buffer [1X PBS containing

Table I. Small interfering RNA sequences used for transfection.

Gene		Sequence (5'-3')
Negative Control	Forward	CCUACGCCACCAAUUUCGU
	Reverse	ACGAAAUUGGUGGCGUAGG
$P2Y_2R$	Forward	GAGGAAGGUGGCUUACCAA
	Reverse	UUGGUAAGCCACCUUCCUC
VEGFR2	Forward	CUCCUAAUGAGAGUUCCUU
	Reverse	AAGGAACUCUCAUUAGGAG

$P2Y_2R$, $P2Y_2$ purinergic receptor; VEGFR2, vascular epithelial growth factor receptor 2.

Table II. Primer sequences for reverse transcription-PCR.

Gene		Primer sequence (5'-3')
hESM-1	Forward	GCCCTTCCTTGGTAGGTAGC
	Reverse	TGTTTCCTATGCCCCAGAAC
$hP2Y_2R$	Forward	GTGCTCTACTTCCTGGCT
	Reverse	CTGAAGTGTTCTGCTCCTAC
hGAPDH	Forward	TCAACAGCGACACCCACTCC
	Reverse	TGAGGTCCACCACCCTGTTG

hESM-1, human endothelial-specific molecule-1; $hP2Y_2R$, human $P2Y_2$ purinergic receptor.

0.1% nonyl phenoxylpolyethoxylethanol-40, 0.1% sodium dodecyl sulfate (SDS) and 1% protease inhibitor cocktail]. After incubation for 1 h on ice, the cell suspension was centrifuged at 16,000 x g for 15 min at 4˚C, and the supernatant was acquired. The proteins from nuclear or cytosolic fractions were extracted using a Nuclear/Cytosol Fractionation Kit (cat. no. K266; BioVision, Inc.) according to the manufacturer's protocol and as described previously (19). The protein concentration was determined using the Bradford method. The extracted proteins (30-60 μg) were subjected to 8% SDS-PAGE and transferred onto polyvinylidene fluoride membranes. The membranes were blocked with 5% non-fat milk in 1X TBS-0.1% Tween 20 for 1 h at room temperature, and then incubated for 1-2 days at 4˚C with the following primary antibodies: Anti-ESM-1 (cat. no. ab103590; 1:1,000; Abcam), anti-phosphor-Src (Try416) cat. no. 6943; 1:1,000; Cell Signaling Technology, Inc.), anti-Src (cat. no. 2109; 1:1,000; Cell Signaling Technology, Inc.), anti-phosphor-VEGFR2 (Tyr1175) (cat. no. 3770; 1:500; Cell Signaling Technology, Inc.), anti-VEGFR2 (cat. no. 2479; 1:1,000; Cell Signaling Technology, Inc.), anti-FoxO1 (cat. no. 2880; 1:1,000; Cell Signaling Technology, Inc.), anti-phosphor-PKC (cat. no. 9375; 1:1,000; Cell Signaling Technology, Inc.), anti-PKC (cat. no. sc-10800; 1:1,000; Santa Cruz Biotechnology, Inc.), anti-phosphor-PAK1 (cat. no. 2601; 1:1,000; Cell Signaling Technology, Inc.), anti-PAK1 (cat. no. 2602; 1:1,000; Cell Signaling Technology, Inc.), anti-phosphor-JNK (cat. no. 9251; 1:1,000; Cell Signaling Technology, Inc.), anti-JNK (cat. no. 9252; 1:1,000; Cell Signaling Technology, Inc.), anti-phosphor-p38 (cat. no. 9211; 1:1,000; Cell Signaling Technology, Inc.), anti-p38 (cat. no. sc-535; 1:1,000; Santa Cruz Biotechnology, Inc.), anti-β-actin (cat. no. MA5-15739; 1:5,000; Thermo Fisher Scientific, Inc.), and anti-proliferating cell nuclear antigen (PCNA; cat. no. sc-25280; 1:1,000; Santa Cruz Biotechnology, Inc.). After primary antibody incubation, the membranes were washed with 1X TBS-0.1% Tween 20 and incubated with 5% non-fat milk in TBS-Tween 20 containing an HRP-conjugated anti-rabbit IgG (cat. no. 111-035-003; 1:5,000; Jackson ImmunoResearch Laboratories, Inc.) or anti-mouse IgG (cat. no. A90-116P; 1:5,000; Bethyl Laboratories, Inc.) for 1 h at room temperature. The immunoreactive bands were visualized by an ECL substrate (cat. no. 170-5061; Bio-Rad Laboratories, Inc.). The relative level of each protein was normalized to the level of β-actin as a loading control. Protein densities were semi-quantified using ChemiDOC™ XRS+ Systems with Image Lab™ Software version 5.2 (Bio-Rad Laboratories, Inc.).

Statistical analysis. All data were statistically analyzed by using GraphPad Prism 7 software (Dotmatics). One-way ANOVA followed by Tukey's post hoc test was conducted to compare group differences. The data are presented as the mean ± SD. $P<0.05$ was considered to indicate a statistically significant difference.

Results

ATP increases ESM-1 mRNA and protein expression in a $P2Y_2R$-dependent manner in RT-R-MDA-MB-231 cells. It was previously reported that RT-R-MDA-MB-231 cells

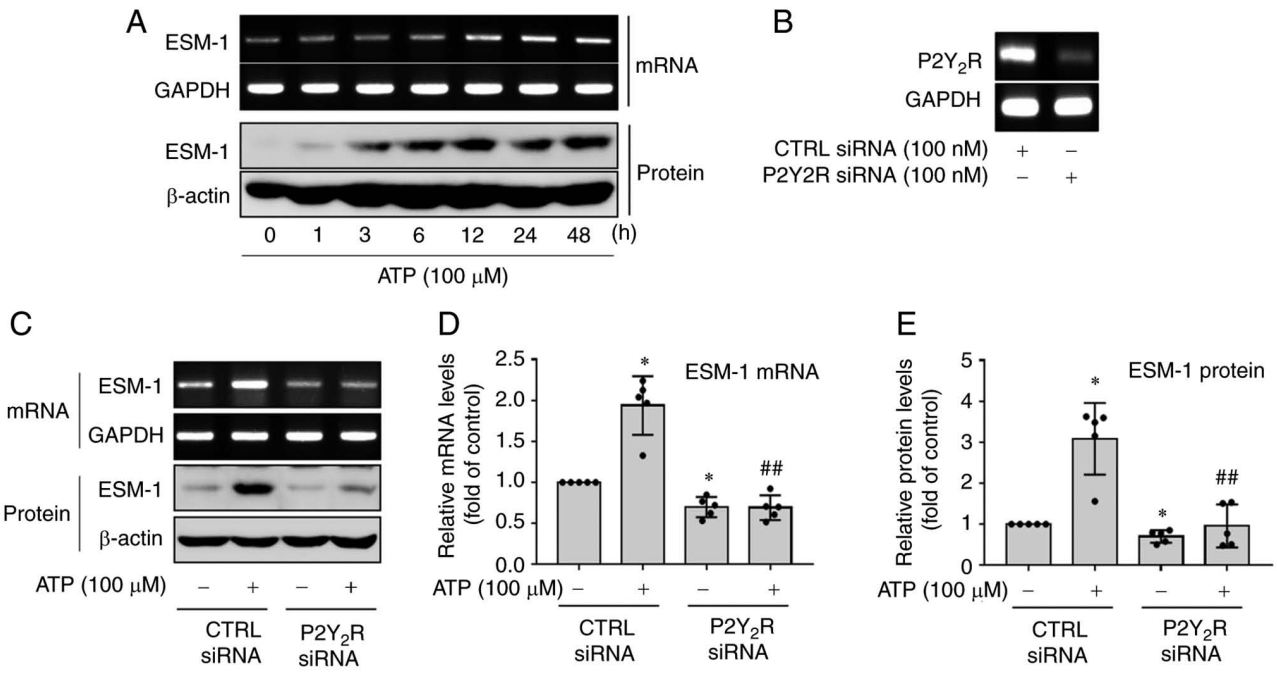

Figure 1. $P2Y_2R$ activation by ATP increased the ESM-1 mRNA and protein expression levels in RT-R-MDA-MB-231 cells. (A) RT-R-MDA-MB-231 cells were treated with 100 μM ATP at the indicated times, and then total RNA and proteins were extracted from the cells for detecting ESM-1 mRNA and protein expression by RT-PCR and western blotting, respectively. (B) RT-R-MDA-MB-231 cells were transfected with 100 nM control or $P2Y_2R$ siRNA. The knockdown efficiency of $P2Y_2R$ expression using $P2Y_2R$ siRNA was determined by RT-PCR. After transfection, the cells were treated with 100 μM ATP for 24 h, and then (C) ESM-1 mRNA and protein expression levels were analyzed by RT-PCR and western blotting, respectively. The relative fold change in (D) mRNA and (E) protein levels were quantified. GAPDH and β-actin used as loading controls in the RT-PCR and western blotting experiments, respectively. The values are presented as the mean ± SD of five independent experiments. *P<0.05 compared with the untreated CTRL siRNA group; $^{\#\#}$P<0.01 compared with the ATP-treated CTRL siRNA group. CTRL, control; ESM-1, endothelial-specific molecule-1; $P2Y_2R$, $P2Y_2$ purinergic receptor; RT-R-MDA-MB-231, radiotherapy resistant MDA-MB-231 cells; RT-PCR, reverse transcription-PCR; siRNA, small interfering RNA.

release more ATP extracellularly than MDA-MB-231 cells (23), and that $P2Y_2R$ is involved in the overexpression of ESM-1 (19). Therefore, in the present study, it was first determined whether the expression of ESM-1 is regulated by extracellular ATP treatment in a $P2Y_2R$-dependent manner in RT-R-MDA-MB-231 cells. Extracellular ATP (100 μM) increased mRNA and protein levels of ESM-1 in a time-dependent manner (Fig. 1A). The increase in ESM-1 expression induced by ATP treatment was significantly inhibited by $P2Y_2R$ siRNA, suggesting that $P2Y_2R$ activation by ATP released from RT-R-MDA-MB-231 cells is involved in the upregulation of ESM-1 (Fig. 1B-E).

ATP-mediated $P2Y_2R$ activation is involved in the transactivation of VEGFR2 via upregulating Src activity. According to a report, ATP-activated $P2Y_2R$ regulates Src activation, which leads to transactivation of epidermal growth factor receptor (EGFR) and induces pulmonary mucoepidermoid carcinoma cell migration (24). Moreover, it has been reported that VEGFR2 is expressed in breast cancer (25) and VEGFR2 activation increases cell proliferation in TNBC cells (26). In addition, VEGFR2 phosphorylation has been demonstrated to decrease by inhibiting Src with PP2, an Src family kinase inhibitor, in TNBC cells (27). Therefore, the relationship between $P2Y_2R$ and VEGFR2 was explored in the present study, with Src as a possible signaling molecule to link the activation of these receptors. In RT-R-MDA-MB-231 cells, ATP treatment increased the phosphorylation of Src (Fig. 2A) and VEGFR2 (Fig. 2B) within 5-10 min, and the activation of Src and VEGFR2 by ATP at 5 min was $P2Y_2R$-dependent (Fig. 2C-E). Furthermore, Src family kinase inhibitor, PP2, treatment abolished the activation of VEGFR2 induced by ATP in RT-R-MDA-MB-231 cells (Fig. 2F-H), indicating that extracellular ATP induces Src activation and transactivation of VEGFR2 via $P2Y_2R$.

ATP treatment increases ESM-1 mRNA and protein expression levels in a VEGFR2-dependent manner in RT-R-MDA-MB-231 cells. It was next determined whether ATP-mediated VEGFR2 activation is involved in ESM-1 expression in RT-R-MDA-MB-231 cells. It is shown in Fig. 3 that increased ESM-1 mRNA and protein levels in response to ATP treatment were suppressed by the knockdown of VEGFR2 in RT-R-MDA-MB-231 cells, suggesting that ATP-mediated VEGFR2 activation is involved in ESM-1 expression.

VEGFR2 is involved in the signaling pathways linked to the $P2Y_2R$-mediated ESM-1 expression in RT-R-MDA-MB-231 cells. It was previously reported that $P2Y_2R$ activated by ATP was involved in PAK1 activation, subsequent JNK and p38 MAPK activation, FoxO1 induction and ESM-1 expression in RT-R-MDA-MB-231 cells (19). It was therefore investigated whether ATP-mediated VEGFR2 activation through $P2Y_2R$ is related to the translocation of FoxO1 to the nucleus from the cytosol and the subsequent activation of upstream signaling molecules, PKC, PAK1, JNK MAPK and p38 MAPK. As expected, ATP induced FoxO1 translocation from the cytosol

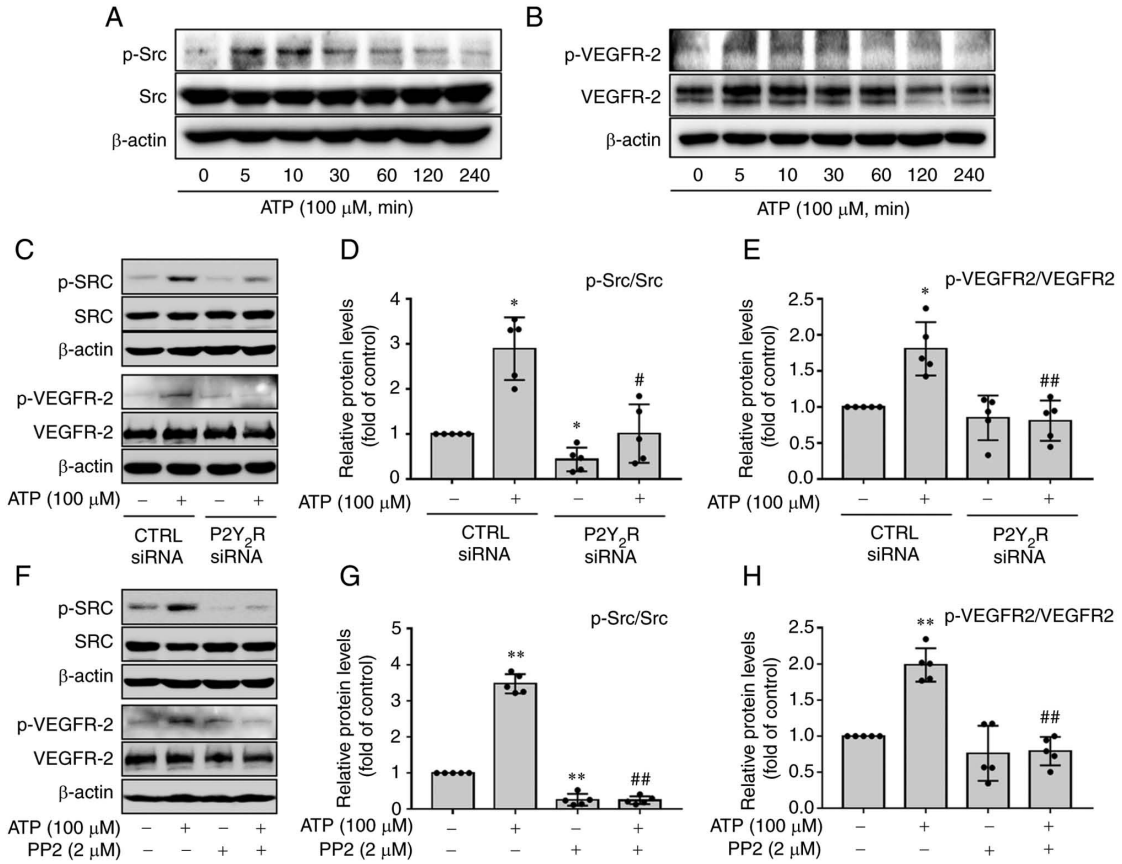

Figure 2. ATP-mediated $P2Y_2R$ activation phosphorylated VEFGR2 via Src activation in RT-R-MDA-MB-231 cells. RT-R-MDA-MB-231 cells were treated with 100 μM ATP in a time-dependent manner. (A) Phosphor- and total-Src, and (B) phosphor-(Tyr1175) and total VEGFR2 levels were analyzed from cell lysates by western blotting. β-actin was used as the loading control. (C-E) RT-R-MDA-MB-231 cells were transfected with 100 nM CTRL or $P2Y_2R$ siRNA and then treated with 100 μM ATP for 5 min. Cells were harvested and (C) phosphor-(Tyr416)/total-Src and phosphor-(Tyr1175)/total-VEGFR2 levels were analyzed from the cell lysates by western blotting. (D and E) β-actin was used as the loading control and the bands were semi-quantified. The values are presented as the mean ± SD of five independent experiments. *P<0.05 compared with the untreated CTRL siRNA group; $^{\#}$P<0.05; $^{\#\#}$P<0.01 compared with the ATP-treated CTRL siRNA group. (F-H) RT-R-MDA-MB-231 cells were treated with 2 μM PP2, a selective Src family inhibitor, for 1 h and then treated with 100 μM ATP for 5 min. The proteins were extracted and (F) phosphor-(Tyr416)/total-Src and phosphor-(Tyr1175)/total-VEGFR2 levels were analyzed by western blotting. (G and H) β-actin was used as the loading control, and the bands were semi-quantified. The values are presented as the mean ± SD of five independent experiments. **P<0.01 compared with the untreated CTRLgroup; $^{\#\#}$P<0.01 compared with the ATP-treated CTRL group. CTRL, control; $P2Y_2R$, $P2Y_2$ purinergic receptor; phosphor, phosphorylated; RT-R-MDA-MB-231, radiotherapy resistant MDA-MB-231 cells; RT-PCR, reverse transcription-PCR; siRNA, small interfering RNA; VEGFR2, vascular epithelial growth factor receptor 2.

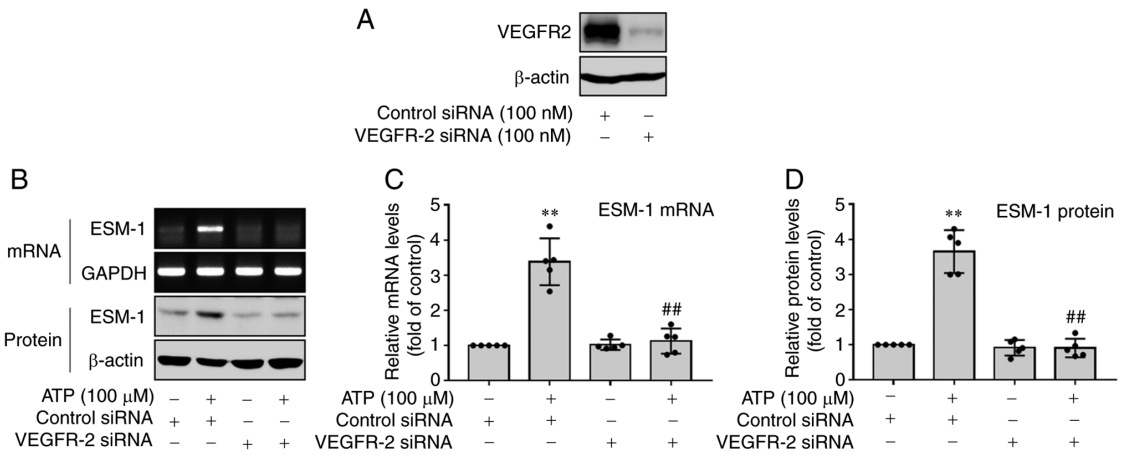

Figure 3. Increase in ESM-1 expression by ATP treatment was inhibited by VEGFR2 knockdown using VEGFR2 siRNA. (A) RT-R-MDA-MB-231 cells were transfected with 100 nM control or VEGFR2 siRNA and transfection efficiency was confirmed by assessing the protein levels by western blotting. (B-D) Control or VEGFR2 siRNA-transfected RT-R-MDA-MB-231 cells were treated with 100 μM ATP for 24 h, and then (B) ESM-1 mRNA and protein expression levels were analyzed by reverse transcription-PCR and western blotting, respectively. (C) GAPDH and (D) β-actin were used as loading controls, respectively, and the bands were semi-quantified. The values are presented as the mean ± SD of five independent experiments. **P<0.01 compared with the untreated control siRNA group; $^{\#\#}$P<0.01 compared with the ATP-treated control siRNA group. ESM-1, endothelial-specific molecule-1; RT-R-MDA-MB-231, radiotherapy resistant MDA-MB-231 cells; siRNA, small interfering RNA; VEGFR2, vascular epithelial growth factor receptor 2.

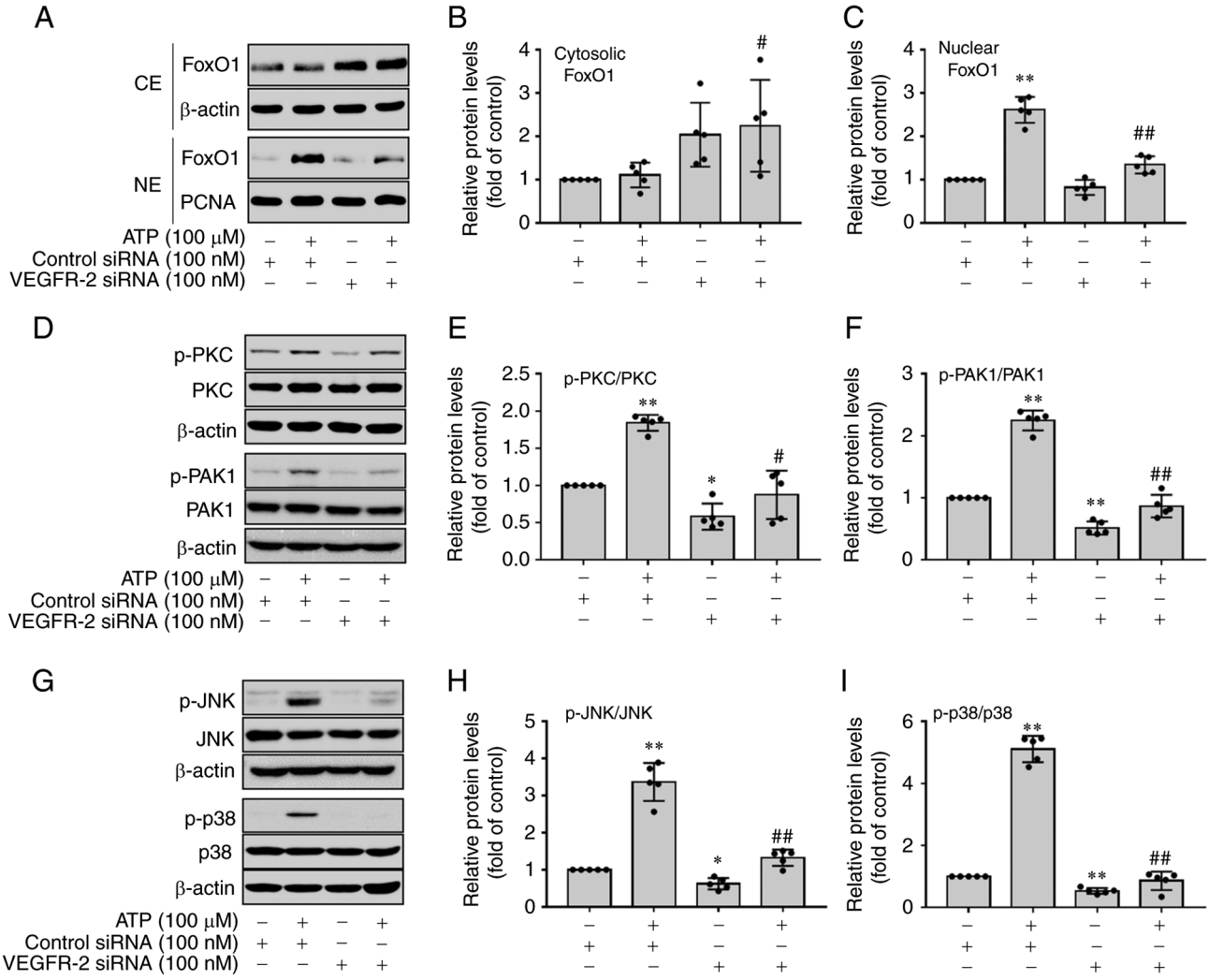

Figure 4. VEGFR2 siRNA-transfected RT-R-MDA-MB-231 cells inhibited activation of the signaling molecules related to the P2Y$_2$R-mediated endothelial-specific molecule-1 expression. (A-C) Control siRNA- or VEGFR2 siRNA-transfected RT-R-MDA-MB-231 cells were treated with 100 μM ATP for 8 h. (A) Then, cytosolic and nuclear fractions were separated from the harvested cells and FoxO1 protein levels in the cytosol or nucleus were analyzed by western blotting. (B) β-actin and (C) PCNA were used as the loading controls, and the bands were semi-quantified. (D-F) Control siRNA- or VEGFR2 siRNA-transfected RT-R-MDA-MB-231 cells were treated with 100 μM ATP for 5 min and then total proteins were obtained from the cell lysates. (D) Phosphor and total PKC or PAK1 levels were analyzed by western blotting. β-actin and (E) total-PKC or (F) total-PAK1 were used as the loading controls, and the bands were semi-quantified. (G-I) Control siRNA- or VEGFR2 siRNA-transfected RT-R-MDA-MB-231 cells were treated for 5 min for JNK detection and 60 min for p38 detection. (G) Then, total proteins were extracted from the cells and phosphor- and total-JNK or phosphor- and total-p38 protein levels were analyzed by western blotting. β-actin and (H) total-JNK or (I) total-p38 were used as the loading controls, and the bands were semi-quantified. The values are presented as the mean ± SD of five independent experiments. *P<0.05, **P<0.01 compared with the untreated control siRNA group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 compared with the ATP-treated control siRNA group. FoxO1, forkhead box O1; JNK, c-Jun N-terminal kinase; PAK1, p21-activated kinase 1; PCNA, proliferating cell nuclear antigen; phosphor, phosphorylated; PKC, protein kinase C; RT-R-MDA-MB-231, radiotherapy resistant MDA-MB-231 cells; siRNA, small interfering RNA; VEGFR2, vascular epithelial growth factor receptor 2.

to the nucleus, which was significantly inhibited by VEGFR2 siRNA (Fig. 4A-C). In addition, PKC, PAK1, JNK MAPK and p38 MAPK phosphorylation by ATP treatment was diminished by transfecting RT-R-MDA-MB-231 cells with VEGFR2 siRNA (Fig. 4D-I). These results suggest that ATP-mediated VEGFR2 activation is related to ESM-1 upregulation in RT-R-TNBC cells through modulation of the related signaling pathway and transcription factor activity.

Discussion

RT is an essential cancer treatment option for most patients with breast cancer at all stages of the disease (before or after lumpectomy) and improves the overall survival rate of patients (28-30). Despite the benefits of RT, some cancer cells in breast (especially in ductal carcinoma and early invasive cancer) and advanced invasive tumors acquire resistance against RT and trigger tumor relapse and metastasis, leading to a poor prognosis (31,32). Thus, RT resistance is fatal for patients with TNBC who only have CT or RT as treatment options due to being unsuitable for hormone therapy, which can dramatically increase mortality (16). Therefore, the therapy limitation of acquiring RT resistance needs to be overcome.

Previous studies demonstrated that RT-R-breast cancer cells, especially RT-R-TNBC cells that were established by repeated fractionated irradiation, exhibited increased tumor growth and metastasis (17,18,23). Moreover, it was determined

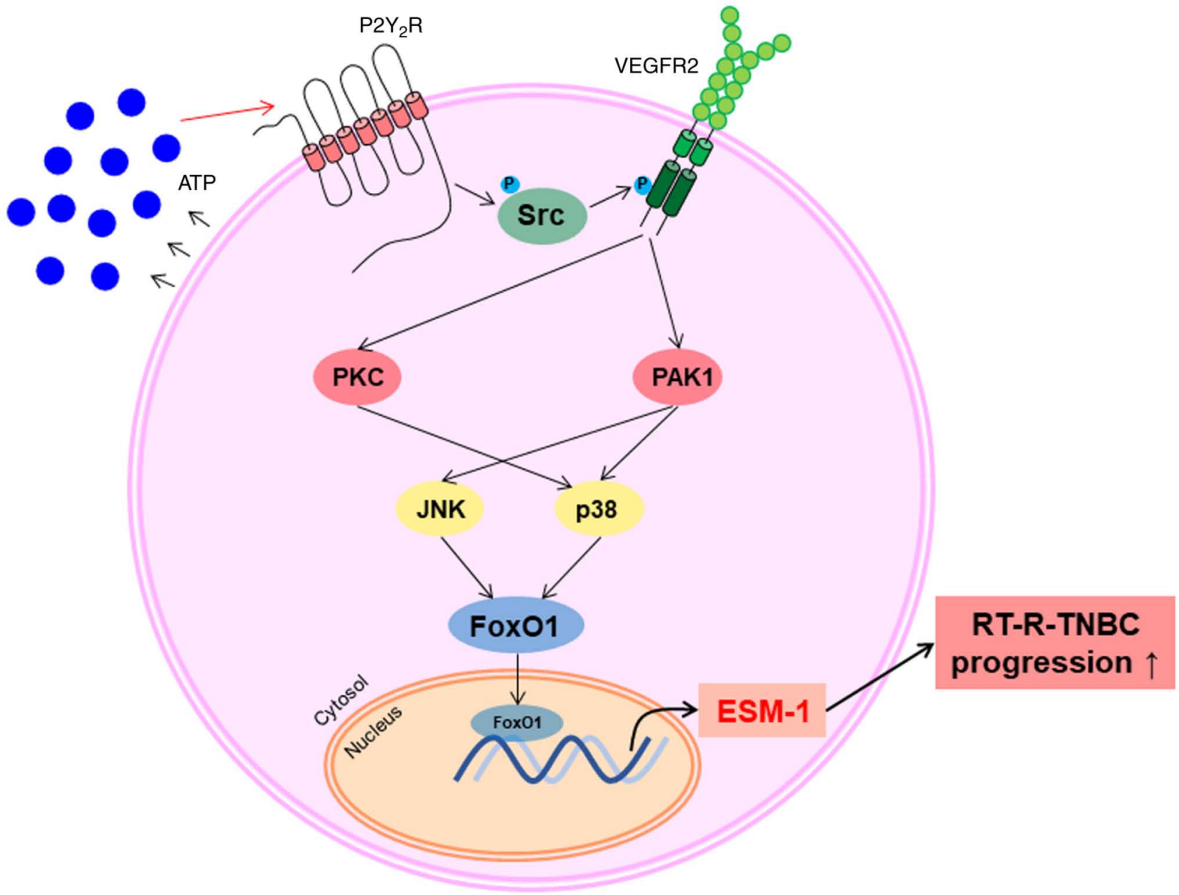

Figure 5. Schematic presentation depicting the proposed $P2Y_2R$-mediated VEGFR2 transactivation via the Src induction of ESM-1 expression. Extracellular ATP released from RT-R-MDA-MB-231 cells activates $P2Y_2R$ and then activated $P2Y_2R$ transactivates VEGFR2 via Src phosphorylation. Phosphorylated VEGF2 mediates PAK1 and PKC activation and subsequent JNK and p38 activation, which results in FoxO1 translocation to the nucleus from the cytosol to induce ESM-1 expression. ESM-1, endothelial-specific molecule-1; FoxO1, forkhead box O1; JNK, c-Jun N-terminal kinase; $P2Y_2R$, $P2Y_2$ purinergic receptor; PAK1, p21-activated kinase 1; PKC, protein kinase C; RT-R-MDA-MB-231, radiotherapy resistant MDA-MB-231 cells; siRNA, small interfering RNA; TNBC, triple negative breast cancer cell; VEGFR2, vascular epithelial growth factor receptor 2.

that ESM-1 is the most upregulated gene in RT-R-TNBC cells compared with TNBC cells, and it is related to increased tumorigenesis of RT-R-TNBC cells (18). In addition, ATP is released from TNBC cells at high levels and is involved in breast cancer growth and metastasis through the activation of $P2Y_2R$ (13,33,34). ATP-mediated $P2Y_2R$ activation upregulates ESM-1 expression through PAK1, PKC, MAPKs, and the transcription factor, FoxO1 (19).

It has been reported that ESM-1 expression is stimulated via the phosphorylation and activation of VEGFR2 (35). VEGF is the predominant angiogenic factor expressed in solid tumors and regulates tumor angiogenesis (36). This growth factor binds to VEGFR, which has two main receptors expressed widely in vascular endothelial cells, VEGFR1 and VEGFR2 (37,38). Although VEGF has ~10-fold higher affinity to VEGFR1 than VEGFR2, the level of phosphorylation of VEGFR1 induced by VEGF is low, and thus, VEGFR1 signaling remains poorly understood (37,38). By contrast, VEGFR2 has been reported as the dominant receptor mediating VEGF proangiogenic activity in endothelial cells (39). VEGF binds to VEGFR2 and induces receptor dimerization and autophosphorylation (40,41). The major phosphorylation sites in VEGFR2 are Tyr951 in the kinase-insert domain, Tyr1054 and Tyr1059 in the tyrosine kinase domain, and Tyr1175 and Tyr1214 in the C-terminus (26). After the phosphorylation of VEGFR2, SH2 domain-containing adaptor proteins are recruited to these phosphorylated residues to mediate the downstream effects of VEGFR2, including cell proliferation, permeability, survival and migration (26). Notably, certain studies have reported that $P2Y_2R$ can transactivate growth factor receptors, including EGFR (42-45) and VEGFR2 (19). In particular, activation of the G protein-coupled $P2Y_2R$ facilitates Src binding to Src homology-3-binding motifs in the $P2Y_2R$ and promotes the interaction and transactivation of VEGFR2, resulting in final vascular cell adhesion molecule-1 expression (19). Therefore, an aim of the present study was to clarify whether $P2Y_2R$ regulates the expression of ESM-1 through VEGFR2 transactivation in RT-R-MDA-MB-231 cells. In the present study, it was demonstrated that extracellular ATP increased VEGFR2 phosphorylation at Tyr1175 within 5-10 min, and this effect was $P2Y_2R$-dependent. Moreover, ATP also increased Src phosphorylation at Tyr416 at 5 min, which was inhibited by $P2Y_2R$ siRNA, and an Src inhibitor, PP2, decreased ATP-mediated VEGFR phosphorylation. These results suggested that $P2Y_2R$ activation by ATP mediates VEGFR2 transactivation through Src phosphorylation in RT-R-MDA-MB-231 cells. Furthermore, the $P2Y_2R$-induced

activation of signaling molecules and transcription factors involved in ESM-1 expression, such as PAK1, PKC, JNK, p38 MAPKs and FoxO1, were inhibited by VEGFR2 siRNA in RT-R-MDA-MB-231 cells. Although phosphoinositide-dependent kinase 1 (PDK1) was reported to be involved in the ESM-1 overexpression in RT-R-MDA-MB-231 cells, $P2Y_2R$ activation by ATP did not influence PDK1 activity (19). Thus, $P2Y_2R$-VEGFR activation also did not affect the phosphorylation of PDK1 in the present study (data not shown). Taken together, we hypothesize that $P2Y_2R$ activated by extracellular ATP released from RT-R-BC cells phosphorylates Src and then transactivates VEGFR2. Phosphor-VEGF2 mediates PAK1 and PKC activation and then JNK and p38 activation, which finally results in FoxO1 translocation to the nucleus from the cytosol to induce ESM-1 expression (Fig. 5). The present study therefore provides a novel strategy to target the $P2Y_2R$-VEGFR2-ESM-1 axis for treating patients with RT-R-TNBC. However, a limitation of this study is the lack of *in vivo* experiments on the effect of targeting the $P2Y_2R$-VEGFR2-ESM-1 axis; we aim to address this in our future research.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HJ performed the experiments, analyzed the data and wrote the manuscript. YSK also conducted the experiments. SWP and SPY contributed to study design and methodology, and revised the manuscript. HJK conceived and designed the study, directed the project, interpreted the results, and wrote and revised the manuscript. HJ, YSK and HJK confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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