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ORIGINAL ARTICLE

Small GTP-binding protein Rap1 mediates EGF and HB-EGF signaling and modulates EGF receptor expression in HTR-8/SVneo extravillous trophoblast cells

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Abstract

Purpose: Extravillous trophoblasts (EVTs) invade the endometrium to establish a feto-maternal interaction during pregnancy. Epidermal growth factor (EGF) and heparin-binding EGF-like growth factor (HB-EGF) stimulate EVT invasion by binding to the EGF receptor (EGFR). We examined the role of the small GTP-binding protein Rap1 in EGF- and HB-EGF-stimulated EVT invasion.

Methods: Expression of Rap1 in the first-trimester placenta was examined by immunohistochemistry. Effect of EGF or HB-EGF on Rap1 activation (GTP-Rap1) and Rap1 knockdown on invasion was assessed in EVT cell line (HTR-8/SVneo). In addition, effect of Rap1 knockdown and Rap1GAP (a Rap1 inactivator) overexpression on the activation of EGF signaling and EGFR expression were examined.

Results: Rap1 was expressed by EVTs, villous cytotrophoblasts, and syncytiotrophoblasts in the placenta. EGF and HB-EGF activated Rap1 and promoted invasion of HTR-8/SVneo, and these effects were inhibited by Rap1 knockdown. The EGF- and HB-EGF-induced phosphorylation of AKT, ERK1/2, p38MAPK, and Src was inhibited by Rap1 knockdown. Furthermore, the knockdown of Rap1 reduced the EGFR protein level. Overexpression of Rap1GAP repressed EGF- and HB-EGF-induced Rap1 activation and reduced EGFR expression.

Conclusion: Rap1 may function as a mediator of EGF and HB-EGF signaling pathways and can modulate EGFR expression in EVTs during placental development.

KEYWORDS
EGF, extravillous trophoblast, HB-EGF, invasion, Rap1

1 | INTRODUCTION

Invasion of human placental trophoblasts into maternal uterine tissues is crucial for normal placental development and successful pregnancy. Extravillous trophoblasts (EVTs), which develop from anchoring villi, migrate and invade into the maternal decidua, replacing the arterial endothelium to establish blood flow to the placental implantation site and enable efficient physiological exchange between the maternal and fetal circulatory systems.[1] Insufficient differentiation and invasion of EVTs into the decidua

results in poor remodeling of the uterine spiral arterioles and a reduction of placental perfusion, both of which are common characteristics of pregnancy-associated complications such as preeclampsia (PE) and fetal growth restriction (FGR).[2] Understanding the mechanisms that control EVT invasion will help to delineate both the phenomenon of placenta formation and the pathogeneses of PE and FGR.

Epidermal growth factor (EGF) and heparin-binding EGF-like growth factor (HB-EGF) stimulate EVT migration and invasion during placentation[3–9] by binding to the membrane-bound EGF receptor (EGFR) and activating subsequent receptor-mediated phosphatidylinositol-3-kinase (PI3K)/AKT and mitogen-activated protein kinase (MAPK) signaling pathways. In human placenta, EGF is expressed in the cytotrophoblasts at 4–5 weeks, of gestation age. At 6–12 weeks of gestation age, the expression of EGF is detected in the syncytiotrophoblasts.[10] HB-EGF has been shown to express in villous and extravillous cytotrophoblasts up to 35 weeks of gestation, but its expression has been reduced in the PE placenta.[4]

EGFR is expressed in villous cytotrophoblasts, syncytiotrophoblasts, and EVT.[11] Reduced expression of EGF and HB-EGF in the placenta is associated with PE.[12]

Ras-associated protein-1 (Rap1), a small GTP-binding protein, is activated by various extracellular stimuli, such as growth factors and cytokines that act on receptor tyrosine kinases and G-protein-coupled receptors.[13,14] The signaling pathways activated by these receptors stimulate guanine nucleotide exchange factors (GEF), leading to conversion of the GDP-bound inactive form of Rap1 to the GTP-bound active form.[14,15] Inactivation of Rap1 is mediated through GTPase-activating proteins (GAPs) such as Rap1GAP, which promote the hydrolysis of bound GTP to GDP.[14,16] Exchange protein directly activated by cAMP (EPAC), also known as cAMP-GEF, is one of the most well-characterized RapGEFs.[17,18] We demonstrated previously that the EPAC-mediated signaling pathways stimulate human trophoblast differentiation and syncytialization,[19] as well as protein kinase A-mediated endometrial stromal cell decidualization, by activating Rap1.[20–22]

As aforementioned, Rap1 is activated by various growth factors, including EGF,[23] neural growth factor,[24] and hepatocyte growth factor,[25] and it plays a vital role in the migration and invasion of a variety of tumor cells, including prostate,[26] pancreatic,[27] breast,[28] thyroid,[29] and melanoma[30] cells. However, the physiological role of Rap1 in EVT invasion has not yet been examined. In this study, we investigated the role of Rap1 in EGF- and HB-EGF-stimulated EVT invasion, mediated through EGFR.

2 | MATERIALS AND METHODS

2.1 | Reagents

Recombinant human EGF and HB-EGF were purchased from R&D Systems, Inc. (Minneapolis, MN, USA). PP2, a Src inhibitor, was obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2 | Immunohistochemistry

Placental tissues were obtained from three women undergoing legal miscarriages at 6–10 weeks of pregnancy. The use of these tissues was approved by the Clinical Research Ethics Committee of Tokyo University of Pharmacy and Life Sciences (#1512), and informed consent was provided by all participants ($n=3$). Placental tissues were embedded in paraffin and sectioned. For immunohistochemistry experiments, the paraffin-embedded sections were deparaffinized and rehydrated. After boiling with 10 mM citrate buffer (pH 6.0) for antigen retrieval, the sections were soaked in 3% H_2O_2 for 30 min and then blocked with 10% normal goat serum diluted in phosphate-buffered saline. Subsequently, the sections were incubated overnight at 4°C with an anti-Rap1 antibody (1:200 dilution; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) or normal rabbit IgG as a negative control. The following morning, the sections were incubated with antirabbit IgG Fab labeled with horseradish peroxidase (Histofine Simple Stain MAX-PO MULTI; Nichirei, Tokyo, Japan) and developed with Histofine Simple Stain DAB solution (Nichirei). The nuclei were counterstained with methyl green.

2.3 | Cell culture

The HTR-8/SVneo cell line was kindly provided by Dr. Charles Graham (Queen's University, Kingston, ON, Canada)[31] and was cultured in RPMI1640 medium supplemented with 5% (v/v) fetal bovine serum, 50 μg/mL penicillin, 50 μg/mL streptomycin, and 100 μg/mL neomycin.

2.4 | Small interfering RNA (siRNA) and plasmid vector transfection

HTR-8/SVneo cells were grown to 50% confluency in 24-well culture plates and then transfected with a nontargeting control siRNA (15 pmol/well; Qiagen, Mississauga, ON, Canada) or a Rap1 siRNA (15 pmol/well; sc-36384D; Santa Cruz Biotechnology, Santa Cruz, CA, USA) using the Lipofectamine RNAiMAX transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After treatment with the siRNAs for 24 h, cells were rinsed with phosphate-buffered saline and cultured in a fresh medium.

Cells were transfected with pEGFP vector or pEGFP-Rap1GAP vector which were provided by Dr. Yoshimi Takai[32] using the Lipofectamine 3000 (Invitrogen).

2.5 | Immunoblot analysis

Cell lysates were prepared in RIPA buffer (Cell Signaling Technology (CST), Danvers, MA, USA). Equal amounts of protein (20 μg) were separated by SDS-PAGE (5–20% gradient gel) and then transferred electrophoretically to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA) using a semidry transfer system (ATTO, Tokyo, Japan) for 60 min at a constant current of 128 mA. The membranes were

then probed with primary antibodies against Rap1 (Millipore), MMP-2 (Proteintech, Rosemont, IL, USA), phospho-AKT (p-AKT; ser-473; CST), AKT (CST), phospho-ERK1/2 (p-ERK1/2; CST), ERK1/2 (CST), phospho-p38MAPK (p-p38MAPK; pT180/pY182; BD Biosciences, San Jose, CA, USA), p38MAPK (BD Biosciences), EGFR (1:2000; CST), enhanced green fluorescent protein (EGFP, 1:2000; Clontech Laboratories, Mountain View, CA), Rap1GAP (Millipore), and GAPDH (5A12; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). After incubation with the appropriate horseradish peroxidase-labeled secondary antibody (1:5000, PI-1000, and PI-2000; Vector Laboratories, Burlingame, CA, USA), immunoreactive bands were visualized using an enhanced chemiluminescence system (Western Lightning; PerkinElmer, Inc., Waltham, MA, USA) and analyzed using an ImageQuant LAS 500 device (semiauto mode; GE Healthcare Japan, Tokyo, Japan). Relative band intensities were assessed by densitometry analysis of the digitalized autographic images using ImageJ software (plug-in: Gel Plot; NIH, Bethesda, MD, USA) and were normalized to the band intensity of GAPDH.

2.6 | Cell viability assay

Cell viability was evaluated using 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium salt (WST-8; Cell Counting Kit-8; Dojindo, Kumamoto, Japan). Briefly, HTR-8/SVneo cells were transfected with control or Rap1 siRNA for 24 h and then incubated with WST-8 for 20 min at 37°C. The staining intensity of the medium was determined by measuring absorbance at 450 nm, and the data were expressed relative to the control value.

2.7 | Cell invasion assay

Invasion assays of HTR-8/SVneo cells were carried out using a transwell system equipped with 8 μm pore-size polycarbonate filters (Chemotaxicell; Kurabo, Osaka, Japan), as described previously.[33] Cells transfected with control or Rap1 siRNA for 24 h were seeded onto the fibronectin-coated upper compartment. The chambers were then placed into 24-well culture plates (lower compartment) containing basal media supplemented with EGF (100 ng/mL) or HB-EGF (100 ng/mL) to stimulate invasion. After incubation for 24 h, noninvading cells remaining on the upper side of the inserts were removed, and the cells that had invaded the lower surface of the filters were fixed with 4% paraformaldehyde. The nuclei of invaded cells were stained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI), and the numbers of cells in five fields of each filter were counted. Data were expressed as the mean ± SEM of at least three independent experiments.

2.8 | Rap1 GTPase activation assay

HTR-8/SVneo cells were stimulated with EGF or HB-EGF, and the level of active Rap1 (GTP-Rap1) was determined using a Rap1 Activation Assay Kit (Millipore).[21] Briefly, cell lysates were incubated with a GST-RalGDS-RBD fusion protein and pulled down with glutathione agarose. The pull-down samples and cell lysates were separated by SDS-PAGE and immunoblotted using an anti-Rap1 antibody to detect GTP-Rap1 and total Rap1.

2.9 | RNA isolation and RT-PCR analysis

Total RNA was extracted from HTR-8/SVneo cells using Isogen reagent (Nippon Gene, Tokyo Japan), according to the manufacturer's instructions. The extracted RNA (100 ng) was reverse-transcribed (55°C, 10 min) and amplified using the iScriptTM One-Step RT-PCR Kit with SYBR Green (Bio-Rad Laboratories, Hercules, CA, USA). The thermal cycling protocol included 1 min at 95°C for initial denaturation, 40 cycles of 10 s at 95°C for denaturation and 30 s at 60°C for annealing and extension, and the melting curve analysis. The reactions were carried out on an iQ5 Real-Time PCR Detection System (Bio-Rad Laboratories). Sense (S) and antisense (AS) primers are 5'-AACTGTGAGGTGGTCCTTGG -3′ (S) and 5'-GTTGAGGGCAATGAGGACAT-3′ (AS) for EGFR (NM_005228.5, PCR product size: 114 bp), 5′- AGCCACATCGCTCAGACA-3′ (S) and 5′- GCCCAATACGACCAAATCC-3′ (AS) for GAPDH (NM_002046.7, PCR product size: 66 bp). Fold changes in the expression level of each gene were calculated using the ΔΔCt method, with GAPDH as an internal control.[19]

2.10 | Statistical analysis

Statistical significance was assessed using a Student's *t*-test or ANOVA followed by the Tukey–Kramer multiple comparisons test, and $p < 0.05$ was considered statistically significant.

3 | RESULTS

3.1 | Immunolocalization of Rap1 in the human placenta and effects of Rap1 knockdown on EGF- or HB-EGF-stimulated trophoblast invasion

Immunohistochemical analysis of the human first-trimester placenta identified Rap1 expression in villous cytotrophoblasts, syncytiotrophoblasts, and EVTs in the cell column (Figure 1A). No significant signal was detected in the IgG negative control. To determine whether Rap1 is associated with EGF- or HB-EGF-mediated signaling pathways in EVTs, human EVT cell line HTR-8/SVneo cells were treated with these factors, and the cell lysates were subjected to a pull-down assay to determine the level of active GTP-bound Rap1. Both EGF and HB-EGF increased the amount of GTP-Rap1 in HTR-8/SVneo cells (Figure 1B). Since both of these growth factors can promote EVT invasion,[5–7,34] the effects of siRNA-mediated knockdown of Rap1 on EGF- or HB-EGF-mediated cell invasion were examined. Western blotting confirmed that transfection of a Rap1 siRNA into HTR-8/SVneo cells

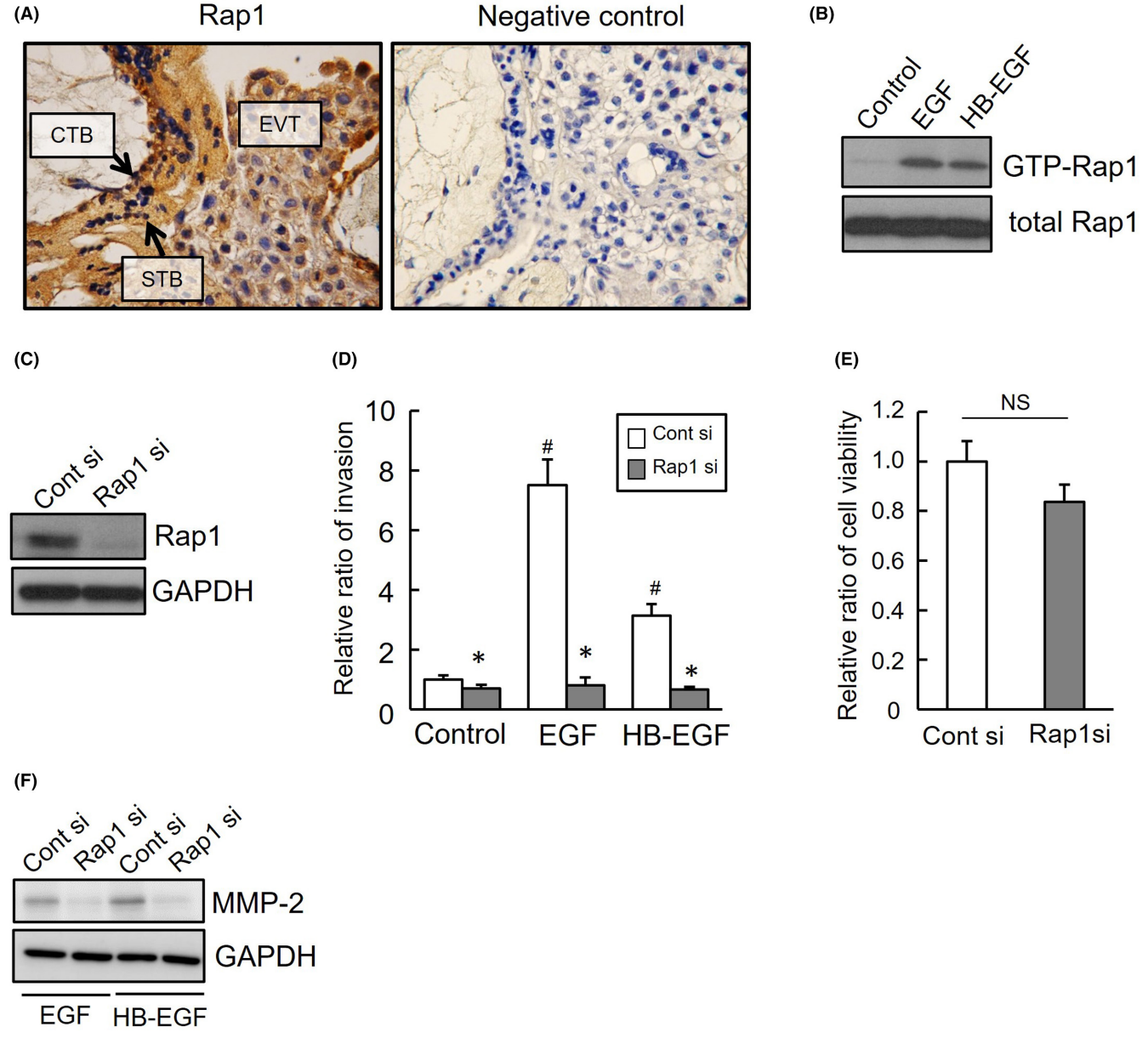

FIGURE 1 Expression of Rap1 in the human placenta and effect of Rap1 knockdown on EGF- or HB-EGF-stimulated HTR-8/SVneo cell invasion. (A) Immunohistochemical analysis of Rap1 in the first-trimester human placenta. (B) Immunoblotting analysis of the levels of total Rap1 and the GTP-bound active form of Rap1 (GTP-Rap1) in HTR-8/SVneo cells. Cells were starved for 6 h and treated with epidermal growth factor (EGF; 100 ng/mL) or heparin-binding EGF-like growth factor (HB-EGF; 100 ng/mL) for 1 min. (C) Immunoblotting analysis of Rap1 in HTR-8/SVneo cells transfected with control (Cont) or Rap1 siRNA. (D) The siRNA-transfected cells described in (C) were plated onto fibronectin-coated transwell chambers and cultured with or without EGF or HB-EGF for 24 h in the presence of 1% FBS. The cells that penetrated to the opposite side of the membrane were counted. (E) WST-8 assay to assess the viabilities of the siRNA-transfected cells described in (C). (D, E) Data are represented as the mean ± SEM of three independent experiments; $^{*}p < 0.01$ versus each Cont si; $^{\#}p < 0.01$ versus Cont si in Control. (F) Cont or Rap1 siRNA-transfected HTR-8/SVneo cells were cultured with EGF (100 ng/mL) or HB-EGF (100 ng/mL) for 24 h in the presence of 1% FBS. Cell lysates were subjected to immunoblotting using an anti-MMP2 antibody.

reduced endogenous Rap1 protein expression (Figure 1C). Treatment of control siRNA-transfected cells with EGF or HB-EGF promoted cell invasion significantly; however, the knockdown of Rap1 impaired these effects significantly (Figure 1D). Knockdown of Rap1 had little effect on the viability of HTR-8/SVneo cells (Figure 1E), suggesting that the inhibitory effect on EVT invasion was not attributable to a reduction in cell viability. It has been reported that MMP-2 participates in trophoblast invasion by degrading the extracellular matrix of the maternal decidua.[35] We examined the effects of Rap1 knockdown on MMP-2 expression in the EGF- or HB-EGF-stimulated HTR-8/SVneo cells (Figure 1F). Knockdown of Rap1 downregulated the MMP-2 protein levels in EGF- or HB-EGF-treated cells (Figure 1F). Overall, these data indicate that Rap1 is involved in EGF- and HB-EGF-stimulated EVT invasion.

3.2 | Knockdown of Rap1 represses EGF- and HB-EGF-mediated intracellular signaling pathways in HTR-8/SVneo cells

To explore the mechanisms underlying the role of Rap1 in EGF- and HB-EGF-induced EVT invasion, we evaluated the effect of Rap1 knockdown on the EGF- and HB-EGF-mediated AKT, ERK1/2, and p38MAPK signaling pathways.[5–7,34] Immunoblot analyses showed that treatment of control siRNA-transfected HTR-8/SVneo cells with EGF or HB-EGF for 20 min induced the phosphorylation of AKT, ERK1/2, and p38MAPK, whereas knockdown of Rap1 repressed these increases (Figure 2A). The basal levels of p-ERK1/2 and p-p38MAPK were also decreased by Rap1 knockdown (Figure 2A). Members of the Src family of nonreceptor tyrosine kinases function as upstream regulators of AKT and MAPK signaling pathways.[36] Therefore, the effects of EGF and HB-EGF stimulation and Rap1 knockdown on Src activation were also investigated. As shown in Figure 2B, the knockdown of Rap1 abolished the EGF- or HB-EGF-mediated activation (phosphorylation) of Src in HTR-8/SVneo cells, suggesting a role of Rap1 as a mediator of Src signaling pathways in EVTs. Moreover, incubation of HTR-8/SVneo cells with PP2, a Src inhibitor, repressed the EGF- and HB-EGF-induced phosphorylation of AKT, ERK1/2, and p38MAPK (Figure 2C) and expression of GTP-Rap1 (Figure 2D) and MMP2 (Figure 2E). Taken together, these

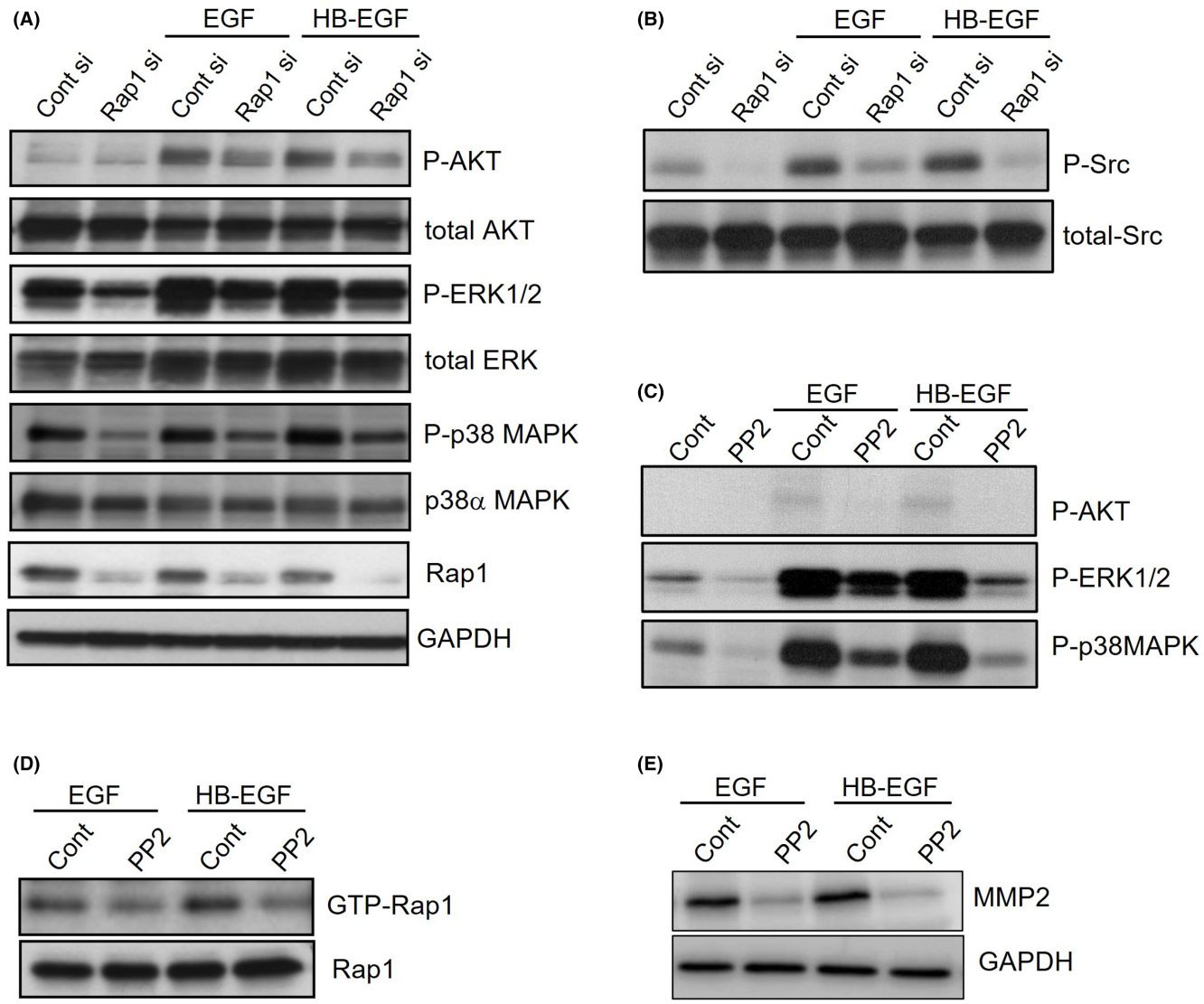

FIGURE 2 The effect of Rap1 knockdown on activation of the EGF and HB-EGF signaling pathways. (A, B) Immunoblot analyses of Rap1, phosphorylated AKT (P-AKT), total AKT, phosphorylated ERK1/2 (P-ERK1/2), total ERK1/2, phosphorylated p38MAPK (P-p38MAPK), p38aMAPK, phosphorylated Src (P-Src), and total Src. HTR-8/SVneo cells transfected with control (Cont) or Rap1 siRNA for 24 h were starved for 6 h and then cultured in the presence or absence of EGF (100 ng/mL) or HB-EGF (100 ng/mL) for 20 min. GAPDH served as an internal control. (C) Immunoblot analyses of P-AKT, P-ERK, and P-p38MAPK. HTR-8/SVneo cells starved for 6 h were treated with a Src inhibitor (PP2, 10 μM) for 1 h and then stimulated with EGF or HB-EGF for 20 min. (D) A GTP-Rap1 pull-down assay of lysates from the cells described in (C). (E) Cells were pretreated with PP2 (10 μM) for 1 h and then cultured with EGF (100 ng/mL) or HB-EGF (100 ng/mL) for 24 h. Immunoblotting of MMP2. (A–E) Representative data from three independent experiments are shown.

observations suggest that Src may regulate the activities of AKT, ERK1/2, p38MAPK, and Rap1 in EGF- and HB-EGF-stimulated cells. Since the knockdown of Rap1 repressed Src activity in the EVTs (Figure 2B), we also speculated that Rap1 can modulate additional upstream sites in the EGF and HB-EGF signaling pathways.

3.3 | Knockdown of Rap1 reduces EGFR protein but not mRNA levels in HTR-8/SVneo cells

As knockdown of Rap1 attenuated EGF- and HB-EGF-stimulated Src, AKT, ERK1/2, and p38MAPK signaling, we investigated whether Rap1 regulates the expression of EGFR, a common receptor for EGF and HB-EGF. The expression level of the EGFR protein was significantly reduced by siRNA-mediated knockdown of Rap1 (Figure 3A). However, the expression level of the EGFR mRNA was not affected by Rap1 knockdown (Figure 3B). These results suggest that Rap1 can function as a posttranscriptional modulator of EGFR in EVTs. We further examined whether EGF or HB-EGF affects EGFR levels in control or Rap1 siRNA transfected cells (Figure 3C). Consistent with the result of Figure 3A, Rap1 knockdown decreased EGFR levels in control cells (Figure 3C). EGF or HB-EGF treatment resulted in the downregulation of EGFR protein levels in control siRNA or Rap1 siRNA transfected cells. This is because that EGF or HB-EGF stimulation causes EGFR degradation by delivery to the lysosomes.[37] As shown in Figure 3D, treatment with PP2 decreased EGFR levels, and EGF or HB-EGF also downregulated EGFR levels in the EVT cell line.

3.4 | Inactivation of Rap1 by Rap1GAP decreases the EGFR protein level in HTR-8/SVneo cells

To confirm that Rap1 activation modulates the EGFR protein level, we transfected HTR-8/SVneo cells with a vector expressing EGFP

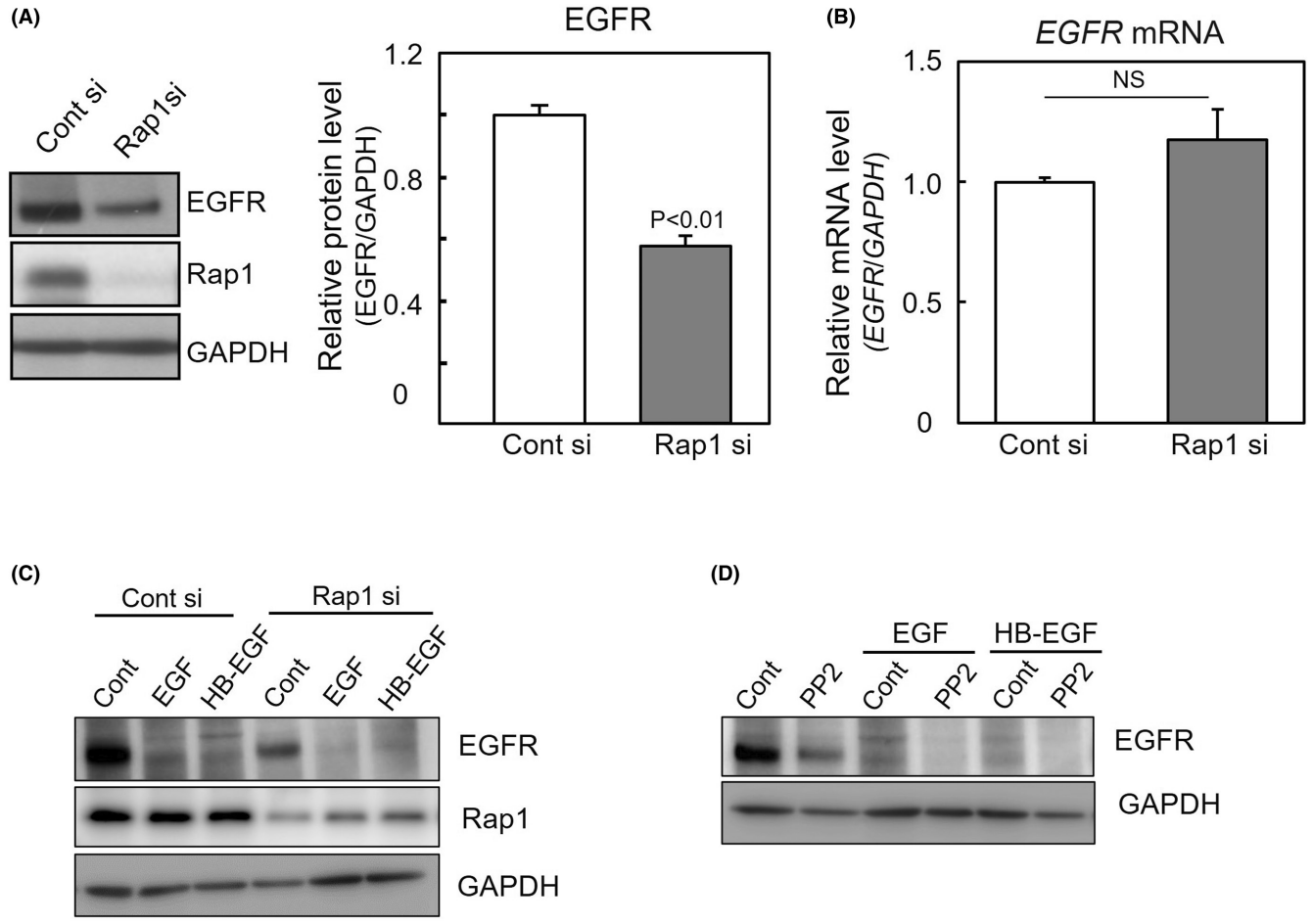

FIGURE 3 The effect of Rap1 knockdown or PP2 on expression levels of the EGFR. HTR-8/SVneo cells were transfected with a control (Cont) or Rap1 siRNA for 24 h and cultured for a further 24 h. (A) Cell lysates were subjected to immunoblotting using an anti-EGFR antibody. Representative data from three independent experiments are shown (left). The relative expression levels of the EGFR protein, normalized to those of GAPDH (right). Data are represented as the mean ± SEM of three independent experiments. (B) Total RNA was subjected to real-time RT-PCR analysis to determine the expression level of the *EGFR* mRNA. Data are represented as the mean ± SEM of four independent experiments. NS, not significant. (C) Cells were transfected with Cont or Rap1 siRNA for 24 h and then cultured with EGF (100 ng/mL) or HB-EGF (100 ng/mL) for 24 h in the presence of 1% FBS. EGFR protein was detected by immunoblotting. (D) Cells were pretreated with PP2 (10 μM) for 1 h and then stimulated with EGF and HB-EGF for 24 h. Immunoblotting of EGFR.

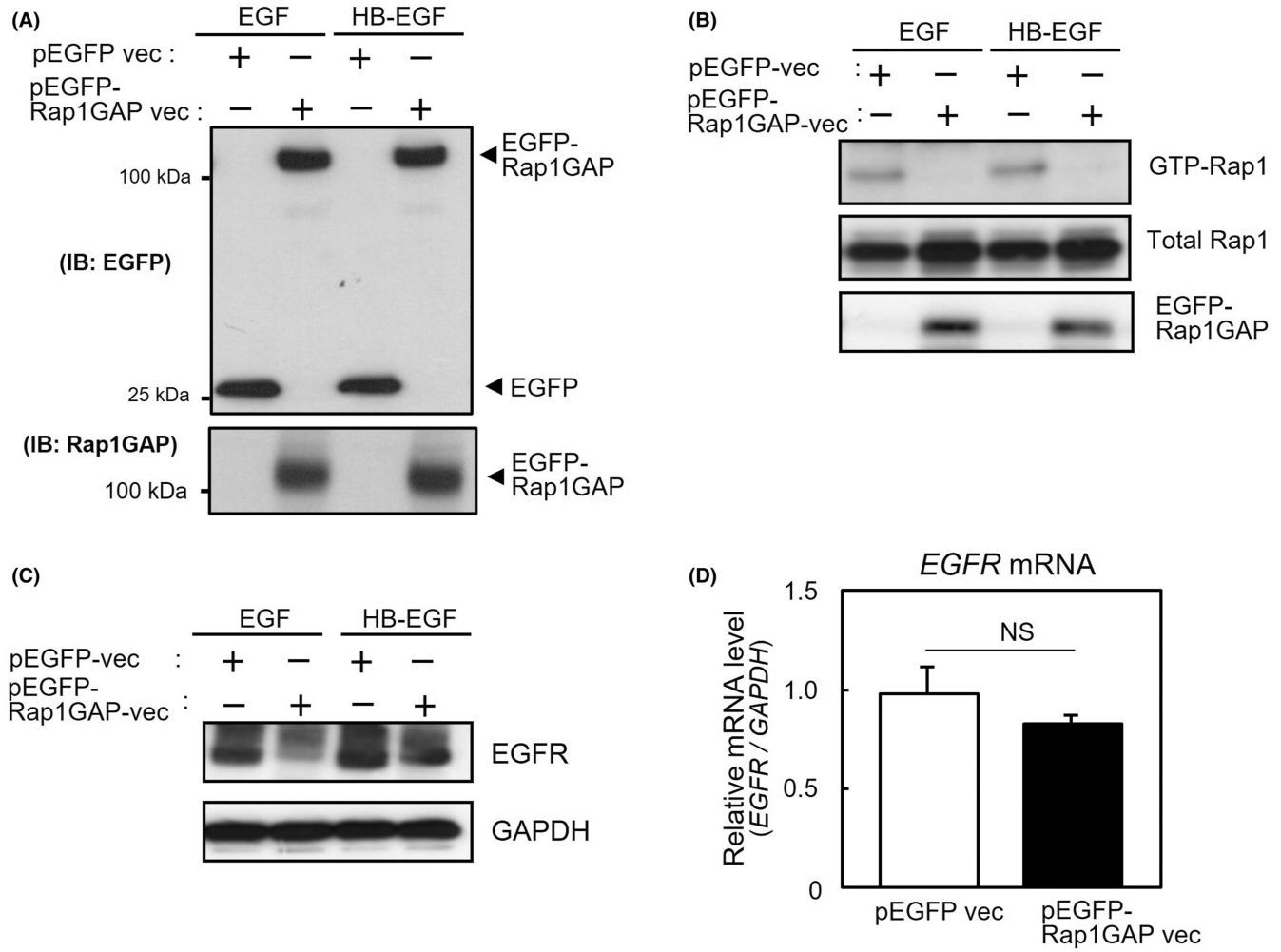

FIGURE 4 The effect of enforced expression of Rap1GAP on the expression level of EGFR. HTR-8/SVneo cells were transfected with a pEGFP or pEGFP-Rap1GAP vector for 48 h, then starved for 6 h, and stimulated with EGF or HB-EGF for 5 min. (A) Cell lysates were subjected to immunoblotting using an anti-EGFP (upper panel) or anti-Rap1GAP (lower panel) antibody. (B) A GTP-Rap1 pull-down assay of the cell lysates to determine the activity of Rap1 in the transfected cells. (C, D) The expression levels of the EGFR protein (C) and mRNA (D) were analyzed by immunoblotting and real-time RT-PCR, respectively. Data from three independent experiments are represented as the mean ± SEM. NS, not significant.

alone (control; pEGFP) or EGFP-labeled Rap1GAP (pEGFP-Rap1GAP). Rap1GAP functions as a negative regulator of Rap1 activity by facilitating the hydrolysis of GTP to GDP.[38] Successful transfection of the pEGFP and pEGFP-Rap1GAP vectors into the cell was confirmed by immunoblotting using an anti-EGFP antibody (Figure 4A, upper panel) and anti-Rap1GAP antibody (Figure 4A, lower panel). Enforced expression of Rap1GAP repressed the EGF- and HB-EGF-induced activation of Rap1 (Figure 4B) and reduced the EGFR protein level in HTR-8/SVneo cells (Figure 4C), without affecting the EGFR mRNA level (Figure 4D). These data lend additional weight to the proposal that active Rap1 can modulate the EGFR protein level in EVTs.

4 | DISCUSSION

In this study, we investigated the role of Rap1 in EGF- and HB-EGF-mediated invasion of EVT cell line. Both EGF and HB-EGF activated Rap1, and knockdown of Rap1 repressed EGF- and HB-EGF-stimulated invasion of an immortalized human EVT cell line. Furthermore, silencing of Rap1 repressed EGF- and HB-EGF-induced phosphorylation of AKT, ERK1/2, and p38MAPK. Although stimulatory effects of EGF- and HB-EGF on trophoblast invasion mediated through EGFR have been reported previously, the results presented here demonstrate the involvement of Rap1 as a critical mediator of EGFR signaling pathways in EVTs (Figure 5). Like Rap1 silencing, PP2-mediated inhibition of Src also repressed EGF- and HB-EGF-induced phosphorylation of AKT, ERK1/2, and p38MAPK, as well as the activation of Rap1. It is reported that EGF stimulates the metastasis of pancreatic carcinoma cells via Src-dependent phosphorylation of p130 Crk-associated substrate (p130CAS), leading to the activation of Rap1.[39] This speculates that Src is an upstream modulator of EGFR-mediated AKT and MAPK signaling pathways in EVTs. However, it is possible that EGF simultaneously activates both Src and Rap1, and they regulate each other in the EVT cell line.

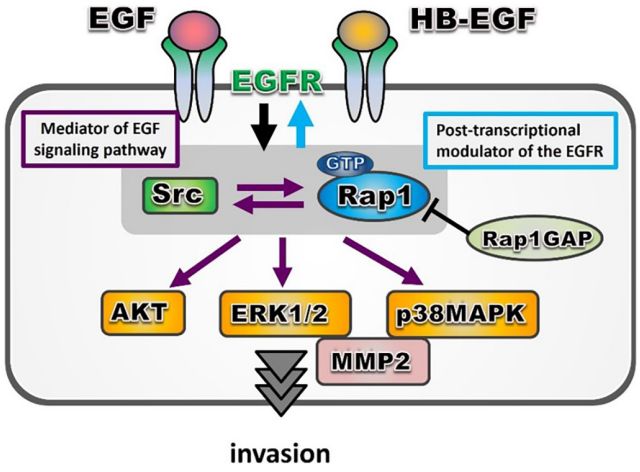

FIGURE 5 Schematic overview of Rap1-mediated EGF and HB-EGF signaling during EVT invasion. Rap1 functions as a mediator of the EGFR signaling pathway and a posttranscriptional regulator of EGFR in extravillous trophoblasts (EVTs).

During their invasion into the maternal decidua, EVTs secrete matrix metalloproteinases (MMPs), metal-dependent endopeptidases capable of degrading the extracellular matrix. MMPs and their regulators, including tissue inhibitors of metalloproteinase (TIMPs), play a critical role in trophoblast invasion. The activation of PI3K/AKT and MAPK pathways in EVTs is necessary for EGF-mediated upregulation of MMP-9, MMP-2, and TIMP-1 expression.[5,40] In this study, we showed that Rap1 knockdown or PP2 treatment downregulated MMP-2 expression in the presence of EGF or HB-EGF. These results suggest that Rap1 plays a role in regulating the MMP-2 expression during EVTs invasion. While inhibition of Src by PP2 decreased the level of GTP-bound active Rap1 in HTR-8/SVneo cells, knockdown of Rap1 also suppressed Src phosphorylation in the presence of EGF or HB-EGF. These results led us to examine whether Rap1 can regulate EGFR expression in EVTs, in addition to its role as a mediator of EGFR signaling. Knockdown of Rap1 decreased EGFR protein but not mRNA levels in EVTs. Furthermore, the inactivation of Rap1 by overexpression of Rap1GAP or PP2 treatment reduced the EGFR protein level. Taken together, these results suggest that Rap1 and Src play a role in the posttranscriptional regulation of EGFR in EVTs as aforementioned (Figure 5).

Although the precise mechanism by which Rap1 regulates EGFR is still unclear, Thalappilly et al.[41] showed that Vav2, a guanine nucleotide exchange factor for Rho GTPases, can regulate EGFR internalization and degradation by interacting with endosome-associated proteins. Knockdown of Vav2 promoted the degradation of EGFR and inhibited the proliferation of HeLa cells.[41] Vav2 interacts with the GTP-binding protein Rap1, and Rap1 is necessary for cell spreading and the accumulation of Vav2 in membrane protrusions at the cell periphery.[42] Since reduced EGFR expression is observed in many pregnancy-related complications, including PE, FGR, and recurrent spontaneous abortion,[43] and EGF and HB-EGF levels are reduced significantly in placentas from PE patients,[12] dysregulation of Rap1 expression or activity may be associated with this pathogenesis. Further studies are required to clarify the role of trophoblastic Rap1 in pregnancy-associated diseases. In addition to its expression in EVTs, an immunostaining analysis of human placental tissues detected Rap1 expression in villous cytotrophoblasts and syncytiotrophoblasts. We demonstrated previously that Rap1 is involved in the cAMP-mediated differentiation and cell fusion (syncytialization) of human trophoblasts.[19] Stimulation of EPAC, a cAMP-GEF that activates Rap1, promoted cAMP-mediated human chorionic gonadotropin production and syncytialization in a human trophoblastic cell line.[19,44] In addition, Chen et al.[45] demonstrated that human placental multipotent mesenchymal stromal cells secrete hepatocyte growth factor, which subsequently increases the level of cAMP in trophoblasts. The increased level of cAMP stimulates the adhesion and migration of trophoblasts via Rap1 activation.[45] Thus, via various growth factors and cAMP signaling pathways, Rap1 is involved in various trophoblast functions, including EVT invasion and differentiation of villous cytotrophoblasts into syncytiotrophoblasts. One of the limitations of the current study is that we used an EVT cell line, HTR-8/SVneo. Although the role of Rap1 should be investigated further using *other cell lines or* primary EVTs, it is worth noting that the HTR-8/SVneo cell line has been used by several placental biologists as a model of EVT invasion. The association of Rap1 in the modulation of EGFR level, its signaling pathway and pathological condition such as PE and FGR would be considered in our subsequent study.

In conclusion, Rap1 functions as a mediator of EGF/HB-EGF-induced AKT, ERK1/2, p38MAPK, and Src phosphorylation via the EGFR signaling pathway and is a posttranscriptional modulator of EGFR during EVT invasion.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

HUMAN RIGHTS STATEMENTS AND INFORMED CONSENT

The study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of the Tokyo University of Pharmacy and Life Sciences (No. 1512). Informed consent was obtained from all subjects involved in the study. Animal studies: n/a.

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