EphrinA1 stimulates cell attachment and inhibits cell aggregation through the EphA receptor pathway in human endometrial carcinoma-derived Ishikawa cells

Haruko Fujii, Hiroshi Fujiwara*, Akihito Horie, Ko Suginami, Yukiyasu Sato, and Ikuo Konishi

Department of Gynecology and Obstetrics, Faculty of Medicine, Kyoto University, Sakyo-ku, Kyoto 606-8507, Japan

*Correspondence address. Tel. +81-75-751-3269; Fax: +81-75-761-3967; E-mail: fuji@kuhp.kyoto-u.ac.jp

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BACKGROUND: Recently, the Eph–ephrinA system was proposed to contribute to the initial interaction between the maternal endometrial epithelium and embryonic trophectoderm. Since the Eph–ephrin interaction can induce adhesive and/or repulsive forces into the cells, we examined the possible role of this system in functional changes in endometrial epithelial cells using endometrial carcinoma-derived Ishikawa cells.

METHODS: The expressions of EphA1, A2 and A4 on Ishikawa cells were examined by RT–PCR and western blotting analyses. The effects of recombinant ephrinA1 on Ishikawa cells were also examined by western blot analysis and cell attachment and aggregation assays.

RESULTS: EphA1, A2 and A4 were expressed on Ishikawa cells. Recombinant ephrinA1 bound to the surfaces of Ishikawa cells and induced phosphorylation of EphA2 and A4. In bovine serum albumin-blocked nitrocellulose-coated dishes, Ishikawa cells remained floating and aggregated with each other. Under these conditions, immobilized ephrinA1 promoted Ishikawa cell attachment with increased tyrosine phosphorylation in focal adhesion kinase. In addition, immobilized ephrinA1 reversibly inhibited Ishikawa cell aggregation. Gene-reduction of EphA1, A2 and A4 by siRNAs attenuated the inhibitory effects of ephrinA1 on cell aggregation, confirming that ephrinA1 affects Ishikawa cell functions through Eph–ephrinA interaction.

CONCLUSIONS: This study demonstrated that the Eph–ephrinA system can promote cell attachment along with intercellular dissociation in Ishikawa cells. These findings suggest that this system can induce functional changes in endometrial epithelial cells.

Key words: Ishikawa cells / cell attachment / cell aggregation / Eph / ephrin

Introduction

The Eph–ephrin system is a unique system that can induce multiple cellular responses such as cell migration, tissue morphogenesis, regulation of angiogenesis and axonal guidance (Gale and Yancopoulos, 1997; Orioli and Klein, 1997; Flanagan and Vanderhaeghen, 1998; Holder and Klein, 1999; Himanen and Nikolov, 2003). Ephrins can be divided into two subclasses: the ephrinA (A-type ephrins) and ephrinB (B-type ephrins) subclasses (Eph Nomenclature Committee, 1997). EphrinA ligands (ephrinA1–A6) are anchored to the cell surface via a glycosylphosphatidylinositol anchor and bind to EphA receptors (class A Eph receptors, EphA1–A9), whereas ephrinB ligands (ephrinB1–B3) have transmembrane and cytoplasmic domains and interact with EphB receptors (class B Eph receptors, EphB1–B6). Class A Eph receptors preferentially bind all A-type ephrins and class B Eph receptors bind all B-type ligands. However, there are some exceptions, as EphA1 primarily binds ephrinA1, EphA4 binds both A- and B-type ligands and ephrinA5 binds EphA receptors as well as EphB2 (Mosch et al., 2010).

Previously, we reported that EphA1 and ephrinA1 are expressed in human blastocysts and endometrial epithelial cells, respectively (Fujiwara et al., 2002), and proposed that ephrinA1 can activate the Eph-bearing embryo through the initial interaction with endometrial epithelium during the implantation process. Later, we found that...
EphA1, A2 and A4 are expressed on murine endometrial epithelial cells, while ephrinA1 and A3 are expressed on the cell surface of trophoderm of murine blastocysts. We also observed that EphA1 reversibly inhibits blastocyst attachment in vitro, suggesting that this system is one of pathways that regulates the duration of the crosstalk period between mother and embryo just before embryo attachment to the endometrium. Consequently, we supposed that the Eph–ephrinA system is involved in the initial step of embryo implantation (Fujii et al., 2006).

Recently, we found that EphA1, A2 and A4 were expressed on the cell surface of human endometrial epithelial cells and endometrial carcinoma-derived Ishikawa cells, while ephrinA1 was expressed on human blastocysts. We further demonstrated that ephrinA1-stimulation significantly promoted the permeability of monolayer culture of Ishikawa cells without affecting cell viability (Fujii et al., 2011). In accordance with these findings, the Eph–ephrin system was reported to induce reduction of the tight junction of epithelial cells via claudin 4 (Tanaka et al., 2011). Consequently, we supposed that the Eph–ephrin system is one of pathways that regulates the duration of the crosstalk in vitro reversibly inhibiting blastocyst attachment.

In the present study, we further examined the effects of the Eph–ephrinA system on the functions of endometrial carcinoma-derived Ishikawa cells.

### Materials and Methods

#### Reagents and cell line

The mouse anti-human EphA1 monoclonal antibody (mAb) and recombinant mouse ephrinA1-human Fc fusion proteins were obtained from the Techno Corp. (Minneapolis, MN, USA). The mouse anti-human EphA2 mAb was obtained from Abcam Ltd. (Cambridge, UK). The rabbit anti-human A2 and A4 polyclonal antibodies (pAbs) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The mouse anti-human focal adhesion kinase (FAK) mAb (clone 4.47) and the mouse anti-phosphotyrosine mAb (4G10) were obtained from Upstate (Lake Placid, NY, USA). The mouse anti-human EphA1 monoclonal antibody (mAb) and recombinant mouse ephrinA1-human Fc fusion protein were clustered by incubation with rabbit anti-human IgG antibody [5 μg anti-human IgG for 50 μg ephrinA1–Fc protein in 1 ml sterile phosphate-buffered saline (PBS)] for 2 h at 4°C. The human Fc portion of Ig was purchased from ICN Pharmaceuticals, Inc. (Aurora, OH, USA) and BioSource International Inc. (Camarillo, CA, USA). The mouse anti-human phosphotyrosine mAb (Y397) mAb, the mouse anti-afadin mAb (AF-6) and rabbit pAb were purchased from Chemicon International Inc., BD Biosciences (Tokyo, Japan) and BioSource International Inc. (Camarillo, CA, USA), respectively. The human Fc portion of Ig was purchased from ICN Pharmaceuticals, Inc. (Aurora, OH, USA). The rabbit anti-human IgG pAb, FITC or horse-radish peroxidase (HRP)-conjugated swine anti-rabbit Ig and FITC-conjugated rabbit or HRP-conjugated goat anti-mouse Ig pAb were purchased from DAKO (Glostrup, Denmark). Mouse IgG1 negative control mAb (X0931, DAKO) and rabbit control Ig (X0903, DAKO) were used as negative controls. Mouse anti-human GAPDH mAb was purchased from Research Diagnostics Inc. (NJ, USA).

Ishikawa 3-H-12 cells, a human endometrial carcinoma-derived cell line, were generously provided by Dr. M. Nishida (Kasumigaura Hospital, Ibaragi, Japan).

#### Binding assay

The binding assay was performed as described previously (Egawa et al., 2003). Briefly, recombinant mouse ephrinA1-human Fc fusion protein was clustered by incubation with rabbit anti-human IgG antibody [5 μg anti-human IgG for 50 μg ephrinA1–Fc protein in 1 ml sterile phosphate-buffered saline (PBS)] for 2 h at 4°C. Then Ishikawa cells were incubated with the preclustered ephrinA1–Fc at a concentration of 500 ng/ml in culture medium at 37°C for 1 h. The cells were collected by centrifugation, fixed with 3% paraformaldehyde, and then washed in HBSS ( Hank’s balanced salt solution) and reacted with FITC-conjugated swine anti-rabbit Ig antibody for 30 min at 4°C to detect the preclustered ephrinA1–Fc that had bound to the cell surface of Ishikawa cells. After washing, the reacted cells were suspended in PBS/glycerin (1:1, vol/vol) and examined under a fluorescence microscope. Preclustered Fc protein was used as a negative control.

#### RNA isolation and RT–PCR analysis

Total RNA, extracted from human endometrial epithelial cell fractions, was reverse-transcribed using a First Strand cDNA Synthesis Kit (Pharmacia, Inc., Piscataway, NJ, USA). Thirty cycles of PCR were performed with human EphA1, A2, A4 or with human ribosomal protein S26 primers (Table I). S26 was used as a positive control to check RNA integrity and for all amplifications, negative controls (without cDNA) were included. PCR products were sequenced to confirm their identity.

#### Cell attachment assay

Twenty-four-well culture plates were coated with nitrocellulose (Nakalai Tesque, Kyoto, Japan) diluted in methanol (50 mg/ml) and air-dried. Recombinant mouse ephrinA1- and human EphA1-human Fc fusion proteins (2.0 μg/cm²) or Fc proteins as controls (0.3 μg/cm²) in PBS were immobilized overnight at 4°C. Non-specific binding was blocked with 1% bovine serum albumin (BSA) for 2 h at 37°C (Huynh-Do et al., 1999). Ishikawa cells suspended in RPMI (Life Technologies, Inc., Grand Island, NY, USA) containing 1% BSA were plated in triplicate in 24 wells coated with immobilized Fc or recombinant proteins (1 × 10⁵ cells/well) and morphological changes in cell attachment were observed in the presence or absence of 1 mM EDTA. To assess the cell attachment

### Table I Primers used for RT–PCR to detect human EphA family.

<table>
<thead>
<tr>
<th>Probe</th>
<th>Sense primer sequences</th>
<th>Antisense primer sequences</th>
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<tbody>
<tr>
<td>EphA1</td>
<td>5′-AAC CTT ATG CCA ACT ACA CC-3′ (1322–1341)</td>
<td>5′-TTC CCC AAA CTC TCC TTC TC-3′ (2009-2028) (Genebank accession number : NM005232)</td>
</tr>
<tr>
<td>EphA2</td>
<td>5′-GCC CGC AAC ATC CTC GCA AA-3′ (2337–2356)</td>
<td>5′-GCA GCC GCA CCC CAA TCC TC-3′ (2948–2967) (Genebank accession number : NM004431)</td>
</tr>
<tr>
<td>EphA4</td>
<td>5′-CAG AAG GAG AGG AGC GAC AG-3′ (2617–2636)</td>
<td>5′-AGG CGC AAG ACG AAG TAA AA-3′ (3071–3090) (Genebank accession number : NM004438)</td>
</tr>
<tr>
<td>S26</td>
<td>5′-GCT CCG TGC TCT CAA GAT GA-3′ (8-27)</td>
<td>5′-TAA ATC GGG GTG GGG GTG TT-3′ (308–327) (Genebank accession number : BC002604)</td>
</tr>
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Cell aggregation assay

To assess the effects of ephrinA1 on cell aggregation, Ishikawa cells suspended in RPMI containing 1% BSA were cultured on immobilized Fc for 60 min in the presence of 1 mM EDTA (control wells). Under this condition, almost all Ishikawa cells remained floating and formed aggregated cell clusters. Then, the aggregated Ishikawa cells were gently transferred to Fc-coated or ephrinA1-coated wells. Morphological changes in cell dissociation were recorded by digital photographs at regular intervals and the numbers of single cells that did not overlap each other were counted. After 30 min, the dispersed Ishikawa cells in ephrinA1-treated dishes were collected again and re-inoculated in an Fc-treated control dish to induce re-aggregation.

Immunoprecipitation and western blotting

Ishikawa cells were lysed in modified RIPA buffer (20 mM Tris–HCl, pH 7.5, 1% Nonidet P-40, 1% deoxycholic acid, 150 mM NaCl) containing protease inhibitors (Complete mini; Roche) and phosphatase inhibitor cocktail (Sigma). Immunoprecipitations were carried out using anti-EphA2 and anti-EphA4 pAbs (2 μg/mg lysate proteins) overnight at 4°C. For the reaction with anti-afadin mAb (AF-6), Ishikawa cells were lysed in HO buffer (50 mM Hepes–NaOH, pH 7.5, 1% Triton X-100, 0.15 M NaCl, 1 mM EGTA, 1.5 mM MgCl2, 10% glycerol).

Whole cell lysates or immunoprecipitated samples were separated on 8% sodium dodecyl sulfate polyacrylamide gels and transferred to Immobilon-P polyvinylidene difluoride membrane (Millipore). Membranes were reacted with anti-phosphotyrosine mAb or anti-phospho-FAK (Y397) mAb and visualized using an ECL Western Blotting System (Pharmacia). After treatment with western blot stripping reagent (Restore™ Western Blot Stripping Buffer, PIERCE, Rockford, IL, USA), the membranes were also re-stained using anti-EphA2 mAb, anti-EphA4 pAb or anti-FAK mAb.

Silencing of endogenous EphA1, A2 and A4 with siRNA

Validated siRNAs for human EphA1 (siRNA/EphA1: SI02223879; GenBank accession number NM005232), EphA2 (siRNA/EphA2: SI00300181; NM004431) and A4 (siRNA/EphA4: SI022233522; NM004438) or for control (siRNA/CTR, sense: UUC UCC CGU GUC ACG UdT dT; antisense: ACG UGA CAC GUU CGG AGA AdT dT) were purchased from Qiagen (Valencia, CA, USA). Combined siRNA(s) at a final concentration of 150 nM was transfected into Ishikawa cells cultured in 60-mm diameter dishes using RNAiFect™ Reagent (Qiagen). Transfected cells were cultured for an additional 48 h before use.

Statistical analysis

The cell numbers of attached Ishikawa cells and the percent of cell numbers of single Ishikawa cells were expressed as means ± SD and were analyzed by one-way analysis of variance, followed by Scheffe’s F-test and paired t-test, respectively. Statistical significance was set at P < 0.05.

Results

Binding of ephrinA1 to the cell surface of Ishikawa cells

The activation of Eph—ephrin signaling is known to require membrane attachment or artificial clustering of the ligands (Davis et al., 1994; Gale and Yancopoulos, 1997). Therefore, to examine the biological reactivity with A class Eph(s) on Ishikawa cells, we used a preclustered ephrinA1–Fc fusion protein for the binding assay. In this assay, the binding of preclustered ephrinA1 was detected on the surfaces of Ishikawa cells (Fig.1A) while preclustered Fc did not bind Ishikawa cells.

The effects of ephrinA1 on activation of EphA2 and A4 in Ishikawa cells

When Ishikawa cells were incubated in wells coated with immobilized ephrinA1, immunoblotting of immunoprecipitated EphA2 and EphA4 using anti-phosphotyrosine mAb demonstrated that EphA2 and EphA4 on Ishikawa cells were tyrosine-phosphorylated within 10 min after ephrinA1-stimulation (Fig. 1B and C).

The effects of ephrinA1 on the cell attachment of Ishikawa cells

When Ishikawa cells were cultured on nitrocellulose-coated dishes treated with 1% BSA, the cells remained floating in media and

Silencing of endogenous EphA1, A2 and A4 with siRNA

Validated siRNAs for human EphA1 (siRNA/EphA1: SI02223879; GenBank accession number NM005232), EphA2 (siRNA/EphA2: SI00300181; NM004431) and A4 (siRNA/EphA4: SI022233522; NM004438) or for control (siRNA/CTR, sense: UUC UCC CGU GUC ACG UdT dT; antisense: ACG UGA CAC GUU CGG AGA AdT dT) were purchased from Qiagen (Valencia, CA, USA). Combined siRNA(s) at a final concentration of 150 nM was transfected into Ishikawa cells cultured in 60-mm diameter dishes using RNAiFect™ Reagent (Qiagen). Transfected cells were cultured for an additional 48 h before use.

Figure 1 Binding of ephrinA1 to Ishikawa cells and its effects on activation of EphA2 and A4 in Ishikawa cells. (A) The binding of ephrinA1 was detected on the cell surface of Ishikawa cells (a and b). Fc did not react with these cells (c and d). Bar indicates 10 μm. Ishikawa cells that were attached on immobilized ephrinA1 were collected and lysed as described in the materials and methods section and subjected to immunoprecipitation by anti-EphA2 (B) and anti-EphA4 (C) pAbs, and then were immunoblotted using anti-phosphotyrosine mAb (upper panels) and anti-EphA2 and anti-EphA4 Abs, respectively (lower panels). EphA2 and EphA4 on Ishikawa cells were tyrosine-phosphorylated (arrows) after 10 min of incubations on immobilized ephrinA1.
aggregated with each other to form large cell masses within 10 min (Fig. 2A-b). In contrast, Ishikawa cells that were incubated on the wells coated with immobilized ephrinA1 did not become aggregated (Fig. 2A-d). On the other hand, Ishikawa cells incubated on the immobilized EphA1 became aggregated (Fig. 2A-c).

In the attachment assay, numbers of cell attachments were significantly increased when Ishikawa cells were incubated in wells coated with immobilized ephrinA1, whereas Ishikawa cells in control and EphA1-coated wells did not show any increase in the number of attached cells (Fig. 2B).

It is well known that integrins induce FAK-phosphorylation. We previously reported that human endometrial epithelial cells and Ishikawa cells express integrin β1 and integrin α3 and α6 on their cell surfaces (Park et al., 2000a, b). Therefore, to estimate contribution of integrins on ephrinA-induced cell attachment, the changes of FAK-phosphorylation were examined. During cell attachment assay, phosphorylation of FAK was increased in Ishikawa cells under ephrinA1-stimulation (Fig. 2C).

Then, we investigated whether or not this reaction is dependent on Ca2+ ions using EDTA. In the presence of 1 mM EDTA, the promotion of cell attachment by immobilized ephrinA1 persisted (Fig. 2D), although the effects were reduced compared with those in the absence of EDTA (Fig. 2D).

The effects of ephrinA1 on cell aggregation in Ishikawa cells

During 60-min incubation in the control wells in the presence of 1 mM EDTA, Ishikawa cells remained floating and formed cell aggregation. These aggregated Ishikawa cells (Fig. 3A-a) were gently transferred to wells coated with ephrinA1 or Fc and changes in morphology were observed during the subsequent 30 min. In the presence of ephrinA1, the aggregated cells gradually became dispersed from each other, attaching to the dishes within 30 min (Fig. 3A-c). In contrast, cell aggregation proceeded on the Fc-coated dishes without adhering to the dishes (Fig. 3A-d). Subsequently, the numbers of floating or attached single Ishikawa cells were significantly higher in the group that was additionally incubated with ephrinA1 compared with those in the control group (Fig. 3B). However, when Ishikawa cells that had been aggregated in the nitrocellulose-coated control dishes were gently transferred to normal culture dishes without nitrocellulose coating, these aggregated cells attached to the dishes as a mass without cell dispersion (Fig. 3A-b).

After a 30-min incubation, the dispersed Ishikawa cells were collected from the ephrinA1-coated wells and were additionally incubated with ephrinA1 or Fc for 30 min. Although Ishikawa cells with ephrinA1 remained single cells, those cultured on the Fc-coated wells gradually aggregated during a 30-min incubation, showing that...
ephrinA1-induced cell attachment and dispersion are reversible reactions (Fig. 3C and D).

The effects of silencing of endogenous EphA1, A2 and A4 with siRNA on cell aggregation in Ishikawa cells

When siRNAs against EphA1, A2 and A4 (siRNA/A1, siRNA/A2 and siRNA/A4) were transfected into Ishikawa cells, reduction of each mRNA expression was observed by RT–PCR (Fig. 4A). Reduction of EphA2 and EphA4 protein expression was also observed by western blotting (Fig. 4B). Since EphA1 protein in Ishikawa cells was not clearly detected by western blotting, we could not confirm EphA1 reduction at the protein level. Ishikawa cells that were transfected with three siRNAs (siRNA/A1/A2/A4-ts-cells) aggregated together in the presence of ephrinA1 (Fig. 4C and D). In contrast, cell aggregation was inhibited by ephrinA1-stimulation in siRNA/CTR-ts-cells.

The effects of ephrinA1 on nectin–afadin system in Ishikawa cells

Then, we examined the involvement of the nectin–afadin system, which is known to be a Ca\(^{2+}\) ion-independent adhesion mechanism (Kikyo et al., 2000). When the aggregated Ishikawa cells, after the initial 60-min incubation on the control dishes, were inoculated on the ephrinA1-coated dishes, the ephrinA1-induced changes in phosphorylation of unknown proteins was clearly observed by immunoprecipitation and western blotting (Fig. 5). However, we could not confirm phosphorylation of nectin isoforms in our system (Kikyo et al., 2000).

Discussion

During the initial process of embryo implantation, the human embryo invades the maternal endometrial stromal tissues as a mass through the epithelial cell layer after attaching to the endometrium. To achieve this process, the intercellular connection among endometrial epithelial cells must be reduced without destroying the critical connection between the embryo-attached epithelial cells and the basement membrane. Previously, we demonstrated that human endometrial epithelial cells and Ishikawa cells expressed mRNA of EphA1, A2, A4 and ephrinA1, A4, A5 using RT–PCR analysis (Fujii et al., 2011). We also observed that ephrinA1-stimulation significantly promoted the permeability of monolayer culture of Ishikawa cells without affecting cell viability, showing that the Eph–ephrinA system can promote intercellular dissociation of Ishikawa cells. From these findings, we proposed that this system plays an important role in the initial step of embryo implantation by opening the endometrial epithelial cell barrier.

In this study, to further elucidate the physiological action of ephrinA1-bearing human blastocysts on EphA-bearing endometrial...
epithelial cells through the Eph–ephrinA system, we examined the effects of immobilized ephrinA1 on cell attachment and aggregation of Ishikawa cells using a cell aggregation assay. Interestingly, on nitrocellulose-coated dishes treated with BSA, cell attachment of Ishikawa cells was inhibited and they gradually aggregated with each other, which may represent lateral cell-to-cell contact of epithelial cells. Under these conditions, immobilized recombinant ephrinA1 inhibited aggregation of Ishikawa cells and induced cell attachment to the dishes. In contrast, immobilized recombinant EphA1 did not show any effects on cell attachment and formed cell aggregation similar to that in controls. We also confirmed that recombinant ephrinA1 can bind to the cell surface of Ishikawa cells and ephrinA1-stimulation can induce EphA2 and EphA4-phosphorylation in Ishikawa cells. We further affirmed the specificity of ephrinA1-stimulation on Ishikawa cells through EphA receptors using siRNA-transfected cells. On the basis of these findings, we concluded that ephrinA ligands induced cell attachment and inhibited cell aggregation in Ishikawa cells through the EphA receptor pathway (Orioli and Klein, 1997; Huynh-Do et al., 1999).

It has been reported that EphA2 activation by ephrinA1 in PC3 cells, a human prostate tumor epithelial-like cell line, decreased cell adhesion and cytoskeletal integrity, coinciding with a transient decrease in FAK phosphorylation (Miao et al., 2000). Later, EphA2 phosphorylation was reported to induce adhesion and spreading in NIH3T3 cells along with FAK phosphorylation, suggesting that EphA2 activation evokes dynamic cytoskeletal reorganization (Carter et al., 2002). Thus, the effects of ephrinA-induced EphA2 activation on FAK-phosphorylation remain controversial. This is probably because the Eph–ephrinA system operates differently among individual cells. This study showed that ephrinA-stimulated Ishikawa cells became able to adhere to the dishes concomitantly with an increase in FAK-phosphorylation. Focal adhesions play a key role in cell attachment (Romer et al., 2006). It is well known that cell attachment by cell adhesion molecules such as integrins was accompanied by an increase in FAK-phosphorylation, leading to various downstream responses. Therefore, our findings suggest that FAK-phosphorylation is involved in ephrinA-induced cell attachment of Ishikawa cells. In general, epithelial cells contact extracellular matrix at the basal site. Therefore, it is reasonable to speculate that adhesion of the cultured epithelial cells to the dishes mainly represents contact between epithelial cells and the extracellular matrix in the basement membrane. Activation of integrin molecules needs the Ca\(^{2+}\) ion. Although the attachment rates were reduced, this cell attachment could be induced even in the presence of 1 mM EDTA. These findings suggest that undefined molecules that do not need the Ca\(^{2+}\) ion are also involved in ephrinA1-induced cell attachment in Ishikawa cells.

Figure 4 Cell aggregation assay after silencing endogenous EphA1, A2 and A4 with siRNAs. When siRNAs against EphA1, A2 and A4 were transfected into Ishikawa cells, reduction of each mRNA and protein expression was confirmed by RT–PCR (A) and western blotting (B), respectively. The lower panels show S26 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as controls. (C) Ishikawa cells that were transfected with the above three siRNAs (siRNA/A1/A2/A3-ts-cells) aggregated with each other even in the presence of ephrinA1 (C-d). In contrast, cell aggregation was significantly inhibited by ephrinA1-stimulation in siRNA/CTR-ts-cells (C-b). (D) The number of Ishikawa cells that remained single following ephrinA1-stimulation was significantly higher in siRNA/CTR-ts-cells, but was not increased in siRNA/A1/A2/A3-ts-cells. *; \(P < 0.05\), **; \(P < 0.01\). Values represent means ± SD.
Similarly, aggregation of Ishikawa cells was induced in control dishes even in the presence of 1 mM EDTA, suggesting that a Ca\(^{2+}\) ion-independent mechanism also affects Ishikawa cell aggregation. The formation of intercellular connections among Ishikawa cells was reduced by ephrinA-stimulation. These findings are in accord with the finding that Eph–ephrin interaction can induce repulsive forces between the contacted cells (Gale and Yancopoulos, 1997). Since similar cell dissociation was observed even in the presence of EDTA, it is suggested that certain Ca\(^{2+}\) ion-independent mechanisms are involved in ephrinA-induced cell dispersion in Ishikawa cells.

To induce or maintain cell–cell connection in epithelial tissues, adherens junctions are important, as are tight junctions. Although it is accepted that cadherins are the main proteins regulating adherens junctions, this family operates in a Ca\(^{2+}\) ion-dependent manner. Therefore, we propose involvement of the nectin–afadin system that can operate in a Ca\(^{2+}\) ion-independent manner. This system also plays an important role in the formation of adherens junctions and is composed of nectin, an adhesive molecule, and afadin, an actin filament binding protein. In this study, we could not confirm the phosphorylated changes in nectin isoforms (Kikyo et al., 2000). However, we observed that ephrinA1-stimulation altered the phosphorylated conditions of unknown proteins with molecular masses of 100 and 150 kDa, which were consistent with nectin or afadin (Takai et al., 2008). Although we could not identify or further characterize these proteins in the present study, this finding suggested that the nectin–afadin system was involved in ephrinA1-induced cell disaggregation in Ishikawa cells.

Previously, we reported that human blastocysts expressed ephrinA1 and EphA1 (Fujii et al., 2002; Fuji et al., 2011). Therefore, the ephrinA-bearing embryo is one of the most plausible candidates to induce exogenous ephrinA signals. Human endometrial epithelial cells construct a tightly connected layer that protects the uterus from pathogenic invasion as a barrier. In general, it is considered that human embryos invade the stromal tissue as a mass (Boyd and Hamilton, 1970; Benirschke and Kaufmann, 2000). During the invasion process through the epithelial cell layer, the embryo must reduce the intercellular connections among epithelial cells while the connection between the embryo-attached epithelial cells and basement membrane should be maintained. In this regard, our finding that ephrinA1-induced cell attachment and inhibited cell aggregation in Ishikawa cells is a favorable change in endometrial epithelial cells for embryo implantation.

Yoshinaga proposed that interaction between the trophectoderm and endometrial epithelial cells via heparin-binding epidermal growth factor (EGF) and EGF-receptor system induces calcium influx into trophoblast cells, resulting in reduction of local calcium concentration at the trophoblast–endometrial interface and relaxation of tight junctions of luminal epithelial cells (Yoshinaga, 1994; Yoshinaga, 2008). This study showed that Ishikawa cells aggregated with each other in control dishes and ephrinA1 inhibited cell aggregation even in the presence of EDTA, suggesting that ephrinA signaling can affect Ishikawa cell function in a Ca\(^{2+}\) ion-independent manner. Consequently, the present study strengthened our hypothesis that Eph–ephrin interaction is one of the important mechanisms by which the human embryo opens the endometrial epithelial cell barrier (Fuji et al., 2011).

As we reported previously, EphA and ephrinA molecules are co-localized in endometrial epithelial cells and Ishikawa cells (Fujii et al., 2002; Fuji et al., 2011). Thus, interaction through ephrinA and EphA molecules is estimated to have already occurred within aggregating Ishikawa cells. However, when we further stimulated the aggregating Ishikawa cells by exogenous ephrinA1-treatment, Ishikawa cells gradually dispersed and attached to the dishes. Therefore, although the precise mechanisms remain unknown, it is reasonable to speculate that exogenously stimulated ephrinA signals induce different or more prominent effects on both molecule-bearing endometrial epithelial cells. Interestingly, Ishikawa cells immediately recovered adhesive ability for homologous cell-to-cell contact just after escaping from exogenous ephrinA-stimulation, showing that these cell aggregations and dissociations were reversible reactions.

Although the expression profiles of Eph–ephrinA molecules are almost similar to those of normal endometrial epithelial cells, Ishikawa cells are established from endometrial carcinoma. Therefore, it is possible that ephrinA1-induced cell attachment and dispersion is related not only to normal epithelial cells, but also to cancer cell behavior. Recent work has suggested that activation of EphA molecules is involved in cancer invasion and metastasis (Taddei et al., 2009; Pasquale, 2010). When the extension of EphA-bearing cancer cell invasion reaches ephrinA-expressing organs such as vessels, EphA molecules on cancer cells can be transiently activated. Accordingly, we should note the possibility that ephrinA-stimulation at the invasion site induces endometrial cancer cell dispersion and attachment, further promoting single cell migration and distant implantation.

In conclusion, this study showed that ephrinA1-stimulation induced cell adhesion to the dishes and inhibited cell-to-cell aggregation in EphA-bearing Ishikawa cells. These findings suggest that this system can induce functional changes in human endometrial epithelial cells. Since the EphA/ephrinA system is expressed in the blastocyst and normal endometrium, the involvement of this system should be
further investigated to clarify the mechanisms underlying the initial step of human embryo implantation.

**Authors’ roles**

H.F., Y.S., A.H. and K.S. performed experiments. H.F. designed this study and wrote paper and I.K. discussed the results.

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