BIOLOGY OF REPRODUCTION (2013) **88**(3):77, 1–11
Published online before print 12 December 2012.
DOI 10.1095/biolreprod.112.100883

Selective Inhibition of Prostaglandin E2 Receptors EP2 and EP4 Inhibits Adhesion of Human Endometriotic Epithelial and Stromal Cells Through Suppression of Integrin-Mediated Mechanisms[1]

JeHoon Lee,[3] Sakhila K. Banu,[3] Robert C. Burghardt,[4] Anna Starzinski-Powitz,[5] and Joe A. Arosh[2,3]

[3]*Reproductive Endocrinology and Cell Signaling Laboratory, Department of Integrative Biosciences, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, College Station, Texas*
[4]*Department of Integrative Biosciences, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, College Station, Texas*
[5]*Molekulare Zellbiologie und Humangenetik, Institut für Zellbiologie und Neurowissenschaft, Frankfurt am Main, Germany*

ABSTRACT

Endometriosis is a chronic gynecological disease of reproductive age women characterized by the presence of functional endometrial tissues outside the uterine cavity. Interactions between the endometriotic cells and the peritoneal extracellular matrix proteins (ECM) are crucial mechanisms that allow adhesion of the endometriotic cells into peritoneal mesothelia. Prostaglandin E2 (PGE_2) plays an important role in the pathogenesis of endometriosis. In previous studies, we have reported that selective inhibition of PGE_2 receptors PTGER2 and PTGER4 decreases survival and invasion of human endometriotic epithelial and stromal cells through multiple mechanisms. Results of the present study indicates that selective inhibition of PTGER2- and PTGER4-mediated PGE_2 signaling 1) decreases the expression and/or activity of specific integrin receptor subunits Itgb1 (beta1) and Itgb3 (beta3) but not Itgb5 (beta5), Itga1 (alpha1), Itga2 (alpha2), Itga5 (alpha5), and Itgav (alphav); 2) decreases integrin-signaling components focal adhesion kinase or protein kinase 2 (PTK2) and talin proteins; 3) inhibits interactions between Itgb1/Itgb3 subunits, PTK2, and talin and PTGER2/PTGER4 proteins through beta-arrestin-1 and Src kinase protein complex in human endometriotic epithelial cells 12Z and stromal cells 22B; and 4) decreases adhesion of 12Z and 22B cells to ECM collagen I, collagen IV, fibronectin, and vitronectin in a substrate-specific manner. These novel findings provide an important molecular framework for further evaluation of selective inhibition of PTGER2 and PTGER4 as potential nonsteroidal therapy to expand the spectrum of currently available treatment options for endometriosis in child-bearing age women.

adhesion, endometriosis, human, integrins, PGE_2

INTRODUCTION

Endometriosis is a chronic inflammatory disease of reproductive-age women characterized by the presence of functional endometrial tissues outside the uterine cavity [1, 2]. The prevalence of the disease is ~10% in childbearing-age women, increasing to 20%–30% in women with subfertility and to 40%–60% in women with dysmenorrhea [1, 2]. The two major symptoms are infertility and pain. In endometriosis patients, concentrations of prostaglandin E2 (PGE_2) in the peritoneal fluid are higher compared to that of endometriosis-free women [3], This increased PGE_2 is thought to promote inflammation, invasion, survival, and pain of endometriosis [4].

Arachidonic acid (AA) is the primary precursor for synthesis of prostaglandins. Cytosolic phospholipase A2 liberates AA from phospholipids. Prostaglandin-endoperoxide synthases 1 (PTGS-1) and 2 (PTGS-2) (also known as cyclooxygenases COX-1 and COX-2, respectively) convert AA into PGH_2 [5]. PGF synthases PGFS-AKR1B1 (earlier known as AKR1B5) and PGFS-AKR1C3 convert PGH_2 into $PGF_{2\alpha}$. PGE synthases PGES-1 (microsomal PGES-1), PGES-2 (microsomal PGES-2), and PGES-3 (cytosolic PGES) covert PGH_2 into PGE_2. Prostaglandin dehydrogenase catabolizes $PGF_{2\alpha}$ and PGE_2 into inactive metabolites 13,14-dihydro-15-keto-prostaglandin F2a and 13,14-dihydro-15-keto prostaglandin E2, respectively [6]. $PGF_{2\alpha}$ and PGE_2 are transported competitively across the cell membrane through prostaglandin transporter-mediated mechanisms [7]. $PGF_{2\alpha}$ and PGE_2 exert their biological effects via seven transmembrane G-protein coupled receptors (GPCRs) [8]. $PGF_{2\alpha}$ acts through the PGF receptor (FP), and activation of FP in turn activates protein kinase C (PKC) and Ca^{2+} cell-signaling pathways [8, 9]. Multifaceted effects of PGE_2 are meditated through PGE_2 receptor 1 (PTGER1), PTGER2, PTGER3, and PTGER4 (also known as EP1, EP2, EP3, and EP4, respectively) by integrating multiple cell-signaling pathways [10–14]. PTGER1 activates PKC and Ca^{2+} pathways. PTGER2 and PTGER4 activate PKA pathway. Activation of PTGER3A, by way of PTGER3D, produces a wide range of complex and opposite actions [8]. Recent studies indicate that PGE_2 transactivates extracellular signal-regulated kinases (ERK1/2), AKT (also known as PKB), nuclear factor-kappa B, and β-catenin pathways through PTGER2 and PTGER4 in cancer [10–14] and endometriosis [15]. PGE_2 promotes adhesion, migration, invasion, proliferation, and survival of several types of cells under physiological and pathological conditions [16, 17].

Data from our laboratory [18] and others [19, 20] have shown that PTGS-2 is more abundantly expressed in ectopic

[1]Supported in part by National Institutes of Health, Eunice Kennedy Shriver National Institute of Child Health & Human Development (NICHD) and Office of Research on Women's Health (1R21HD065138-01A1 and 1R21HD066248-01A1 to J.A.A.).
[2]Correspondence: Joe A. Arosh, Department of Integrative Biosciences, College of Veterinary Medicine & Biomedical Sciences, Mail Stop: TAMU 4458, Texas A&M University, College Station, Texas 77843.
E-mail: jarosh@cvm.tamu.edu

Received: 26 March 2012.
First decision: 12 April 2012.
Accepted: 5 December 2012.

eISSN: 1529-7268 http://www.biolreprod.org
ISSN: 0006-3363

endometria compared to eutopic endometria from women with or without endometriosis during the proliferative phase of the menstrual cycle. Selective PTGS-2 inhibitor rofecoxib at 25 mg/day for 6 mo effectively suppresses the pelvic pain symptoms in endometriosis patients [21]. The gonadotropin-releasing hormone agonist leuprolide acetate at 3.75 mg for 1 mo decreases the expression of PTGS-2 along with P450 aromatase in eutopic endometria of endometriosis patients [22]. PGE_2 stimulates de novo estrogen biosynthesis from endometriotic lesions and in turn estradiol increases PTGS-2 expression and PGE_2 production [1]. In vitro studies indicate that the selective PTGS-2 inhibitor celecoxib prevents growth and survival of primary cultured eutopic endometrial epithelial cells from endometriosis patients [23]. In animal models for endometriosis, celecoxib decreases establishment of endometriosis and number and size of endometriotic implants in the rat [24], and the selective PTGS-2 inhibitor NS-398 induces regression of endometriotic implants through caspase-3-dependent apoptosis in hamster [25]. Recently, we have found that selective inhibition of PTGER2 and PTGER4 inhibits migration, invasion, growth, and survival of human endometriotic cells [15, 26, 27]. These results together suggest the importance of PTGS-2, PGE_2, and PTGER2 and PTGER4 pathways in the pathogenesis of endometriosis.

Interaction between endometriotic cells and peritoneal extracellular matrix (ECM) proteins occur through crucial mechanisms that allow adhesion, migration, and invasion of the endometriotic cells into peritoneal mesothelia and submesothelia [28–34]. Adhesion of endometriotic epithelial and stromal cells to peritoneum is mediated through integrin-ECM protein interactions. Integrins are single transmembrane cell surface-adhesion receptors that are made of heterodimers of non-covalently associated α- and β-subunits. There are 18 integrin α-subunits and eight β-subunits, which can combine into 24 different heterodimers that recognize distinct but overlapping sets of extracellular ligands [35–38]. Human peritoneum is composed of collagen I, fibronectin, vitronectin, laminin, and collagen IV ECM proteins. Collagen I, fibronectin, and vitronectin are the major components of connective tissues, and laminin and collagen IV are the major components of basement membrane [33, 39–43]. Collagen, vitronectin, fibronectin, and laminins bind with Itga2b1 (α2β1), Itgavb3 (αvβ3), Itga5b1 (α5β1), and Itga3b1 (α3β1) integrin receptors, respectively, and also with many other integrin receptors [35–38].

PGE_2 regulates the expression and activity of αv-, α5-, β1-, and β3-integrin subunits and thereby promotes adhesion of metastatic cancer cells and endothelial cells [44–47]. However, the PGE_2-mediated molecular and cellular mechanisms that control the expression and activity of integrins and their interactions with ECM proteins of the peritoneum and that are involved in the pathogenesis of endometriosis have not been elucidated. The objective of the present study were to determine functional interaction between PGE_2 signaling and adhesion of human endometriotic epithelial and stromal cells to ECM and to unravel the underlying molecular and cellular mechanisms. The results of the present study indicate that loss of function of EP2- and EP4-mediated PGE_2 signaling inhibits adhesion of human endometriotic epithelial and stromal cells through integrin-mediated mechanisms.

MATERIALS AND METHODS

Materials

The reagents used in this study were purchased from the following suppliers: Prestained protein markers and Bio-Rad assay reagents and standards (Bio-Rad Laboratories, Hercules, CA); Protran BA83 nitrocellulose membrane (Whatman Inc., Sanford, ME); Pierce ECL (Pierce Biotechnology, Rockford, IL); protease inhibitor cocktail tablets, that is, complete ethylenediaminetetraacetic acid (EDTA)-free and PhosStop tablets (Roche Applied Biosciences, Indianapolis, IN); antibiotic-antimycotic reagents and trypsin–EDTA (Invitrogen Life Technologies Inc, Carlsbad, CA); Blue X-Ray film (Phenix Research Products, Hayward, CA); fetal bovine serum (HyClone, Logan, UT); and tissue culture dishes and plates (Corning Inc., Corning, NY); ECM adhesion assay kits Cytoselect collagen I, laminin (Cell Biolabs Inc., San Diego, CA); and InnoCyte collagen IV, fibronectin, and vitronectin (EMD Biosciences, Gibbstown, NJ). EP2 and EP4 small interfering RNA (siRNA), siGLO RISC-free siRNA, and DharmaFect-1 were obtained from Dharmacon Inc. (Lafayette, CO). Antagonists/inhibitors for EP2 (AH6809), EP4 (AH23848) were purchased from Sigma-Aldrich (St. Louis, MO). EP2 and EP4 antibodies were purchased from Cayman Chemicals (Ann Arbor, MI). All the other antibodies used in this study were purchased from Cell Signaling Technology (Danvers, MA), Chemicon International (Billerica, MA), or Santa Cruz Biotechnology (Santa Cruz, CA) except β-actin monoclonal antibody (Sigma-Aldrich) and goat anti-rabbit or anti-mouse immunoglobulin G (IgG) conjugated with horseradish peroxidase (Kirkegaard & Perry Laboratories, Gaithersburg, MA). The rest of the chemicals used were molecular biological grade and were obtained from Fisher Scientific (Pittsburgh, PA) or Sigma-Aldrich.

Human Endometriotic Cells and Culture

Immortalized human endometriotic epithelial cells 12Z and stromal cells 22B used in this study were derived from active red peritoneal endometriosis lesions during the proliferative phase of the menstrual cycle from women [48]. The 12Z and 22B cells share several phenotypic and molecular characteristics of primary cultured endometriotic cells [48]. Accumulating information from our and other laboratories indicates that 12Z and 22B cells mimic the active/progressive phase of endometriosis [15, 26, 27, 48–50]. Importantly, a xenograft of a mixed population of 12Z and 22B cells into the peritoneal cavity of nude mice is able to proliferate, attach, invade, reorganize, and establish peritoneal endometriosis-like lesions that are histomorphologically similar to those found in spontaneous peritoneal endometriotic tissue in women [51]. These well-characterized 12Z and 22B cells were cultured in Dulbecco modified Eagle medium F-12 (DMEM/F12) without special steroid treatment containing 10% fetal bovine serum (FBS) and penicillin (100 units/ml), streptomycin (100 μg/ml), and amphotericin-B (2.5 μg/ml) in a humidified 5% CO_2 and 95% air at 37°C as described previously [15, 18, 26, 52]. At 70%–80% confluency, the cells were cultured in DMEM/F12 with 2% dextran-charcoal-treated FBS and treated with EP inhibitors (EP-I) for EP2 (AH6809, 75 μM) and EP4 (AH23848, 50 μM) for 24h. These inhibitors competitively bind with the respective EP2 or EP4 receptors and inhibit their activations [9, 53, 54] but not their expressions [15]. The doses for these inhibitors were selected based on the dose response experiments that we published previously [26, 27, 51].

EP2/EP4 siRNA

Knockdown of EP2 and EP4 genes were performed as described previously [15]. The 12Z and 22B cells (3.0×10^5/well) were cultured in antibiotic-free DMEM/F12 with 10% FBS in six-well tissue culture plates. At 70%–80% confluency, cells were used for EP2 and EP4 knockdown experiments using SMART-pool siRNA duplex delivered by DharmaFect-1 as described previously [15, 18, 27] and per the manufacturer's instructions (Dharmacon Inc., Lafayette, CO). As an internal control, MOCK siRNA was performed using the transfection procedure in the absence of siRNA. Briefly, siRNA duplexes (100 nM/well) and DharmaFect-1 (3 μl/well) were diluted in 50 μl antibiotic- and serum-free DMEM/F12 medium separately and mixed gently and incubated for 5 min at room temperature. Afterward, EP2 and EP4 siRNA and DharmaFect-1 were mixed (total volume, 100 μl) and incubated at room temperature for 20 min. Then, 100 μl of siRNA:DharmaFect-1 complex was diluted with 2 ml antibiotic-free media with 10% FBS and added to the well. After 24 h, the medium was replaced with fresh DMEM/F12 with 10% FBS and incubated for an additional 24 h. Fluorescence-labeled siGLO RISC-free siRNA was transfected separately, and the transfection efficiency was estimated using a fluorescence microscope. A transfection efficiency of more than 80% was considered optimal for further experiments. The efficiency of siRNA on silencing of EP2 and EP4 proteins was assessed by Western blot analysis at 72 h posttransfection. The knockdown efficiency was 70%–80% in both 12Z and 22B cells as we reported previously [15].

ECM Adhesion Assay

In vitro ECM-cell adhesion assays were performed using Cytoselect collagen I, laminin, InnoCyte collagen IV, fibronectin, and vitronectin precoated

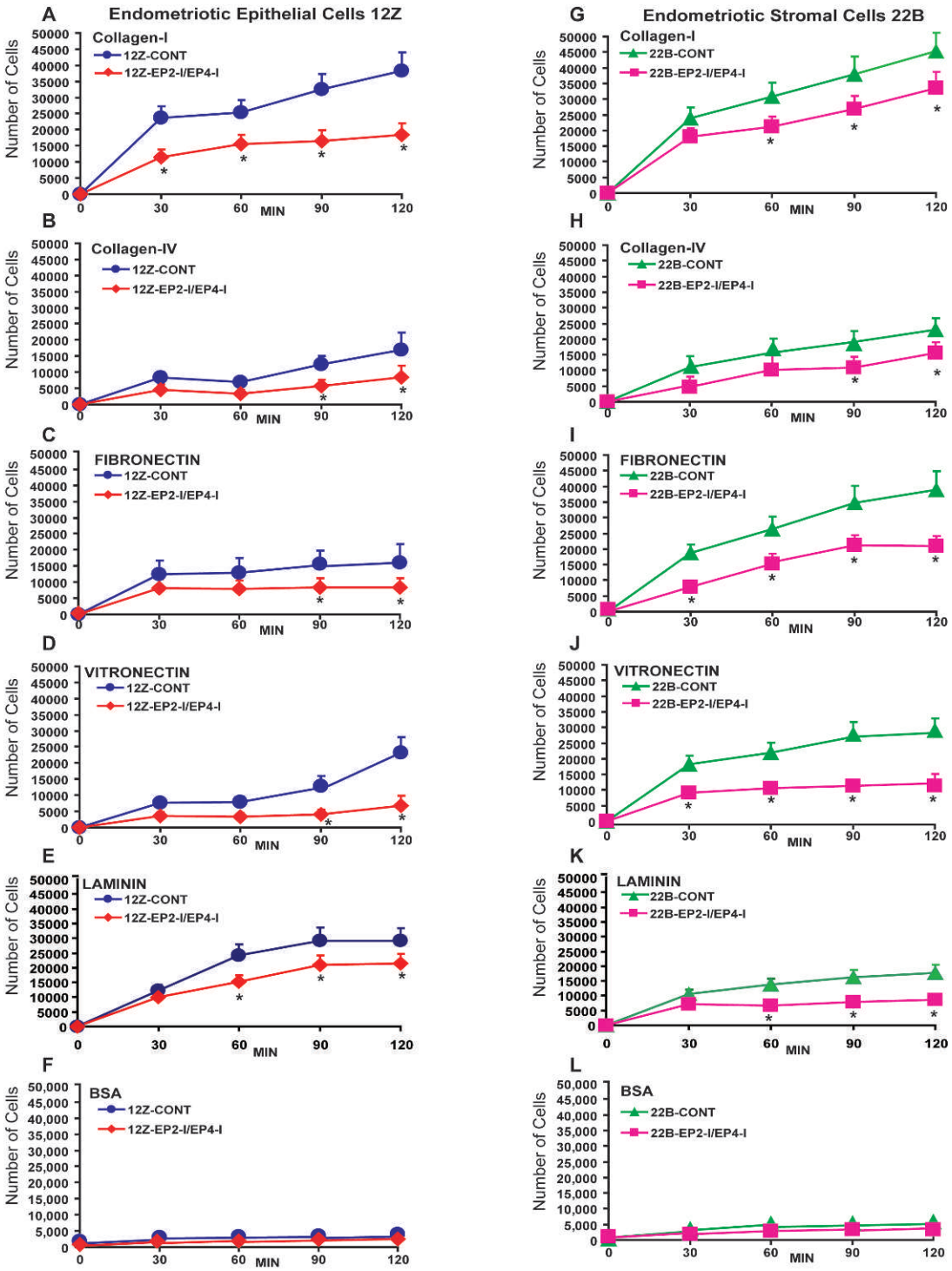

FIG. 1. Selective inhibition of EP2 and EP4 decreased adhesion of human endometriotic epithelial and stromal cells. Effects of pharmacological inhibition of EP2 and EP4 on the temporal adhesion of endometriotic epithelial cells 12Z (**A**–**F**) and endometriotic stromal cell 22B (**G**–**L**) on various ECM proteins. Bovine serum albumin (BSA) was used as an internal control. The adhesion assays were performed as described in *Materials and Methods*. *Control (CONT) versus inhibition of EP2 and EP4 (EP2-I/EP4-I), $P < 0.05$. The 12Z and 22B cells were treated with EP inhibitors (EP-I) of EP2 (AH6809, 75 μM) and EP4 (AH23848, 50 μM) for 24 h. Numerical data are expressed as mean ± SEM of three experiments.

adhesion assay kits following the manufacturer's protocols. Briefly, 12Z and 22B cells were cultured and treated with inhibitors for EP2 (AH6809, 75 μM) and EP4 (AH23848, 50 μM) as described above. After 20 h, the cells were harvested and resuspended in serum-free DMEM/F12 media and plated on collagen I, collagen IV, fibronectin, vitronectin, or laminin precoated plates at concentration of 100 000 cells/well and incubated at 37°C and 5% CO_2 for 0, 30, 60, 90, and 120 min. Bovine serum albumin (BSA) was used as internal control. For siRNA experiments, the cells were harvested after 72 h, and adhesion assay was performed as described above. The number of cells adhered to the specific ECM protein were measured using a fluorescence plate reader at an excitation wavelength of ~480–485 nm and emission wavelength of ~520 nm.

Protein Extraction and Western Blot Analysis

Total protein was isolated from endometriotic cells and immunoblot/Western blot analysis was performed as described previously [15, 18]. Briefly,

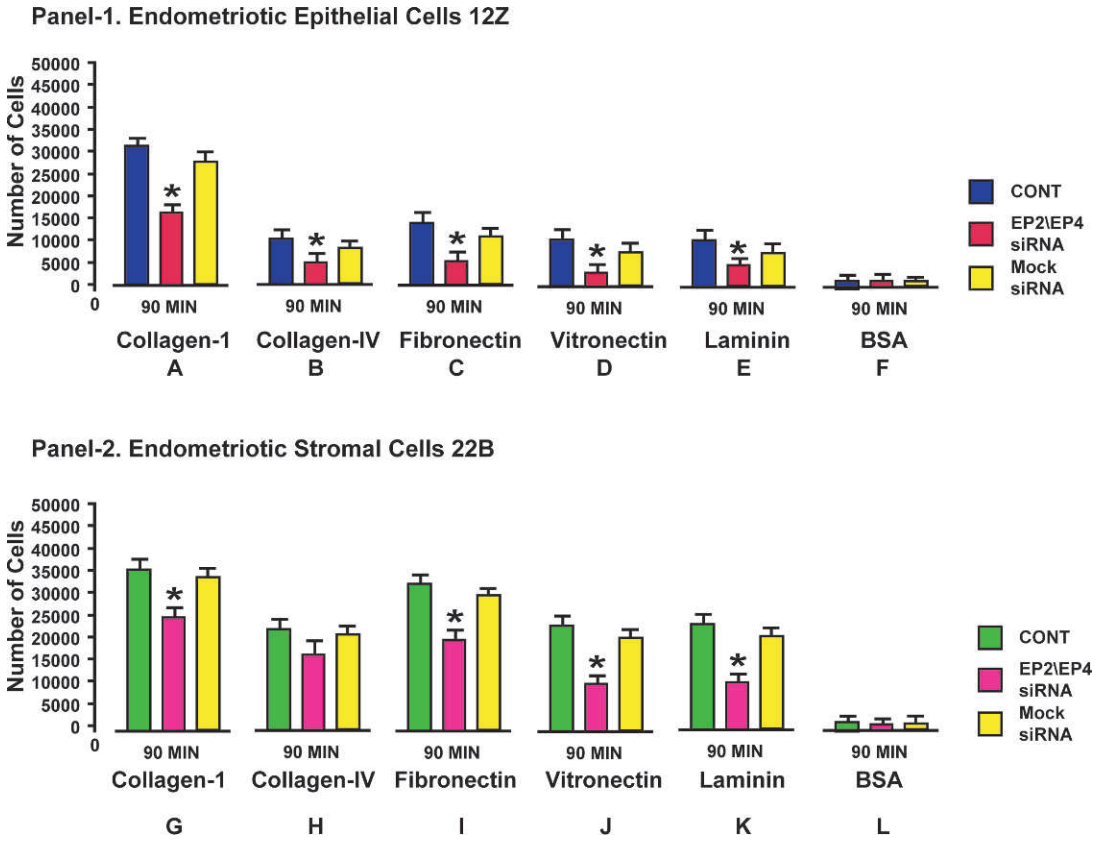

FIG. 2. Knockdown of EP2 and EP4 decreased adhesion of human endometriotic epithelial and stromal cells. Effects of knockdown of EP2 and EP4 genes on adhesion of endometriotic epithelial cells 12Z (**Panel-1**, **A**–**F**) and endometriotic stromal cell 22B (**Panel-2**, **G**–**L**) on various ECM proteins. Bovine serum albumin (BSA) was used as an internal control. EP2 and EP4 knockdown were performed using SMART-pool siRNA duplex delivered by DharmaFect-1. After 72 h of siRNA treatment, the adhesion assays were performed as described in *Materials and Methods*. *Control (CONT) versus inhibition of EP2 and EP4 (EP2/EP4 siRNA), $P < 0.05$. Numerical data are expressed as mean ± SEM of three experiments.

the cells were harvested using 1% trypsin-EDTA and pelleted. The cell lysates were sonicated in sonication buffer consisting of 20 mM Tris-HCl, 0.5 mM EDTA, 100 μM diethyldithiocarbamate (DEDTC), 1% Tween, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail tablets, that is, complete EDTA-free (1 tablet/50 ml) and PhosStop (1 tablet /10 ml). Sonication was performed using a Microson ultrasonic cell disruptor (Microsonix Incorporated, Farmingdale, NY). Protein concentration was determined using the Bradford method [55] and a Bio-Rad Protein Assay kit. Protein samples (75 μg) were resolved using 7.5%, 10%, or 12.5% SDS-PAGE. Chemiluminescent substrate was applied according to the manufacturer's instructions (Pierce Biotechnology). The blots were exposed to Blue X-Ray film, and densitometry of the autoradiograms was performed using an Alpha Imager (Alpha Innotech Corporation, San Leandro, CA).

Immunoprecipitation

The 12Z and 22B cells were cultured, treated, harvested, and then total cell lysates were prepared as described above. Immunoprecipitation (IP) was carried out using protocols provided by Santa Cruz Biotechnology and Cell Signaling Technology. In brief, total cell lysate (1 mg, ~120–125 μl) was precleared by incubating with ExactaCruZ F preclearing matrix (50 μl/ml) (Santa Cruz Biotechnology) for 30 min at 4°C [15]. The precleared cell lysate (~120–125 μl) was incubated with rabbit monoclonal anti-human t-Src antibody (1 μg/ml) (Cell Signaling Technology) overnight at 4°C and then further incubated with immunoprecipitation ExactraCruZ F IP matrix (50 μl/ml) (Santa Cruz Biotechnology) overnight at 4°C. Protein-antibody IP complexes were precipitated and washed three times using 500 μl of IP buffer: 20mM Tris-HCl, 0.5mM EDTA, 100 μM DEDTC, 1% Tween, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail tablets, that is, complete EDTA-free (1 tablet/50 ml) and PhosStop (1 tablet /10 ml). Finally, the protein-antibody IP complexes were resuspended in 40 μl of 2× SDS sample buffer, boiled at 100°C for 5 min, and then resolved in 10% SDS PAGE gel [15]. Rabbit IgG was immunoprecipitated and used as an internal control.

Statistical Analyses

Statistical analyses were performed using general linear models of Statistical Analysis System (Cary, NC). The effects of inhibition of EP receptors on cell adhesion and expression levels of different proteins and degree of protein-protein interactions in 12Z and 22B cells were analyzed by one-way analysis of variance (ANOVA) followed by Tukey-Kramer HSD test. The numerical data are expressed as mean ± SEM. Statistical significance was considered at $P < 0.05$.

RESULTS

Selective Inhibition of EP2 and EP4 Decreased Adhesion of Human Endometriotic Cells

The substrate-specific and temporal adhesion of human endometriotic epithelial cells 12Z and stromal cells 22B was determined. The adhesion ability of 12Z cells decreased ($P < 0.05$) in the following order: collagen I > laminin > collagen IV > vitronectin > fibronectin. The adhesion ability of 22B cells decreased ($P < 0.05$) in the following order: collagen I > fibronectin > vitronectin > collagen IV > laminin. Inhibition of EP2 and EP4 decreased the adhesion of 12Z cells on collagen I at 30 min, laminin at 60 min, and collagen IV, vitronectin, and fibronectin at 90–120 min (Fig. 1). On the other hand, inhibition of EP2 and EP4 decreased ($P < 0.05$) the adhesion of 22B cells on vitronectin and fibronectin at 30 min and collagen I at 60 min, but did not inhibit adhesion of 22B cells on collagen IV and laminin (Fig. 1).

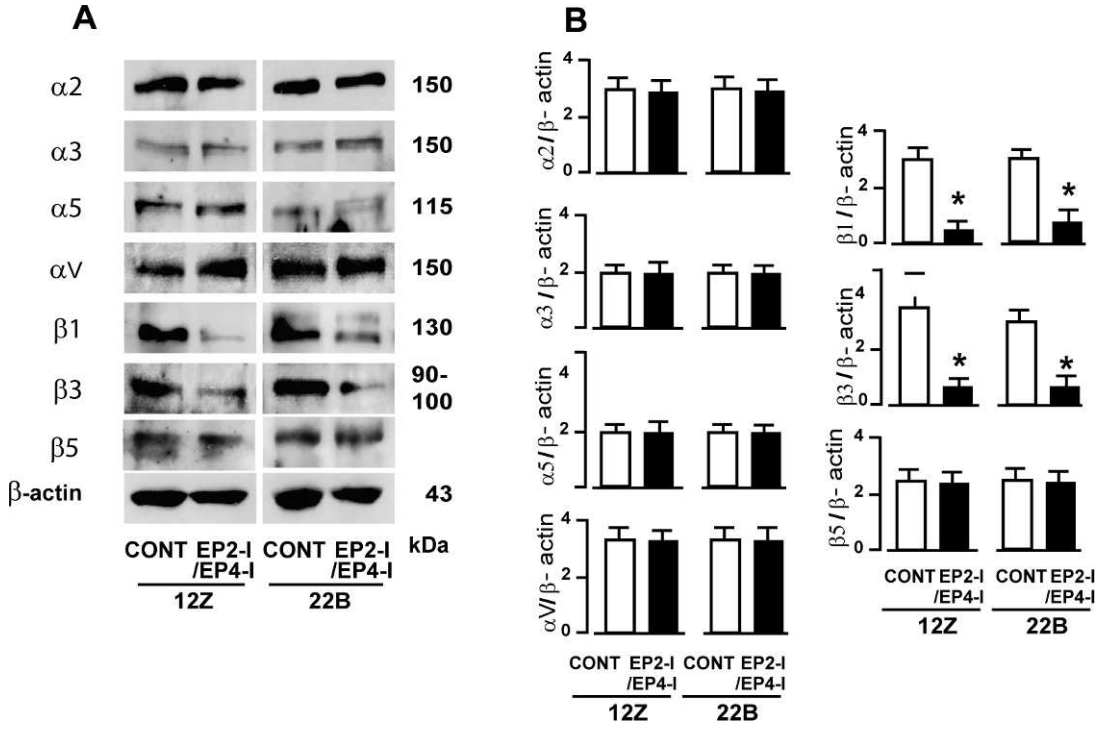

FIG. 3. Selective inhibition of EP2 and EP4 decreased the expression of integrin proteins in human endometriotic epithelial and stromal cells. **A**) Western blot analysis of integrin α- and β-subunit proteins in endometriotic epithelial cells 12Z and stromal cells 22B; β-actin was measured as the internal control protein. **B**) Densitometry of the Western blots. The 12Z and 22B cells were treated with EP inhibitors (EP-I) of EP2 (AH6809, 75 μM) and EP4 (AH23848, 50 μM) for 24 h. The experiments were performed as described in *Materials and Methods*. *Control versus EP2-I/EP4-I, $P < 0.05$. Numerical data are expressed as mean ± SEM of three experiments.

To complement the pharmacological effects of EP2 and EP4 inhibition, we used a siRNA approach to knock down EP2 and EP4 genes to confirm their role in adhesion of 12Z and 22B cells. Silencing of EP2 and EP4 genes decreased ($P < 0.05$) adhesion of 12Z cells with collagen I, collagen IV, fibronectin, and vitronectin (Fig. 2) and adhesion of 22B cells with collagen I, fibronectin, and vitronectin at 90 min (Fig. 2). The effects of pharmacological inhibition of EP2 or EP4 were similar to those of knockdown of EP2 and EP4 genes on adhesion of 12Z and 22B cells to ECM. Therefore, pharmacological inhibition of EP2 and EP4 was selected for the follow-up experiments.

Selective Inhibition of EP2 and EP4 Decreased Expression of Integrin Receptor β-Subunit Proteins in Human Endometriotic Cells

We determined the effects of inhibition of EP2 and EP4 on the expression of substrate-specific integrin receptor α- and β-subunits. The results indicated that selective inhibition of EP2 and EP4 decreased (2- to 3-fold, $P < 0.05$) the expression of the β1- and β3-subunits whereas it did not decrease the expression of β5-, α2-, α3-, α5-, and αv-subunits in 12Z and 22B cells (Fig. 3).

Selective Inhibition of EP2 and EP4 Decreased Expression of Focal Adhesion Kinase and Talin Proteins in Human Endometriotic Cells

Focal adhesion kinase (FAK) and talin are the important proteins involve in integrin inside-out and outside-in signaling. Therefore, we determined whether selective inhibition of EP2 and EP4 affects the expression/activation of talin and FAK proteins. The results indicated that selective inhibition of EP2 and EP4 decreased the expression of talin protein and phosphorylation of FAK protein at tyrosine-397 in 12Z and 22B cells (Fig. 4).

Interaction Between EP2 and EP4 and β-Subunits, FAK, and Talin Proteins in Human Endometriotic Cells

We determined whether EP2 and EP4 protein directly interacted with β-subunits and FAK and talin proteins. Immunoprecipitation/Western blot analysis (Fig. 5) indicated that selective inhibition of EP2 and EP4 decreased (2- to 3-fold, $P < 0.05$) the direct interaction between EP2/EP4 and β1/β3-subunit proteins and FAK and talin proteins in 12Z and 22B cells.

Interaction Between EP2 and EP4 and β-Subunits of Integrin Receptors Is Meditated Through Src/β-Arrestin 1 Protein Complex in Human Endometriotic Cells

In a previous study, we reported that EP2 and EP4 interacts with tyrosine kinase receptors intracellularly through a Src/β-arrestin 1 protein complex and selective inhibition of EP2 and EP4 protein decreased the expression of Src and β-arrestin 1 proteins [15]. It has been shown that Src kinase phosphorylates β-subunits of integrin intracellularly directly, which is necessary for inside-out integrin activation [35–38, 56–58]. Therefore, we determined whether EP2 and EP4 could interact with the β-subunit through Src/β-arrestin 1 protein complex. Immunoprecipitation/Western blot analysis (Fig. 6) indicated that selective inhibition of EP2 and EP4 decreased (2- to 3-fold, $P < 0.05$) the interaction between EP2/EP4 and β1/β3-

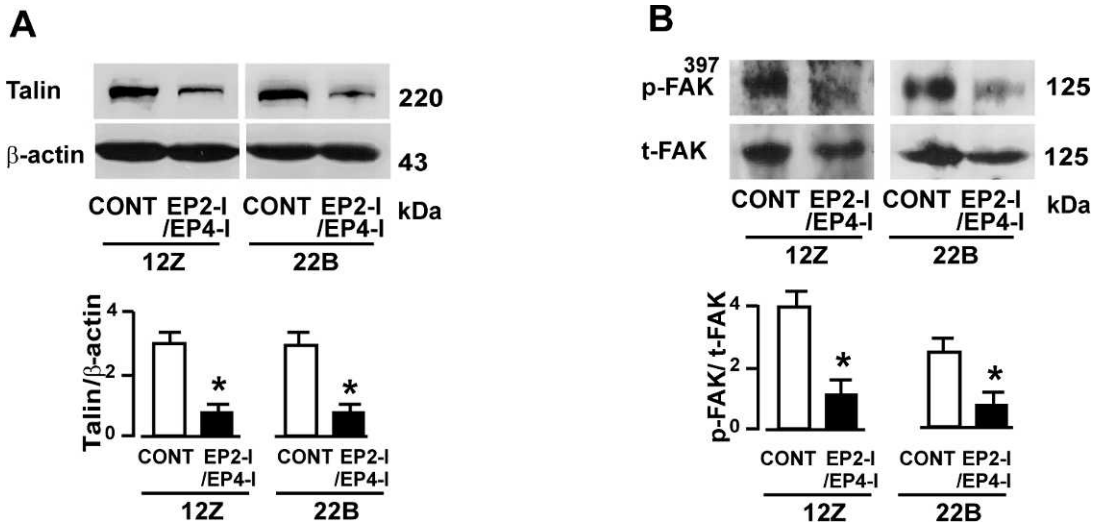

FIG. 4. Selective inhibition of EP2 and EP4 decreased the expression of talin and FAK proteins in human endometriotic epithelial and stromal cells. Densitometry of Western blot analysis of talin (**A**) and FAK (**B**) proteins in endometriotic epithelial cells 12Z and stromal cells 22B; β-actin was measured as the internal control protein. The 12Z and 22B cells were treated with EP inhibitors (EP-I) of EP2 (AH6809, 75 μM) and EP4 (AH23848, 50 μM) for 24 h. The experiments were performed as described in *Materials and Methods*. *Control versus EP2-I/EP4-I, $P < 0.05$. Numerical data are expressed as mean ± SEM of three experiments.

subunit proteins through Src/β-arrestin 1 in 12Z and 22B cells.

Interaction Between EP2 and EP4 and Talin and FAK Proteins Is Mediated Through Src/β-Arrestin 1 Protein Complex in Human Endometriotic Cells

Src/β-subunit/talin complex recruits FAK protein, which activates intracellular signal transduction pathways associated with integrin outside-in signaling [35, 37, 38]. In addition, activation/phosphorylation of FAK protein downstream phosphorylates ERK and AKT proteins [59–61]. Therefore, we determined whether inhibition of EP2 and EP4 impaired the interactions between EP2/EP4 and FAK or talin protein through Src/β-arrestin 1 complex. Immunoprecipitation/Western blot analysis (Fig. 7) indicated that selective inhibition of EP2 and EP4 decreased (2- to 3-fold, $P < 0.05$) the interaction between talin and β1- or β3-subunits through Src/β-arrestin 1 complex and in turn decreased (2- to 3-fold, $P < 0.05$) the

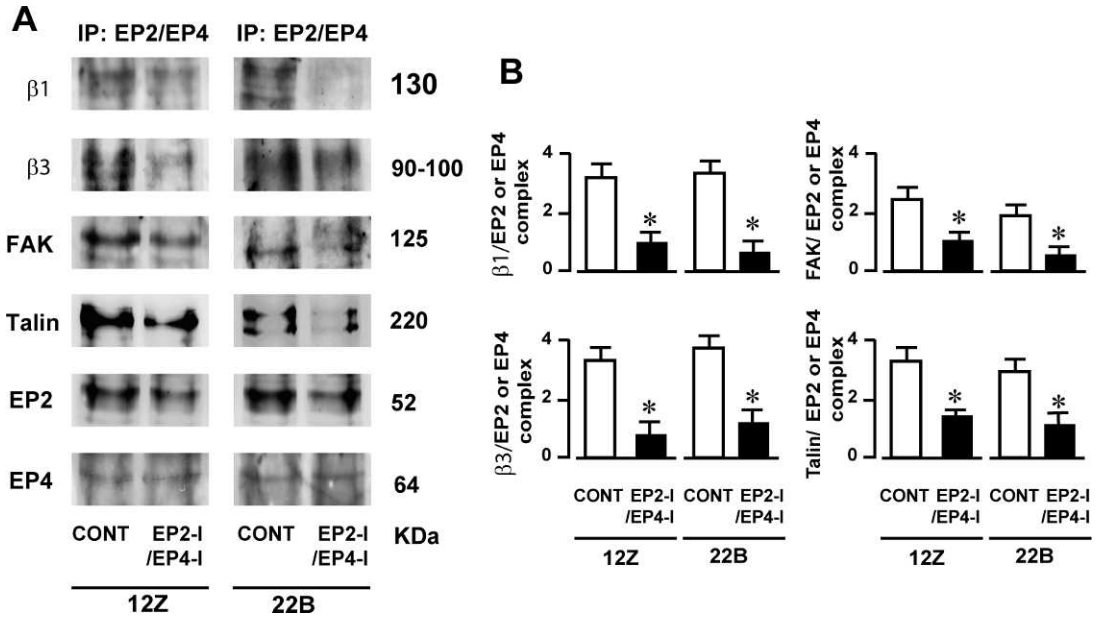

FIG. 5. Selective inhibition of EP2 and EP4 decreased their direct interaction with β1- and β3-subunits of integrin receptors and FAK and talin proteins in human endometriotic epithelial and stromal cells.**A**) Immunoprecipitation (IP)/Western blot analysis. EP2 and EP4 proteins were together immunoprecipitated, and their interactions with β1- and β3-subunits, FAK, and talin proteins were analyzed by Western blots. EP2 and/or EP4 protein was measured as the input control. **B**) Densitometry of IP/Western blots. The interaction was expressed as the ratio between β1, β3, talin, or FAK and EP2 or EP4 protein. The 12Z and 22B cells were treated with EP inhibitors (EP-I) of EP2 (AH6809, 75 μM) and EP4 (AH23848, 50 μM) for 24 h. The experiments were performed as described in *Materials and Methods*. *Control versus EP2-I/EP4-I, $P < 0.05$. Numerical data are expressed as mean ± SEM of three experiments.

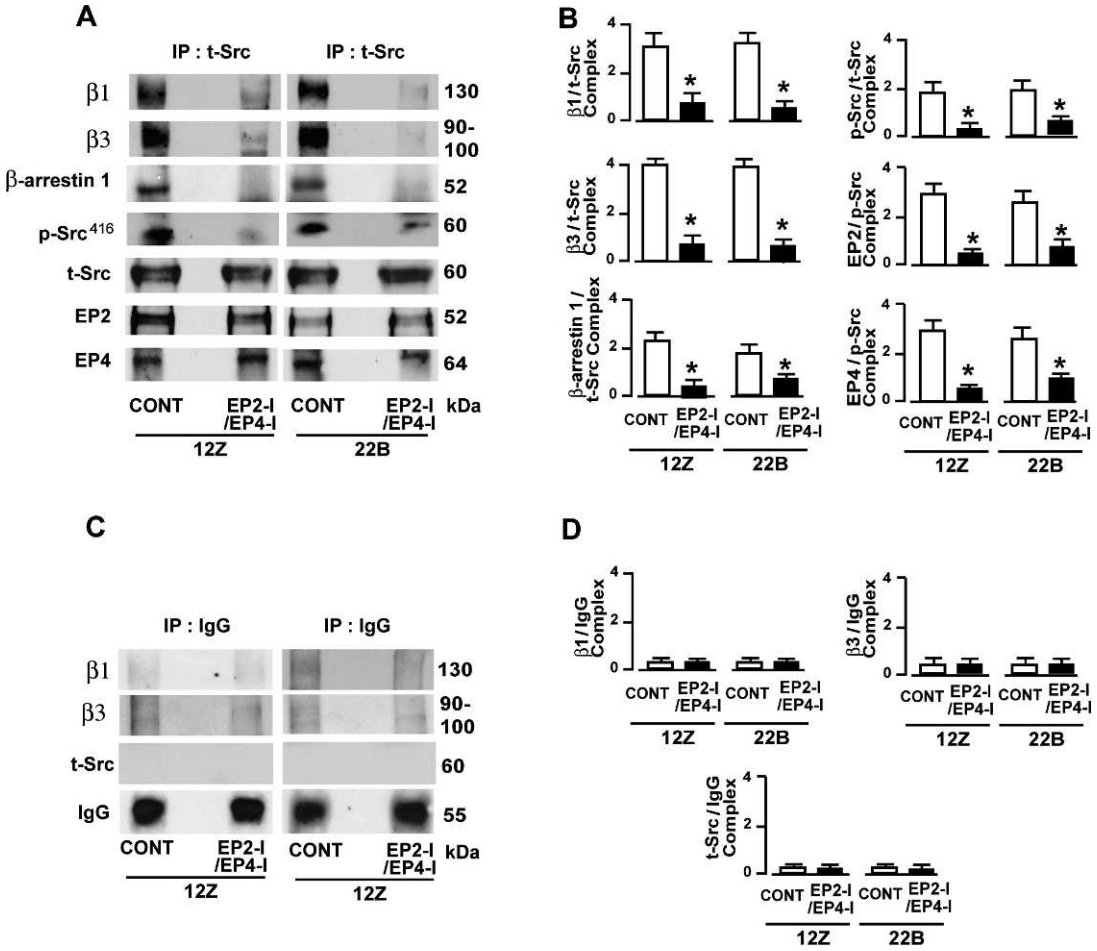

FIG. 6. EP2 and EP4 interact with β-subunit of integrin receptors through Src/ β-arrestin 1 complex in human endometriotic epithelial and stromal cells. **A**) Immunoprecipitation (IP)/Western blot analysis. Total Src protein was immunoprecipitated and its interactions with β1- and β3-subunits, β-arrestin 1, and EP2 and EP4 proteins were analyzed by Western blots. t-Src or p-Src protein was measured as the input control. **B**) Densitometry of IP/Western blot analysis. The interaction was expressed as the ratio between β1, β3, β-arrestin 1, EP2, or EP4 and t-Src or p-Src proteins. **C**) As an internal control, IgG protein was immunoprecipitated, and its interactions with β1, β1, and t-Src proteins were analyzed by Western blots. **D**) In the densitometry analysis, the interaction was expressed as a ratio between β1, β3, or t-Src and IgG proteins. The 12Z and 22B cells were treated with EP inhibitors (EP-I) of EP2 (AH6809, 75 μM) and EP4 (AH23848, 50 μM) for 24 h. The experiments were performed as described in *Materials and Methods*. *Control versus EP2-I/ EP4-I, $P < 0.05$. Numerical data are expressed as mean ± SEM of three experiments.

interaction between talin, p-FAK, p-ERK1/2, and p-AKT proteins in 12Z and 22B cells.

DISCUSSION

Human peritoneal mesothelium is a contiguous layer of cells resting on an ECM base that is composed of collagen I, fibronectin, vitronectin, laminin, and collagen IV [33, 39–43]. The results of the present study indicate that endometriotic epithelial cells 12Z and stromal cells 22B distinctly attach to ECM proteins in an epithelial-stromal cell- and ECM substrate-specific temporal pattern. Importantly, 12Z and 22B cells exhibited an equal degree of adhesion toward collagen I. Stromal cells 22B showed higher adhesion toward stromal components of ECM such as vitronectin and fibronectin. In contrast, epithelial cells 12Z displayed a higher degree of adhesion toward epithelial components of ECM such as collagen IV and laminin.

The EP2 inhibitor (AH6809, 75 μM) and EP4 inhibitor (AH23848, 50 μM) competitively bind with the respective EP receptors and inhibit their activations [9, 53, 54]. We reported that AH6809 and/or AH23848 inhibited the growth of 12Z and 22B cells whereas the more selective EP1 antagonist SC19220 did not inhibit the growth of 12Z and 22B cells. Thus, we concluded that PGE$_2$ promoted growth, survival, and invasion of 12Z and 22B cells through EP2 and EP4 but not through EP1 or EP3 [15]. A siRNA approach was used to verify that the observed pharmacological effects of AH6809 and AH23848 are primarily due to inhibition of EP2 and EP4. Our data indicated that effects of pharmacological inhibition of EP2 or EP4 were similar to those of the knockdown of EP2 and EP4 genes on adhesion of 12Z and 22B cells to ECM and thus confirmed an exclusive inhibitory role of EP2 and EP4.

Integrin receptor subunits α2, α3, αv, α5, β1, and β3 are expressed in glandular epithelium and stroma of eutopic and ectopic endometria [31, 32, 34, 62–67]. In order to understand the molecular mechanisms through which inhibition of EP2 and EP4 decreased the adhesion of 12Z and 22B cells to ECM, the expression of ECM substrate-specific integrin receptors was determined. The results of the present study indicated that selective inhibition of EP2 and EP4 decreased the expression of β1- and β3-subunits but not the expression of the β5-, α2-, α3-, α5-, and αv-subunits in both 12Z and 22B cells.

Although α- and β-subunits are needed for functional integrin receptors, the cytoplasmic domain of the β-subunit of integrin interacts directly with talin protein and indirectly to

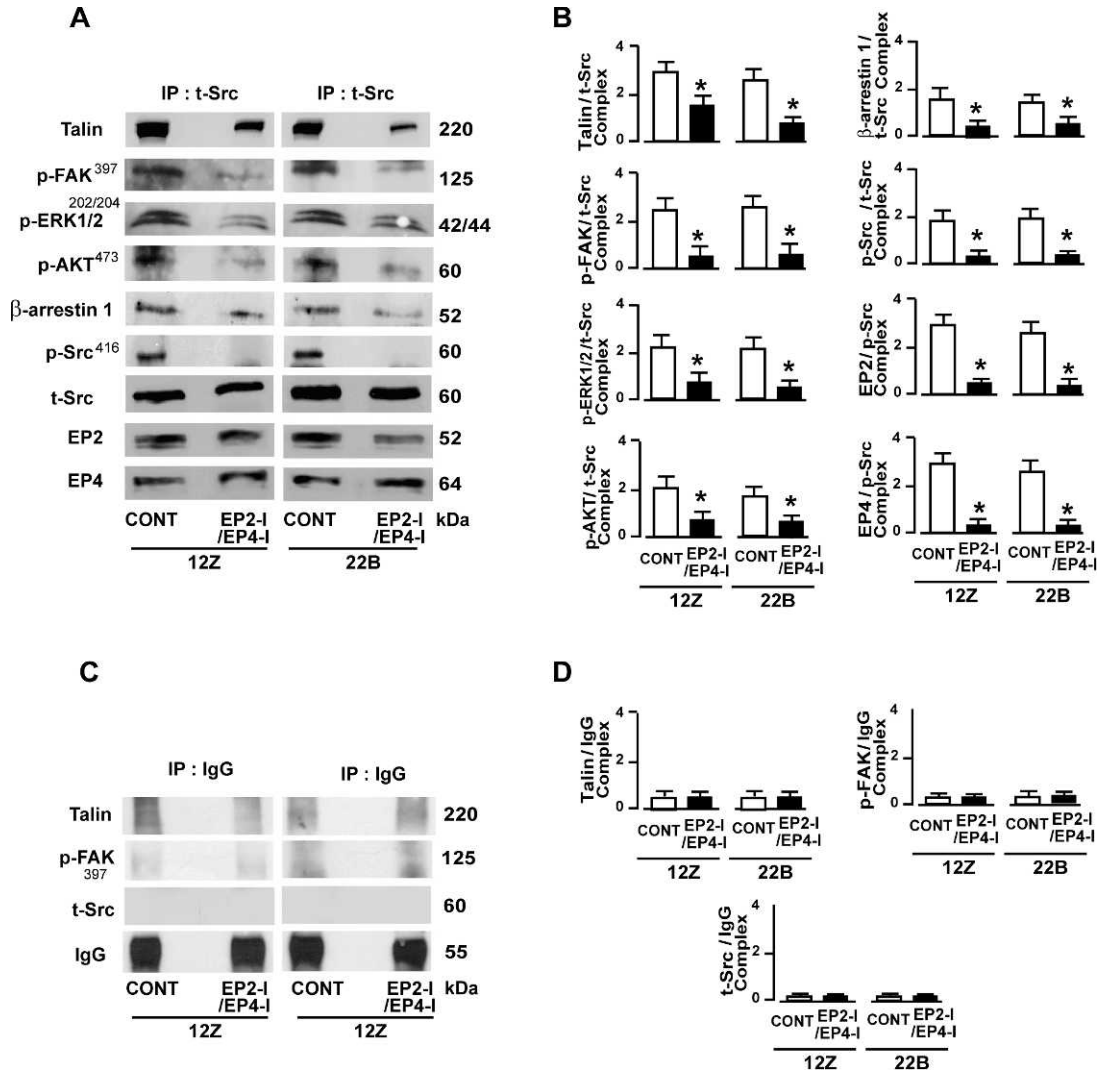

FIG. 7. EP2 and EP4 interact with p-FAK, P-ERK1/2, and P-AKT through Src/ β-arrestin 1 complex in human endometriotic epithelial and stromal cells. **A**) Immunoprecipitation (IP)/Western blot analysis. Total Src protein was immunoprecipitated, and its interactions with β-arrestin 1, talin, FAK, ERK1/2 and AKT, and EP2 and EP4 proteins were analyzed by Western blots. **B**) Densitometry of IP/Western blot analysis. The interaction was expressed in ratio between talin, p-FAK, pERk1/2, p-AKT, β-arrestin, EP2, or EP4 and t-Src or p-Src proteins. **C**) As an internal control, IgG protein was immunoprecipitated, and its interaction with talin, p-FAK, and t-Src were analyzed by Western blots. **D**) In the densitometry analysis, the interaction was expressed as a ratio between talin, p-FAK, or t-Src and IgG proteins. The 12Z and 22B cells were treated with EP inhibitors (EP-I) of EP2 (AH6809, 75 μM) and EP4 (AH23848, 50 μM) for 24 h. The experiments were performed as described in *Materials and Methods*. *Control versus EP2-I/EP4-I, $P < 0.05$. Numerical data are expressed as mean ± SEM of three experiments.

other linkage adapter proteins such as α-actinin, vinculin, and paxillin through talin during the process of cell adhesion [35–38, 57]. This protein complex recruits FAK, which acts as a phosphorylation-regulated signaling protein that is important for adhesion turnover, cell migration, and integrin receptor cross-talk with the MAPK [68] and AKT [69] pathways. This process is known as integrin-activated outside-in signaling. Disruption of any of the components outside-in signaling compromises linkage between integrin receptors and the actin cytoskeleton and thus perturbs the adhesion and migration of cells [35–38, 57]. Therefore, we determined the interaction between EP2/EP4 and outside-in signaling components. The results of the present study indicated that selective inhibition of EP2 and EP4 decreased the expression of talin protein and dephosphorylated FAK protein and decreased the interactions between Src, talin, FAK, pERK1/2, and p-AKT proteins in 12Z and 22B cells.

In addition to outside-in signaling, integrins can regulate their affinity for the ECM by inside-out signaling. Inside-out integrin activation can be controlled by the conformation of talin, phosphorylation of β-subunits, and interaction of talin with β-subunits [35–38, 57, 70]. Mutation of tyrosine to phenylalanine in the NxxY motif of β1 abolished its phosphorylation through Src and perturbed its binding ability to the ECM. In addition, phosphorylation of FAK is impaired in cells that lack Src kinase [71, 72]. These studies together suggest that phosphorylation of NxxY of β-integrin tail intracellularly by Src kinase is one of the major regulatory mechanisms. Therefore, we determined the interaction between EP2/EP4 and integrin inside-out signaling components. The results of the present study indicated that selective inhibition of EP2 and EP4 decreased the interaction between EP2/EP4 and β-subunits of integrins through the Src/β-arrestin 1 complex in 12Z and 22B cells.

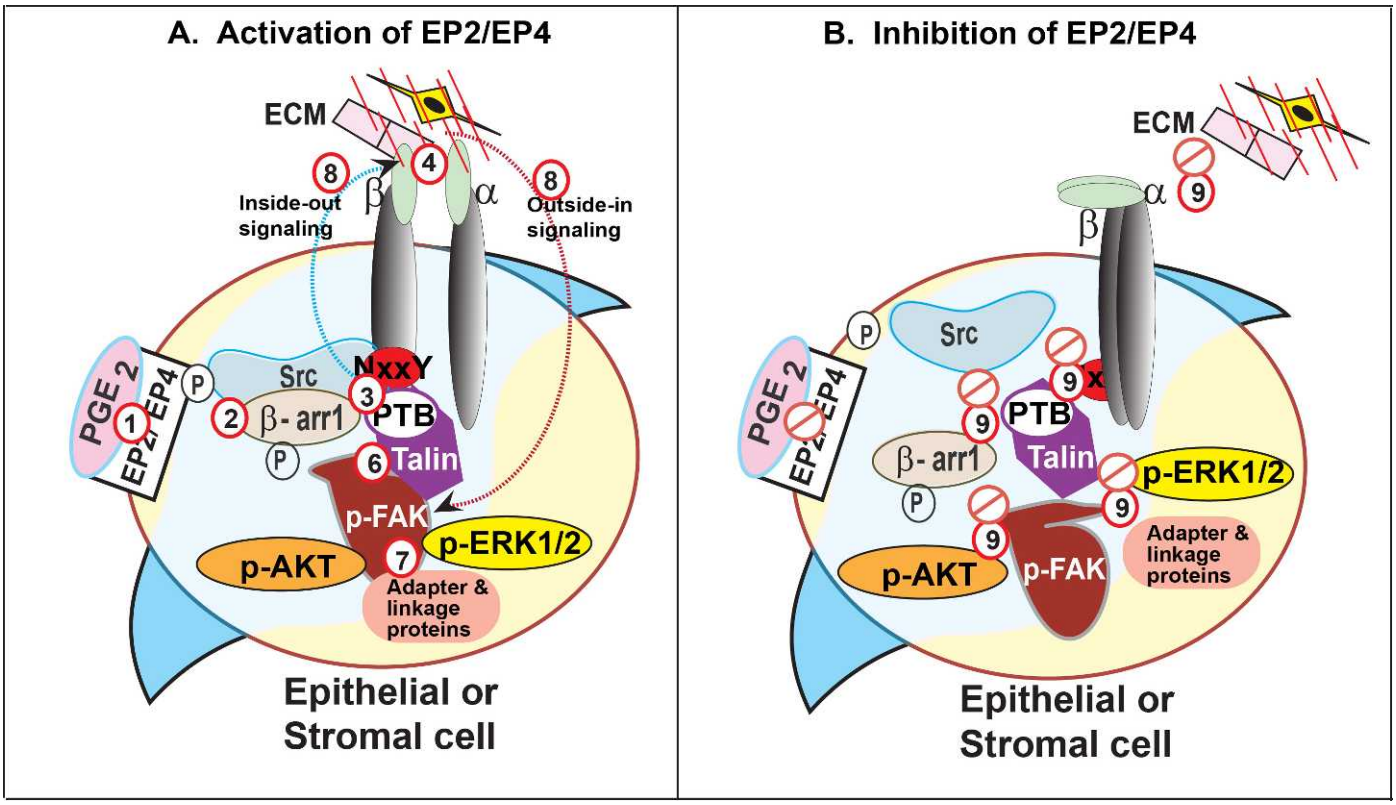

FIG. 8. Mechanisms through which EP2- and EP4-mediated PGE$_2$ signaling promotes and loss of function of EP2 and EP4 inhibits adhesion of human endometriotic epithelial and stromal cells on ECM substrates. **1**) PGE$_2$ binds EP2 and EP4 receptors. **2**) Activation of EP2/EP4 causes phosphorylation and activation of p-Src416 and p-β-arrestin 1 (p-β-arr1). **3**) Activated/phosphorylated c-Src/β-arrestin 1 phosphorylates NxxY motifs of cytoplasmic domain of β-subunits; this could facilitate the interaction between NxxY motifs of cytoplasmic domain of β-subunits and the phosphotyrosine-binding (PTB) domain of talin. **4**) This leads to conformational changes in talin and β-subunits that increase ligand-binding affinity of integrin receptor for extracellular matrix (ECM) substrate. **5**) The activation of integrin receptor by ECM results in phosphorylation of focal adhesion kinase (FAK) protein, recruiting it into the Src/β-subunit/talin complex. **6**) Phosphorylated FAK recruits linkage adapter proteins kindling, α-actinin, vinculin, and paxillin into the cytoplasmic face of ligand-bound integrins. *Note:* This adapter protein complex was not determined in the present study. **7**) In addition, activation of FAK phosphorylates ERK1/2 and AKT proteins. These complexes are responsible for connecting integrins to the actin cytoskeleton and regulation of cell adhesion, migration, and differentiation. **8**) Thus, EP2- and EP4-mediated PGE$_2$ signaling activates integrin inside-out and outside-in signaling in human endometriotic epithelial and stromal cells, thereby promoting adhesion of endometriotic cells with peritoneum. **9**) Loss of function of EP2 and EP4 disassembles integrin conformation and linkage mechanisms and disturbs integrin outside-in and inside-out signaling in 12Z and 22B cells, thereby decreasing adhesion of human endometriotic epithelial and stromal cells with specific ECM substrates.

We determined protein-protein interactions by immunoprecipitation. Selective inhibition of EP2/EP4 decreased the overall expression of β1, β3, FAK or talin protein. The decreased interaction between EP2/EP4 and β1, β3, FAK, or talin protein could be due to either overall decreased mean expression of β1, β3, FAK, and talin proteins or due to their decreased functional activity. GPCR transactivates tyrosine kinase receptors through intracellular Src-dependent signaling mechanisms [73, 74]. Recruitment of β-arrestin 1 to activated GPCRs is associated with the activation of Src [75]. In quiescent cells, β-arrestin 1 protein is mainly localized diffusely in the cytosol. In stimulated cells, upon agonist stimulation of GPCRs, the β-arrestin 1 is phosphorylated and translocated from the cytosol to the membrane fraction and interacts with GPCRs [74]. PGE$_2$ increases the interactions between EP4 and β-arrestin 1 and Src signaling complex intracellularly and thereby transactivates EGFR and AKT signaling, which is critical for the regulation of colorectal carcinoma cell migration [13]. Our previous studies have shown that selective inhibition of EP2 and EP4 decreases the expression of Src and β-arrestin 1 proteins and their interactions and thereby inhibits EP2/EP4 interactions with matrix metalloproteinases (MMPs) in 12Z and 22B cells. The results of the present study along with previous findings [13, 73–75] indicate that the loss of function of EP2 and EP4 inhibits inside-out signaling by disturbing association between the Src/β-arrestin 1, talin, and β-subunit proteins and inhibits outside-in signaling by disturbing integrin linkage protein-protein interactions between Src/β-arrestin 1, talin, FAK, p-ERK1/2, and p-AKT, thereby inhibiting integrin-mediated adhesion of 12Z and 22B cells to specific ECM substrates (see Fig. 8).

Recently, we have shown that selective inhibition of EP2 and EP4 suppresses cell survival pathways, activates intrinsic apoptotic pathways, deregulates cell cycle machinery, and inhibits MMP-mediated invasion of 12Z and 22B cells, thereby inhibiting survival, growth, and invasion of endometriotic ells [26, 27, 51]. The results of the present and previous studies [15, 26] indicate that selective inhibition of EP2 and EP4 might provide an opportunity to block adhesion, invasion, growth, and survival pathways in the pathogenesis of endometriosis. These novel findings provide an important molecular framework for further evaluation of selective inhibition of EP2 and EP4 in primary cell culture and xenograft models as a potential nonsteroidal therapy to expand the spectrum of currently available treatment options for endometriosis in child-bearing age women.

In summary, selective inhibition of EP2 and EP4 1) inhibits adhesion of 12Z and 22B cells to collagen I, vitronectin, fibronectin, collagen IV, and laminin in a substrate- and epithelial-stromal cell-specific manner; 2) decreases the expression of β1- and β3-subunits whereas it does not decrease the expression of β5-, α2-, α5-, αv-, and α3-subunits; 3) decreases the expression of talin protein and dephosphorylate FAK protein; 4) inhibits the interaction between EP2/EP4 and β-subunits of integrins through Src/β-arrestin complex; and 5) inhibits the interaction between Src, talin, FAK, pERK1/2, and p-AKT proteins in 12Z and 22B cells. These results suggest that selective inhibition of EP2 and EP4 antagonizes integrin linked outside-in and inside-out signaling pathways and thereby reduces adhesion of human endometriotic epithelial cells 12Z and stromal cells 22B to ECM proteins.

REFERENCES

1. Bulun SE. Endometriosis. N Engl J Med 2009; 360:268–279.
2. Giudice LC, Kao LC. Endometriosis. Lancet 2004; 364:1789–1799.
3. De Leon FD, Vijayakumar R, Brown M, Rao CV, Yussman MA, Schultz G. Peritoneal fluid volume, estrogen, progesterone, prostaglandin, and epidermal growth factor concentrations in patients with and without endometriosis. Obstet Gynecol 1986; 68:189–194.
4. Wu MH, Shoji Y, Chuang PC, Tsai SJ. Endometriosis: disease pathophysiology and the role of prostaglandins. Expert Rev Mol Med 2007; 9:1–20.
5. Smith WL, Dewitt DL. Prostaglandin endoperoxide H synthases-1 and −2. Adv Immunol 1996; 62:167–215.
6. Tai HH, Ensor CM, Tong M, Zhou H, Yan F. Prostaglandin catabolizing enzymes. Prostaglandins Other Lipid Mediat 2002; 68–69:483–493.
7. Schuster VL. Molecular mechanisms of prostaglandin transport. Annu Rev Physiol 1998; 60:221–242.
8. Narumiya S, Sugimoto Y, Ushikubi F. Prostanoid receptors: structures, properties, and functions. Physiol Rev 1999; 79:1193–1226.
9. Coleman RA, Smith WL, Narumiya S. International Union of Pharmacology classification of prostanoid receptors: properties, distribution, and structure of the receptors and their subtypes. Pharmacol Rev 1994; 46: 205–229.
10. Cha YI, DuBois RN. NSAIDs and cancer prevention: targets downstream of COX-2. Annu Rev Med 2007; 58:239–252.
11. Fujino H, Regan JW. Prostaglandin F(2alpha) stimulation of cyclooxygenase-2 promoter activity by the FP(B) prostanoid receptor. Eur J Pharmacol 2003; 465:39–41.
12. Jabbour HN, Sales KJ. Prostaglandin receptor signalling and function in human endometrial pathology. Trends Endocrinol Metab 2004; 15: 398–404.
13. Buchanan FG, Gorden DL, Matta P, Shi Q, Matrisian LM, DuBois RN. Role of beta-arrestin 1 in the metastatic progression of colorectal cancer. Proc Natl Acad Sci U S A 2006; 103:1492–1497.
14. Castellone MD, Teramoto H, Williams BO, Druey KM, Gutkind JS. Prostaglandin E2 promotes colon cancer cell growth through a Gs-axin-beta-catenin signaling axis. Science 2005; 310:1504–1510.
15. Banu SK, Lee J, Speights VO Jr, Starzinski-Powitz A, Arosh JA. Selective inhibition of prostaglandin E2 receptors EP2 and EP4 induces apoptosis of human endometriotic cells through suppression of ERK1/2, AKT, NFkB and b-catenin pathways and activation of intrinsic apoptotic mechanisms. Mol Endocrinol 2009; 23:1291–1305.
16. Wang D, Dubois RN. Eicosanoids and cancer. Nat Rev Cancer 10: 181–193.
17. Wang D, Dubois RN. Prostaglandins and cancer. Gut 2006; 55:115–122.
18. Banu SK, Lee J, Speights VO, Starzinski-Powitz A, Arosh JA. Cyclooxygenase-2 regulates survival, migration and invasion of human endometriotic cells through multiple mechanisms. Endocrinology 2008; 149:1180–1189.
19. Chishima F, Hayakawa S, Sugita K, Kinukawa N, Aleemuzzaman S, Nemoto N, Yamamoto T, Honda M. Increased expression of cyclooxygenase-2 in local lesions of endometriosis patients. Am J Reprod Immunol 2002; 48:50–56.
20. Ota H, Igarashi S, Sasaki M, Tanaka T. Distribution of cyclooxygenase-2 in eutopic and ectopic endometrium in endometriosis and adenomyosis. Hum Reprod 2001; 16:561–566.
21. Cobellis L, Razzi S, De Simone S, Sartini A, Fava A, Danero S, Gioffre W, Mazzini M, Petraglia F. The treatment with a COX-2 specific inhibitor is effective in the management of pain related to endometriosis. Eur J Obstet Gynecol Reprod Biol 2004; 116:100–102.
22. Kim YA, Kim MR, Lee JH, Kim JY, Hwang KJ, Kim HS, Lee ES. Gonadotropin-releasing hormone agonist reduces aromatase cytochrome P450 and cyclooxygenase-2 in ovarian endometrioma and eutopic endometrium of patients with endometriosis. Gynecol Obstet Invest 2009; 68:73–81.
23. Olivares C, Bilotas M, Buquet R, Borghi M, Sueldo C, Tesone M, Meresman G. Effects of a selective cyclooxygenase-2 inhibitor on endometrial epithelial cells from patients with endometriosis. Hum Reprod 2008; 23:2701–2708.
24. Matsuzaki S, Canis M, Darcha C, Dallel R, Okamura K, Mage G. Cyclooxygenase-2 selective inhibitor prevents implantation of eutopic endometrium to ectopic sites in rats. Fertil Steril 2004; 82:1609–1615.
25. Laschke MW, Elitzsch A, Scheuer C, Vollmar B, Menger MD. Selective cyclo-oxygenase-2 inhibition induces regression of autologous endometrial grafts by down-regulation of vascular endothelial growth factor-mediated angiogenesis and stimulation of caspase-3-dependent apoptosis. Fertil Steril 2007; 87:163–171.
26. Lee J, Banu SK, Rodriguez R, Starzinski-Powitz A, Arosh JA. Selective blockade of prostaglandin E2 receptors EP2 and EP4 signaling inhibits proliferation of human endometriotic epithelial cells and stromal cells through distinct cell cycle arrest. Fertil Steril 2010; 93:2498–2506.
27. Lee J, Banu SK, Subbarao T, Starzinski-Powitz A, Arosh JA. Selective inhibition of prostaglandin E2 receptors EP2 and EP4 inhibits invasion of human immortalized endometriotic epithelial and stromal cells through suppression of metalloproteinases. Mol Cell Endocrinol 2011; 332: 306–313.
28. Osteen KG, Yeaman GR, Bruner-Tran KL. Matrix metalloproteinases and endometriosis. Semin Reprod Med 2003; 21:155–164.
29. Pitsos M, Kanakas N. The role of matrix metalloproteinases in the pathogenesis of endometriosis. Reprod Sci 2009; 16:717–726.
30. Zhou HE, Nothnick WB. The relevancy of the matrix metalloproteinase system to the pathophysiology of endometriosis. Front Biosci 2005; 10: 569–575.
31. Klemmt PA, Carver JG, Koninckx P, McVeigh EJ, Mardon HJ. Endometrial cells from women with endometriosis have increased adhesion and proliferative capacity in response to extracellular matrix components: towards a mechanistic model for endometriosis progression. Hum Reprod 2007; 22:3139–3147.
32. Regidor PA, Vogel C, Regidor M, Schindler AE, Winterhager E. Expression pattern of integrin adhesion molecules in endometriosis and human endometrium. Hum Reprod Update 1998; 4:710–718.
33. Witz CA, Montoya-Rodriguez IA, Cho S, Centonze VE, Bonewald LF, Schenken RS. Composition of the extracellular matrix of the peritoneum. J Soc Gynecol Investig 2001; 8:299–304.
34. Witz CA, Takahashi A, Montoya-Rodriguez IA, Cho S, Schenken RS. Expression of the alpha2beta1 and alpha3beta1 integrins at the surface of mesothelial cells: a potential attachment site of endometrial cells. Fertil Steril 2000; 74:579–584.
35. Askari JA, Buckley PA, Mould AP, Humphries MJ. Linking integrin conformation to function. J Cell Sci 2009; 122:165–170.
36. Harburger DS, Calderwood DA. Integrin signalling at a glance. J Cell Sci 2009; 122:159–163.
37. Humphries JD, Byron A, Humphries MJ. Integrin ligands at a glance. J Cell Sci 2006; 119:3901–3903.
38. Legate KR, Fassler R. Mechanisms that regulate adaptor binding to beta-integrin cytoplasmic tails. J Cell Sci 2009; 122:187–198.
39. Heyman L, Kellouche S, Fernandes J, Dutoit S, Poulain L, Carreiras F. Vitronectin and its receptors partly mediate adhesion of ovarian cancer cells to peritoneal mesothelium in vitro. Tumour Biol 2008; 29:231–244.
40. Kenny HA, Krausz T, Yamada SD, Lengyel E. Use of a novel 3D culture model to elucidate the role of mesothelial cells, fibroblasts and extracellular matrices on adhesion and invasion of ovarian cancer cells to the omentum. Int J Cancer 2007; 121:1463–1472.
41. Lessan K, Aguiar DJ, Oegema T, Siebenson L, Skubitz AP. CD44 and beta1 integrin mediate ovarian carcinoma cell adhesion to peritoneal mesothelial cells. Am J Pathol 1999; 154:1525–1537.
42. Strobel T, Cannistra SA. Beta1-integrins partly mediate binding of ovarian cancer cells to peritoneal mesothelium in vitro. Gynecol Oncol 1999; 73: 362–367.
43. Witz CA, Thomas MR, Montoya-Rodriguez IA, Nair AS, Centonze VE, Schenken RS. Short-term culture of peritoneum explants confirms attachment of endometrium to intact peritoneal mesothelium. Fertil Steril 2001; 75:385–390.
44. Attur MG, Dave MN, Clancy RM, Patel IR, Abramson SB, Amin AR. Functional genomic analysis in arthritis-affected cartilage: yin-yang

regulation of inflammatory mediators by alpha 5 beta 1 and alpha V beta 3 integrins. J Immunol 2000; 164:2684–2691.
45. Dormond O, Bezzi M, Mariotti A, Ruegg C. Prostaglandin E2 promotes integrin alpha Vbeta 3-dependent endothelial cell adhesion, rac-activation, and spreading through cAMP/PKA-dependent signaling. J Biol Chem 2002; 277:45838–45846.
46. Kajimoto M, Ichiyama T, Ueno Y, Shiraishi M, Hasegawa M, Furukawa S. Enhancement of activated beta1-integrin expression by prostaglandin E2 via EP receptors in isolated human coronary arterial endothelial cells: implication for the treatment of Kawasaki disease. Inflamm Res 2009; 58: 224–228.
47. Waldner C, Schror K, Heering P. COX-2 dependent PGE(2) downregulates alpha(v) integrin expression via the EP(3) receptor in cultured mesangial cells. J Clin Pathol 2004; 57:553–555.
48. Zeitvogel A, Baumann R, Starzinski-Powitz A. Identification of an invasive, N-cadherin-expressing epithelial cell type in endometriosis using a new cell culture model. Am J Pathol 2001; 159:1839–1852.
49. Banu SK, Lee J, Satterfield MC, Spencer TE, Bazer FW, Arosh JA. Molecular cloning and characterization of prostaglandin transporter in ovine endometrium: role of mitogen activated protein kinase pathways in release of prostaglandin F2 alpha. Endocrinology 2008; 149:219–231.
50. Wu Y, Starzinski-Powitz A, Guo SW. Trichostatin A, a histone deacetylase inhibitor, attenuates invasiveness and reactivates E-cadherin expression in immortalized endometriotic cells. Reprod Sci 2007; 14: 374–382.
51. Banu SK, Starzinski-Powitz A, Speights VO, Burghardt RC, Arosh JA. Induction of peritoneal endometriosis in nude mice using human immortalized endometriosis epithelial and stromal cells: a potential experimental tool to study molecular pathogenesis of endometriosis in human. Fertil Steril 2009; 91:2199–2209.
52. Banu SK, Lee J, Starzinski-Powitz A, Arosh JA. Gene expression profiles and functional characterization of human immortalized endometriotic epithelial and stromal cells. Fertil Steril 2008; 90:972–987.
53. Woodward DF, Pepperl DJ, Burkey TH, Regan JW. 6-Isopropoxy-9-oxoxanthene-2-carboxylic acid (AH 6809), a human EP2 receptor antagonist. Biochem Pharmacol 1995; 50:1731–1733.
54. Crider JY, Griffin BW, Sharif NA. Endogenous EP4 prostaglandin receptors coupled positively to adenylyl cyclase in Chinese hamster ovary cells: pharmacological characterization. Prostaglandins Leukot Essent Fatty Acids 2000; 62:21–26.
55. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72:248–254.
56. Legate KR, Wickstrom SA, Fassler R. Genetic and cell biological analysis of integrin outside-in signaling. Genes Dev 2009; 23:397–418.
57. Moser M, Legate KR, Zent R, Fassler R. The tail of integrins, talin, and kindlins. Science 2009; 324:895–899.
58. Vicente-Manzanares M, Choi CK, Horwitz AR. Integrins in cell migration—the actin connection. J Cell Sci 2009; 122:199–206.
59. Fong YC, Liu SC, Huang CY, Li TM, Hsu SF, Kao ST, Tsai FJ, Chen WC, Chen CY, Tang CH. Osteopontin increases lung cancer cells migration via activation of the alphavbeta3 integrin/FAK/Akt and NF-kappaB-dependent pathway. Lung Cancer 2009; 64:263–270.
60. Thamilselvan V, Craig DH, Basson MD. FAK association with multiple signal proteins mediates pressure-induced colon cancer cell adhesion via a Src-dependent PI3K/Akt pathway. FASEB J 2007; 21:1730–1741.
61. Webb DJ, Donais K, Whitmore LA, Thomas SM, Turner CE, Parsons JT, Horwitz AF. FAK-Src signalling through paxillin, ERK and MLCK regulates adhesion disassembly. Nat Cell Biol 2004; 6:154–161.
62. Giannelli G, Sgarra C, Di Naro E, Lavopa C, Angelotti U, Tartagni M, Simone O, Trerotoli P, Antonaci S, Loverro G. Endometriosis is characterized by an impaired localization of laminin-5 and alpha3beta1 integrin receptor. Int J Gynecol Cancer 2007; 17:242–247.
63. Koks CA, Groothuis PG, Dunselman GA, de Goeij AF, Evers JL. Adhesion of menstrual endometrium to extracellular matrix: the possible role of integrin alpha(6)beta(1) and laminin interaction. Mol Hum Reprod 2000; 6:170–177.
64. Lessey BA, Castelbaum AJ. Integrins and implantation in the human. Rev Endocr Metab Disord 2002; 3:107–117.
65. Lessey BA, Castelbaum AJ, Wolf L, Greene W, Paulson M, Meyer WR, Fritz MA. Use of integrins to date the endometrium. Fertil Steril 2000; 73: 779–787.
66. Rai V, Hopkisson J, Kennedy S, Bergqvist A, Barlow DH, Mardon HJ. Integrins alpha 3 and alpha 6 are differentially expressed in endometrium and endometriosis. J Pathol 1996; 180:181–187.
67. Sillem M, Prifti S, Monga B, Arslic T, Runnebaum B. Integrin-mediated adhesion of uterine endometrial cells from endometriosis patients to extracellular matrix proteins is enhanced by tumor necrosis factor alpha (TNF alpha) and interleukin-1 (IL-1). Eur J Obstet Gynecol Reprod Biol 1999; 87:123–127.
68. Lerea KM, Venjara AY, Olson SC, Kelly MR. Threonine phosphorylation of integrin beta3 in calyculin A-treated platelets is selectively sensitive to 5′-iodotubercidin. Biochim Biophys Acta 2007; 1773:185–191.
69. Kirk RI, Sanderson MR, Lerea KM. Threonine phosphorylation of the beta 3 integrin cytoplasmic tail, at a site recognized by PDK1 and Akt/PKB in vitro, regulates Shc binding. J Biol Chem 2000; 275:30901–30906.
70. Wegener KL, Partridge AW, Han J, Pickford AR, Liddington RC, Ginsberg MH, Campbell ID. Structural basis of integrin activation by talin. Cell 2007; 128:171–182.
71. Law DA, DeGuzman FR, Heiser P, Ministri-Madrid K, Killeen N, Phillips DR. Integrin cytoplasmic tyrosine motif is required for outside-in alphaIIbbeta3 signalling and platelet function. Nature 1999; 401:808–811.
72. Sakai T, Zhang Q, Fassler R, Mosher DF. Modulation of beta1A integrin functions by tyrosine residues in the beta1 cytoplasmic domain. J Cell Biol 1998; 141:527–538.
73. Buchanan FG, Wang D, Bargiacchi F, DuBois RN. Prostaglandin E2 regulates cell migration via the intracellular activation of the epidermal growth factor receptor. J Biol Chem 2003; 278:35451–35457.
74. Pierce KL, Lefkowitz RJ. Classical and new roles of beta-arrestins in the regulation of G-protein-coupled receptors. Nat Rev Neurosci 2001; 2: 727–733.
75. Luttrell LM, Ferguson SS, Daaka Y, Miller WE, Maudsley S, Della Rocca GJ, Lin F, Kawakatsu H, Owada K, Luttrell DK, Caron MG, Lefkowitz RJ. Beta-arrestin-dependent formation of beta2 adrenergic receptor-Src protein kinase complexes. Science 1999; 283:655–661.