Regulation of tight junctions by sex hormones in normal human endometrial epithelial cells and uterus cancer cell line Sawano

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Abstract The number of patients with uterine endometrial carcinoma, the cause of which involves sex hormones, has recently been growing rapidly because of increases in life expectancy and obesity. Tight junction proteins claudin-3 and −4 are receptors of *Clostridium perfringens* enterotoxin (CPE) and increase during endometrial carcinogenesis. In the present study of normal human endometrial epithelial (HEE) cells and the uterus cancer cell line Sawano, we investigate changes in the expression of tight junction proteins including claudin-3 and −4, the fence and barrier functions of the tight junction and the cytotoxic effects of CPE by sex hormones. In primary cultured HEE cells, treatment with progesterone (P4) but not estradiol (E2), induced claudin-1, −3, −4 and −7 and occludin, together with the downregulation of the barrier function but not the fence function. In Sawano cells, claudin-3 and −4 were upregulated by E2 but not by P4, together with a disruption of both the barrier and fence function. In primary cultured HEE cells, claudin-3 and −4 were localized at the apicalmost regions (tight junction areas) and no cytotoxicity of CPE was observed. In Sawano cells, claudin-3 and −4 were found not only in the apicalmost regions but also at the basolateral membrane and the cytotoxicity of CPE was enhanced by E2. Thus, tight junctions are physiological regulated by sex hormones in normal HEE cells during the menstrual cycle suggesting that safer and more effective therapeutic methods targeting claudins in uterine cancer can be developed.

Keywords Endometrial epithelial cells · Uterus · Cancer · Tight junctions · Estrogen · Progesterone · Signal transduction · Human

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Introduction

Uterine endometrial carcinoma is one of the most common malignancies in the female genital tract and the number of patients with this disease has recently been growing rapidly (Duong et al. 2011; Duncan et al. 2012). This is attributed to changes of reproductive behavior (Evans et al. 2011), improvement in life expectancy, increased obesity (Bray et al. 2005) and the spread of hormone-replacement therapy (Parkin 2011). Accordingly, useful new biomarkers for diagnosis and

new molecular targets for therapeutic approaches must be developed to improve the poor conventional outcome of the disease.

In several human cancers, including endometrial cancer, some tight junction protein claudins are abnormally regulated and therefore represent promising molecular targets for diagnosis and therapy (Pan et al. 2007; Gadducci et al. 2008; Szabó et al. 2009). The claudin family, which consists in at least 27 members, is solely responsible for the formation of tight junction strands and for the regulation of tight junction barrier and fence functions (van Meer et al. 1986; Schneeberger and Lynch 1992; Gumbiner 1993; Cereijido et al. 1998; Mineta et al. 2011). In addition, the downregulation of the fence function contributes to the loss of epithelial cell polarity in cancer (Tokunaga et al. 2007).

The upregulation of claudin-3 and −4, which are the receptors of *Clostridium perfringens* enterotoxin (CPE), is observed during endometrial carcinogenesis (Pan et al. 2007). The 35-kDa polypeptide CPE binds to its receptor and then the CPE causes changes in membrane permeability via the formation of a complex on the plasma membrane followed by the induction of apoptosis (McClane and Chakrabarti 2004). The development of a novel tumor-targeted therapy for uterine cancers is anticipated by using a claudin-targeting molecule (Santin et al. 2007; Gadducci et al. 2008). However, the regulatory mechanisms of claudin-based tight junctions remain unknown, even in normal human endometrial epithelial (HEE) cells. Thus, analyses of the regulation of tight junction molecules, including claudins, in normal HEE cells in vitro are essential to develop safer and more effective diagnostic and therapeutic methods targeting claudins in uterine cancer.

Normal HEE cells display a typical polarized phenotype during the proliferative phase of the menstrual cycle, which is controlled by 17β-estradiol (E2) and during the secretory phase, which is predominantly governed by progesterone (P4), when the epithelial cells become receptive for implantation (Lessey 1998). The redistribution of desmosomes and adherens junctions in HEE cells in vivo is observed during the menstrual cycle (Buck et al. 2012). On the other hand, inhibition of the mitotic activity of estrogen exerted by P4 is known to be of clinical relevance as unopposed or increased estrogen action in women is a well-established risk factor for endometrial cancer (Key and Pike 1988). However, the mechanisms of sex hormonal regulation of tight junctions in normal HEE cells and uterine endometrial cancers remain unknown.

In the present study, primary cultured HEE cells and the uterine cancer cell line Sawano have been treated with E2 and P4 in order to investigate the detailed mechanisms of the sex hormonal regulation of tight junctions in normal HEE cells and uterine endometrial cancer cells. We have also investigated changes in the expression of tight junction proteins, including claudin-3 and −4, the fence and barrier functions of the tight junction and the cytotoxic effects of CPE.

Materials and methods

Antibodies, activators and inhibitors

Rabbit polyclonal anti-occludin and anti-claudin-1, −3, −4 and −7 antibodies and mouse monoclonal anti-occludin (OC-3F10) and anti-claudin-4 (3E2C1) antibodies were obtained from Zymed Laboratories (San Francisco, Calif., USA). A rabbit polyclonal anti-actin antibody was obtained from Sigma-Aldrich (St. Louis, Mo., USA). A mouse monoclonal anti-E-cadherin (36/E-cadherin) antibody was obtained from BD Biosciences Pharmingen (San Diego, Calif., USA). Mouse monoclonal anti-cytokeratin7 (CK7, OV-TL 12/13) and anti-bromodeoxyuridine (BrdU) antibodies and horseradish-peroxidase-conjugated anti-rabbit and anti-mouse IgG were obtained from Dako (Tokyo, Japan). A rabbit polyclonal anti-vimentin (H-84) antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, Calif., USA). Alexa-Fluor-594-phalloidin, Alexa-Fluor-488 (green)-conjugated anti-rabbit and anti-mouse IgG and Alexa-Fluor-594 (red)-conjugated anti-rabbit and anti-mouse IgG antibodies were purchased from Molecular Probes (Eugene, Ore., USA). E2 and P4 were purchased from Sigma. Mitogen-activated protein kinase (MAPK) inhibitor U0126, phosphatidyl inositol 3-kinase (PI3K) inhibitor LY294002, hedgehog inhibitor cyclopamine and glycogen synthase kinase-3β (GSK-3β) inhibitor SB216763 were purchased from Calbiochem-Novabiochem (San Diego, Calif., USA).

Isolation and culture of HEE cells and stromal cells

Human endometrial tissues were obtained from patients with benign diseases (e.g., myoma uteri or adenomyosis) who underwent a hysterectomy at the Sapporo Medical University hospital. Informed consent was obtained from all patients and the study was approved by the ethics committee of Sapporo Medical University. Human endometrial tissues were minced into pieces of 2 to 3 mm^3 in volume and washed with phosphate-buffered saline (PBS) containing 100 U/ml penicillin and 100 μg/ml streptomycin (Lonza Walkersville, Walkersville, Md., USA) three times. These minced tissues were digested in 10 ml Hanks' balanced salt solution with 0.5 μg/ml DNase I and 0.04 mg/ml Liberase (Roche, Basel, Switzerland) and then incubated, with the bubbling of mixed O_2 gas containing 5.2 % CO_2, at 37 °C for 20–30 min. The dissociated tissues were subsequently filtered with 300-μm mesh followed by filtration with 40-μm

mesh (Cell Strainer, BD Biosciences, San Jose, Calif., USA). Stromal cells were removed by filtration and the remaining cells were backwashed and collected as epithelial cells. After centrifugation at 1000 g for 2 min, isolated cells were cultured in bronchial epithelial basal medium (BEBM, Lonza Walkersville) containing 4 % fetal bovine serum (FBS; CCB, Nichirei Bioscience, Tokyo, Japan) and supplemented with BEGM SingleQuots (Lonza Walkersville; including 0.4 % bovine pituitary extract, 0.1 % insulin, 0.1 % hydrocortisone, 0.1 % gentamicin, amphotericin-B [GA-1000], 0.1 % retinoic acid, 0.1 % transferrin, 0.1 % triiodothyronine, 0.1 % epinephrine, 0.1 % human epidermal growth factor), 100 U/ml penicillin, 100 µg/ml streptomycin and 50 µg/ml amphotericin-B on 35- and 60-mm culture dishes or 24-well tissue culture plates (Corning Glass Works, Corning, N.Y., USA) that had been coated with rat tail collagen (500 µg dried tendon/ml 0.1 % acetic acid). Following the above protocol, tissue dissociation and cell isolation were repeated for the same sample three or four times. The cells were placed in a humidified 5 % CO_2:95 % air incubator at 37 °C.

The epithelial cells were treated with P4 and/or E2. Some cells were pretreated with the inhibitors U0126, LY294002, cyclopamine and SB216763 at 30 min before treatment with P4.

Cell line culture and treatment

The human endometrioid endometrial cancer cell line Sawano (RCB1152) was purchased from RIKEN Bio-Resource Center (Tsukuba, Japan). Sawano cells were maintained with minimal essential medium (MEM; Sigma-Aldrich) supplemented with 15 % dialyzed FBS (Invitrogen, Carlsbad, Calif., USA). The medium contained 100 U/ml penicillin, 100 µg/ml streptomycin and 50 µg/ml amphotericin-B. Sawano cells were plated on 35- and 60-mm culture dishes, which were coated with rat tail collagen (500 µg dried tendon/ml in 0.1 % acetic acid) and incubated in a humidified 5 % CO_2 incubator at 37 °C.

The Sawano cells were then treated with P4 and/or E2. Some cells were pretreated with the inhibitors U0126, LY294002, cyclopamine and SB216763 30 min before treatment with E2.

Immunohistochemistry

Human endometrial tissues from the proliferative and secretory phases were frozen in Neg-50 (Richard-Allan Scientific, Kalamazoo, Mich., USA). Serial sections (7–8 µm thick) were cut with a cryostat (Leica CM1850, Heidelberg, Germany) and placed on MAS-coated slides (Matsunami, Tokyo, Japan). The sections were incubated with rabbit polyclonal claudin-1, −3, or −7 antibodies or mouse monoclonal claudin-4 or occludin antibodies (1:100 dilution) at

room temperature for 1 h. After being washed with PBS, the sections were incubated with Alexa-488 (green)-conjugated anti-rabbit IgG or Alexa-584 (red)-conjugated anti-mouse IgG antibodies (1:200) at room temperature for 1 h. 4,6-Diamidino- 2-phenylindole (DAPI; Sigma-Aldrich) was used for counterstaining of nuclei in the cells. The specimens were examined by using an epifluorescence microscope (Olympus, Tokyo, Japan).

Transmission electron microscopy

The tissues were fixed in 2.5 % glutaraldehyde/0.1 M PBS (pH 7.3) overnight at 4 °C, postfixed in 2 % osmium tetroxide in the same buffer, dehydrated in a graded ethanol series and embedded in Epon 812. Ultrathin sections were then cut on a Sorvall Ultramicrotome MT-5000. The sections were stained with uranyl acetate followed by lead citrate and examined at 80 kV with a transmission electron microscope (H7500; Hitachi, Tokyo, Japan).

RNA isolation, reverse transcription plus the polymerase chain reaction and real-time poolymerase chain reaction analysis

Total RNA was extracted and purified by using TRIzol (Invitrogen). Total RNA (1 µg) was reverse-transcribed into cDNA by using a mixture of oligo (dT) and Superscript II reverse transcriptase according to the manufacturer's recommendations (Invitrogen). Synthesis of each cDNA was performed in a total volume of 20 µl for 50 min at 42 °C and terminated by incubation for 15 min at 70 °C. The polymerase chain reaction (PCR) was performed in the 20-µl total mixture containing 100 pM primer pairs, 1.0 µl of the 20-µl total reverse transcription (RT) product, PCR buffer, dNTPs and Taq DNA polymerase according to the manufacturer's recommendations (Takara, Kyoto, Japan). Amplifications were carried out for 25–40 cycles, depending on the PCR primer pair, with cycle times of 15 s at 96 °C, 30 s at 55 °C and 60 s at 72 °C. The final elongation time was 7 min at 72 °C. Of the total 20-µl PCR product, 7 µl was analyzed by 1 % agarose gel electrophoresis with ethidium bromide staining and standardized by using a GeneRuler 100-bp DNA ladder (Fermentas, Ontario, Canada). The PCR primers used for claudin-1, −3, −4 and −7, occludin, estrogen receptor alpha (ER-alpha), progesterone receptor (PR) and glucose-3-phosphate dehydrogenase (G3PDH) by RT-PCR had the following sequences: claudin-1 (sense 5′-AACGCGGGGCTGCAGCTGTTG-3′ and anti-sense 5′-GGATAGGGCCTTGGTGTTGGGT-3′), claudin-3 (sense 5′-TGCTGTTCCTTCTCGCCGCC-3′ and antisense 5′-CTTAGACGTAGTCCTTGCGG-3′), claudin-4 (sense 5′-AGCCTTCCAGGTCCTCAACT-3′ and antisense 5′-AGCAGCGAGTCGTACACCTT-3′), claudin-7 (sense 5′-AGCATAATTTTCATCGTGG-3′ and antisense 5′-

GAGTTGGACTTAGGGTAAGAGCG-3′), occludin (sense 5′-TCAGGGAATATCCACCTATCACTTCAG-3′ and anti-sense 5′-CATCAGCAGCAGCCATGTACTCTTCAC-3′), ER-α (sense 5′-GCAGACAGGGAGCTGGTTCA-3′ and antisense 5′-AGAGATGCTCCATGCCTTTG-3′), PR (sense 5′-TTACCATGTGGCAGATCCCACAG-3′ and antisense 5′-ACCATCCCTGCCAATATCTTGGG-3′) and G3PDH (sense 5′-ACCACAGTCCATGCCATCAC-3′ and antisense 5′-TCCACCACCCTGTTGCTGTA-3′).

Real-time PCR detection was performed by using a TaqMan Gene Expression Assay kit with a StepOnePlus real-time PCR system (Applied Biosystems, Foster City, Calif., USA). The amount of 18S ribosomal RNA (rRNA) mRNA (Hs99999901) in each sample was used to standardize the quantity of the mRNAs of claudin-3 (Hs00265816) and claudin-4 (Hs00533616). The relative mRNA-expression levels between the control and treated samples were calculated by the difference of the threshold cycle [comparative C_T ($\Delta\Delta C_T$) method] and presented as the average of triplicate experiments with a 95 % confidence interval.

Western blot analysis

For Western blot of total cell lysates, the dishes were washed with PBS, after which 300 μl sample buffer (1 mM NaHCO$_3$, 2 mM phenylmethylsulfonylfluoride) was added to the 60-mm culture dishes. The cells were scraped and collected in microcentrifuge tubes and then sonicated for 10 s. The protein concentrations of samples were determined by using a BCA Protein Assay Reagent Kit (Pierce Chemical, Rockford, Ill., USA). Aliquots of 15 μg protein/lane for each sample were separated by electrophoresis in 4–20 % SDS polyacrylamide gels (Cosmo Bio, Tokyo, Japan). After electrophoretic transfer to nitrocellulose membranes (Immobilon; Millipore, Billerica, Mass., USA), the membranes were saturated with blocking buffer (TRIS-buffered saline [TBS] with 0.1 % Tween 20 and 4 % skim milk) for 30 min at room temperature and incubated with rabbit polyclonal anti-occludin, anti-claudin-1, −3, −4, or −7 (1:1000) antibodies or mouse monoclonal anti-E-cadherin (1:2000) antibody for 1 h at room temperature. The membranes were incubated with horseradish-peroxidase-conjugated anti-rabbit and anti-mouse IgG at room temperature for 1 h. The immunoreactive bands were detected by using an enhanced chemiluminence Western blotting analysis system (GE Healthcare, Little Chalfont, UK).

Immunocytochemistry

The cells were grown on 35-mm glass-base dishes (Iwaki, Chiba, Japan) coated with rat tail collagen and incubated with each medium. They were then fixed with cold acetone and ethanol (1:1) at 20 °C for 10 min. After being washed with PBS, the cells were incubated with mouse monoclonal anti-occludin, anti-claudin-4, anti-cytokeratin-7, anti-vimentin, or anti- E-cadherin, or rabbit polyclonal anti-claudin-1 or −3 antibodies (1:100) at room temperature for 1 h and then with Alexa-Fluor-488 (green)-conjugated anti-rabbit IgG (1:200), Alexa-Fluor-594 (red)-conjugated anti-mouse IgG (1:200), or Alexa-Fluor-594 (red)-phalloidin (1:100) at room temperature for 1 h. Nuclei in the cells were counterstained with DAPI (Sigma-Aldrich). The specimens were examined by using an epifluorescence microscope (Olympus, Tokyo, Japan) or a confocal laser scanning microscope (LSM510; Carl Zeiss, Jena, Germany).

BrdU assay

To label cells in the S phase, BrdU (3 μg/ml, BD Biosciences) was added to the medium for 2 h. The cells were fixed with cold acetone and ethanol (1:1) at 20 °C for 10 min. After being rinsed in PBS, the cells were preincubated with 2 N HCl at room temperature for 30 min before incubation with mouse monoclonal anti-BrdU (1:100) or rabbit polyclonal anti-claudin-3 (1:100) antibodies at room temperature for 1 h. The cells were incubated with Alexa-Fluor-488 (green)-conjugated anti-mouse IgG (1:200) or Alexa-Fluor-594 (red)-conjugated anti-rabbit IgG (1:200) at room temperature for 1 h. Nuclei in the cells were counterstained with DAPI (Sigma-Aldrich). The BrdU-positive nuclei were counted by using an epifluorescence microscope (Olympus).

Continuous on-line measurements of transepithelial electrical resistance

Cells were cultured to confluence on the inner chambers of 12-mm Transwell 0.4-μm pore-size filters (Corning Life Science). Transepithelial electrical resistance (TER) was monitored by using a cellZscope (nanoAnalytics, Germany), a computer-controlled automated multiwell device (24 wells). The values are expressed in standard units of ohms per square centimeter and are presented as the mean ± SD of triplicate experiments.

Measurement of permeability

To determine the paracellular flux, the cells were cultured on 12-mm Transwell 0.4-μm pore-size filters (Corning) and then a medium containing fluorescein isothiocyanate (FITC)-labeled dextran (MW: 4 kDa, Sigma) was added to the inner chamber. Samples were collected from the outer chamber at 60 min and measured with a Wallac 1,420 multilabeled counter (PerkinElmer, Turku, Finland).

Diffusion of BODIPY-sphingomyelin

For measurement of the tight junctional fence function, we used diffusion of BODIPY (boron-dipyrromethene)-sphingomyelin

with some modification. Sphingomyelin/bovine serum albumin (BSA) complexes (5 nM) were prepared in P buffer (10 nM HEPES, pH 7.4, 1 mM sodium pyruvate, 10 mM glucose, 3 mM $CaCl_2$, 145 mM NaCl) by using a BODIPY fluorophore conjugated to sphingomyelin (BODIPY-FL-sphingomyelin; Molecular Probes) and defatted BSA. Cells plated on glass-bottomed microwell plates (Mat Tek, Ashland, Mass., USA) were loaded with BODIPY-sphingomyelin/BSA complex for 2 min on ice, after which time they were rinsed with cold MEM and mounted in MEM on a glass slide. The samples were analyzed by using a confocal laser scanning microscope (LSM510; Carl Zeiss, Jena, Germany). All micrographs shown were generated within the first 5 min of analysis.

CPE treatment and MTT assay

For purification of CPE, the bacterial DNA fragment encoding the full-length CPE gene was amplified by PCR (primer 1, 5′-AGATGTTAATCATATGATGCTTAGTAACAATTTAAATCC-3′; primer 2, 5′-AGGATCCTTAAAATTTTTGAAATAATATTAATAAGGG-3′). The PCR products were digested with the restriction enzymes *Nde*I/*Bam*HI and

Fig. 1 Immunohistochemical staining for cytokeratin-7 (**a**), claudin-1 (**c**, **h**), claudin−3 (**d**, **i**), claudin−4 (**e**, **j**), claudin −7 (**f**, **k**) and occludin (**g**, **l**) in human endometrium in the proliferative and secretory phases (*CLDN* claudin, *OCLN* occludin, *CK7* cytokeratin-7). *Bars* 50 μm. Transmission electron microscopic (TEM) image (**b**) in human endometrium in the proliferative phase. *Bar* 250 nm
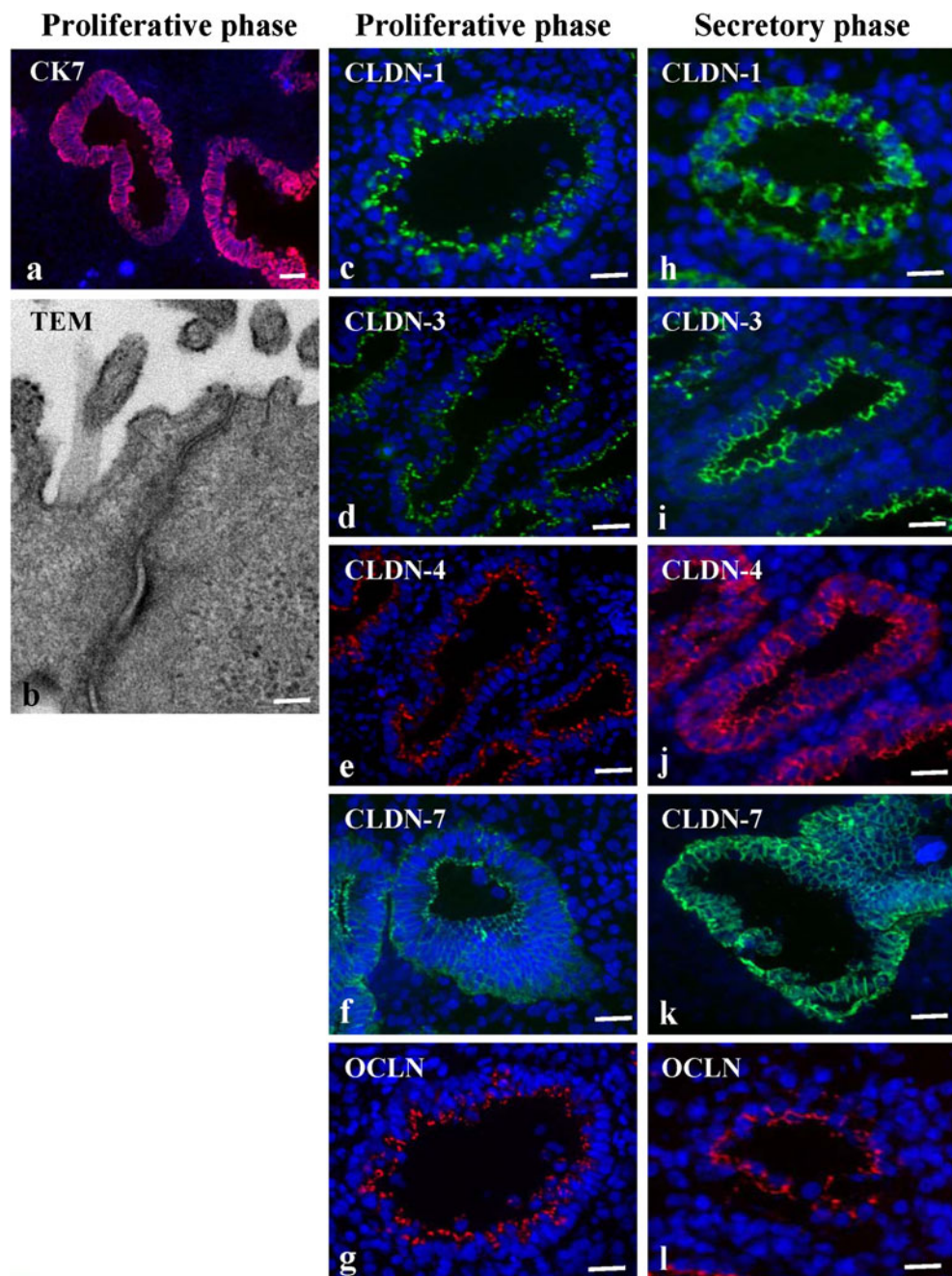
cloned into an *Nde*I/*Bam*HI-digested pet 16 (Novagen) expression vector to generate an in-frame NH2-terminus His-tagged CPE expression plasmid, pet 16-(His) 5-CPE. His-tagged CPE toxin was prepared from pet 16-(His) 5-CPE-transformed *Escherichia coli* BL-21 (DE 3). Transformed bacteria were grown overnight at 37 °C, after which CPE protein expression was induced for 3 h with 1 mM isopropyl β-D-thio-galactoside. The cells were then harvested, resuspended in 20 mM TRIS-HCl (pH 7.9) binding buffer and lysed by sonication. The fusion protein was

isolated from the supernatant on a His-Bind column (Novagen). After treatment with 10-column volumes of binding buffer and six-column volumes of wash buffer, His-tagged CPE was eluted with 0.5 M NaCl, 20 mM TRIS-HCl (pH 7.9) and dialyzed (Mr 3,500 cutoff dialysis tubing) against PBS overnight. The cells plated on 24-well tissue-culture plates (BD Labware, Franklin Lakes, N.J., USA) were treated with 0.5–2 µg/ml CPE for 1 h. Cell survival was evaluated with the colorimetric assay by using an MTT Cell Growth Assay Kit (Millipore) according to

Fig. 2 Phase-contrast (**a**, **b**) and immunocytochemical staining for cytokeratin-7 (**c**) and vimentin (**d**) in human endometrial epithelial (HEE) cells (**a**, **c**) and stromal cells (**b**, **d**) in primary culture. *Bars* 10 µm. Reverse transcription with the polymerase chain reaction (RT-PCR), (**e**) for claudin-1, −3, −4 and −7, occludin, estrogen receptor alpha, progesterone receptor and glucose-3-phosphate dehydrogenase in HEE cells and stromal cells in primary culture. Western blotting (**f**) for claudin-1, −3, −4 and −7, occludin, E-cadherin and actin in HEE cells in primary culture. Immunocytochemical staining for claudin-1 (**g**), claudin−3 (**h**), claudin−4 (**i**), claudin−7 (**j**), occludin (**k**) and E-cadherin (**l**) in HEE cells in primary culture. *Bars* 20 µm (*Epi* epithelial cells, *Str* stromal cells, *CK7* cytokeratin-7, *CLDN* claudin, *OCLN* occludin, *E-cad* E-cadherin, *ERα* estrogen receptor alpha, *PR* progesterone receptor, *G3PDH* glucose-3-phosphate dehydrogenase)
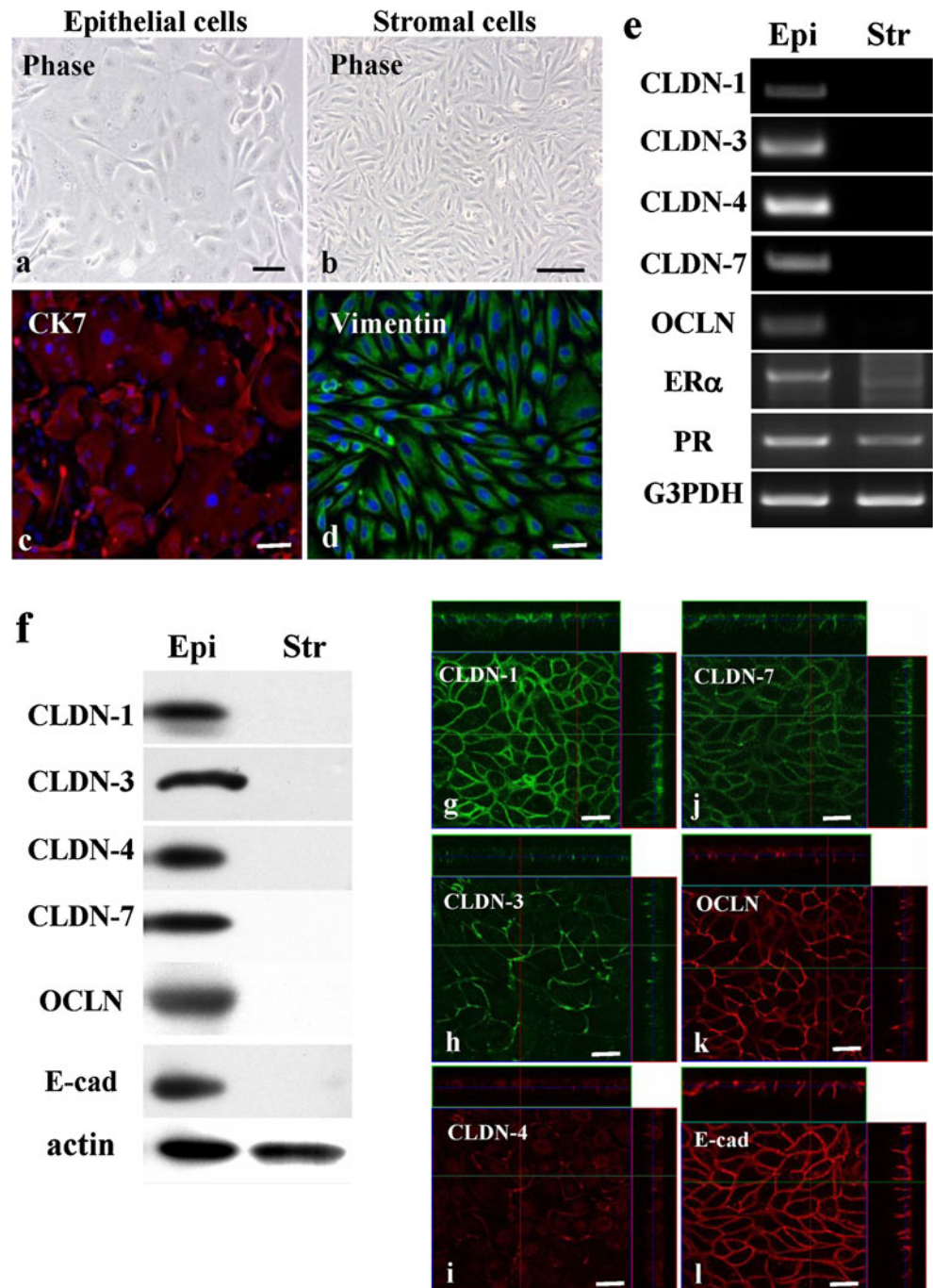
the manufacturer's recommendations. The ratio of absorbance was calculated and presented as the mean±SD of triplicate experiments.

Data analysis

Signals were quantified by using Scion Image Beta 4.02 Win (Scion, Frederick, Mass., USA). Each set of results shown is representative of at least three separate experiments. Results are given as means ± SE. Differences between groups were tested by analysis of variance followed by a post-hoc test and an unpaired two-tailed Student's t-test and considered to be significant when $P<0.05$.

Results

Distribution of tight junction proteins in the proliferative and secretory phases of HEE cells

Adherens junction proteins in HEE cells are known to be redistributed during the menstrual cycle (Buck et al. 2012). To investigate the distribution of tight junction proteins in HEE cells during the menstrual cycle, we performed immunostaining for claudin-1, −3, −4 and −7 and occludin.

In the proliferative phase of HEE cells, in which CK7 was detected and tight junction structures were observed (Fig. 1a, b), claudin-1, −3 and −4 and occludin were localized in the

Fig. 3 Western blotting (**a**) for claudin-1, −3, −4 and −7, occludin and actin in HEE cells treated with 100 μM 17β-estradiol (*E2*) and/or 100 μM progesterone (*P4*) for 24 h (*C* control). Western blotting (**b**) for claudin-1, −3, −4, −7, occludin and actin in HEE cells treated with 1–100 μM P4 for 24 h. Immunocytochemical staining for claudin-1 (**c**, **d**), claudin−3 (**e**, **f**), claudin−4 (**g**, **h**), claudin−7 (**i**, **j**), occludin (**k**, **l**) and F-actin (**m**, **n**) in HEE cells treated with 100 μM P4 for 24 h. *Bars* 40 μm (*CLDN* claudin, *OCLN* occludin)
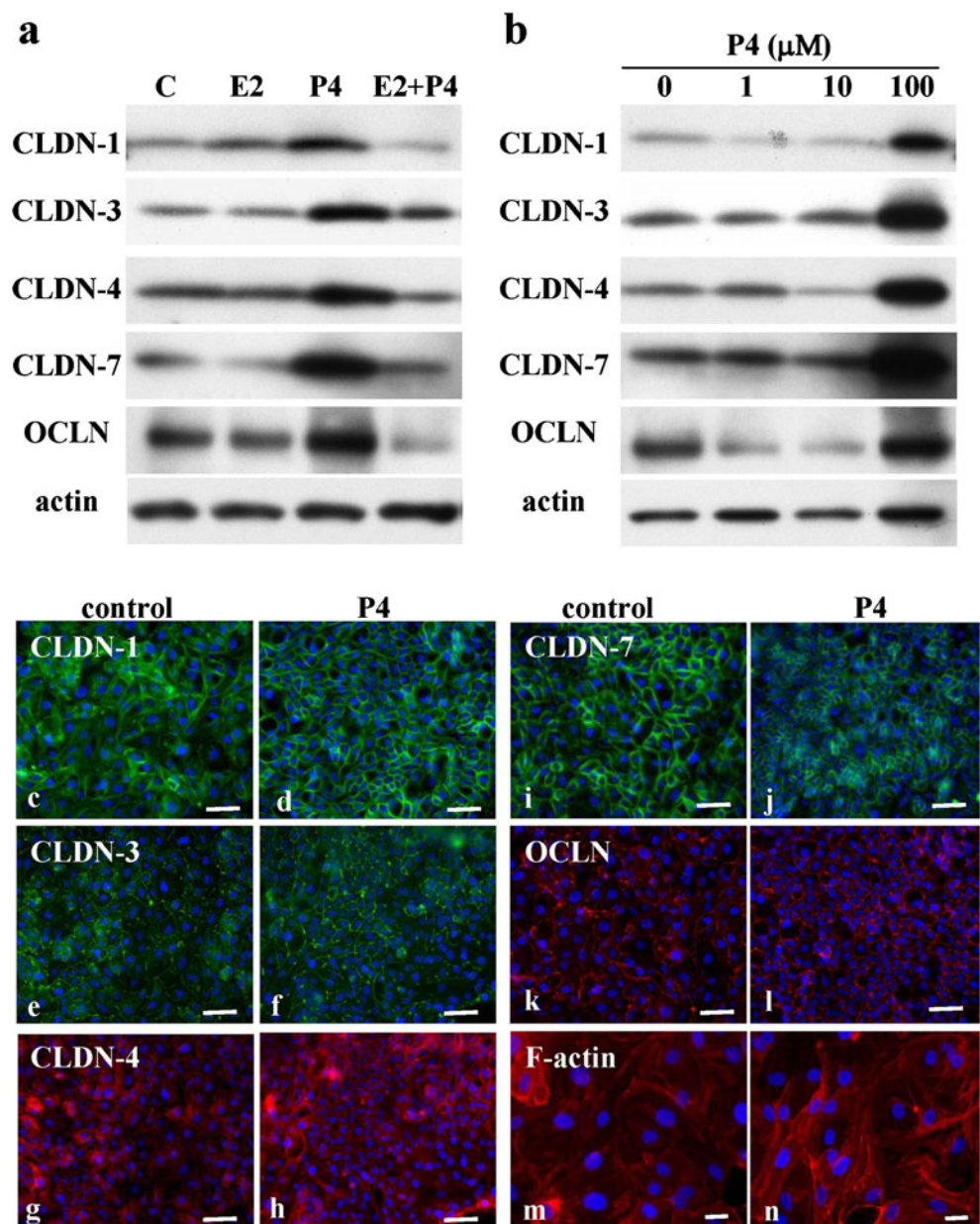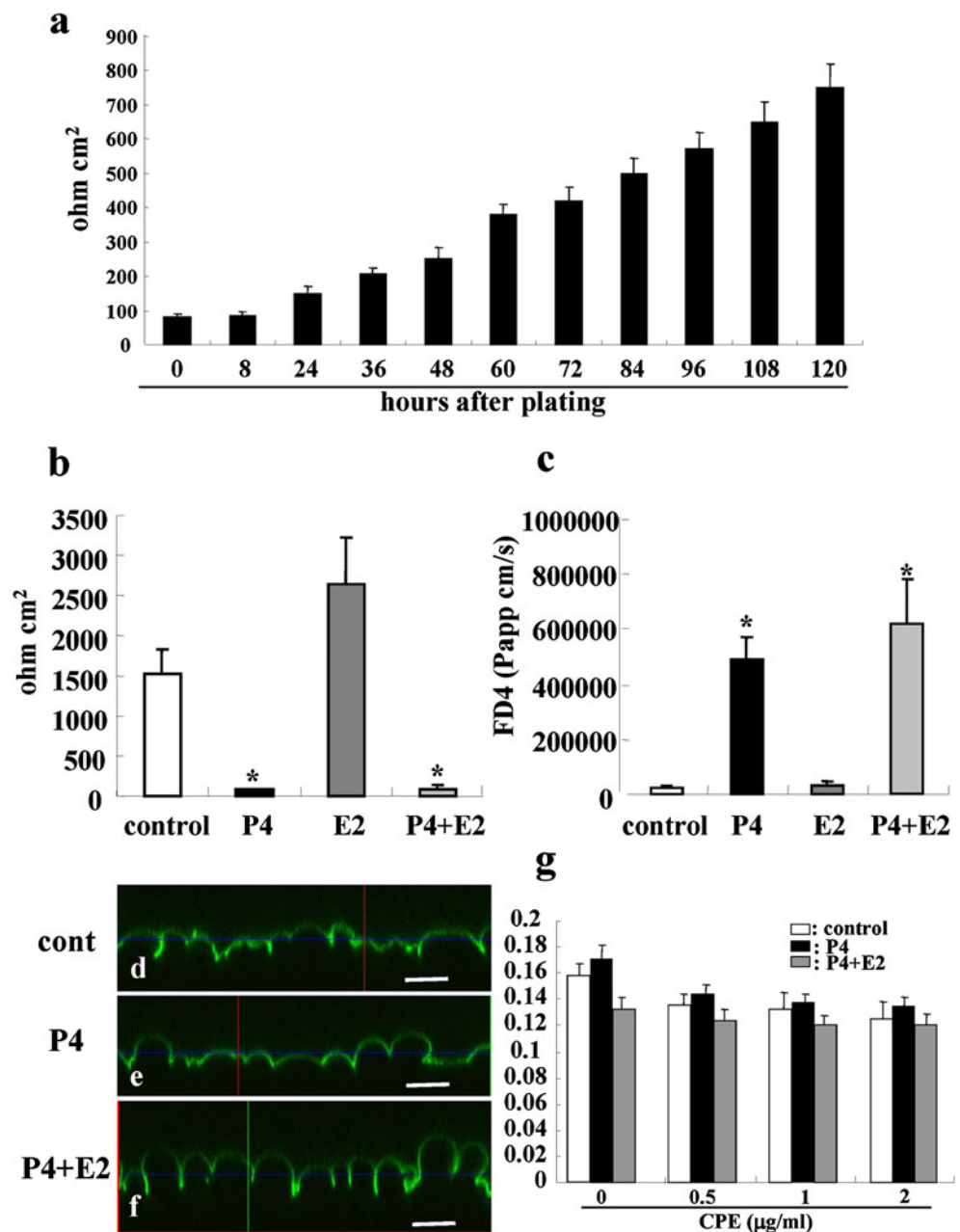
subapical region of the cells, whereas claudin-7 was observed not only in the subapical region but also throughout the lateral membrane (Fig. 1c–g). In the secretory phase, claudin-1, −3 and −4 were redistributed to the lateral membrane, whereas occludin was maintained in the subapical region (Fig. 1h–l).

Characterization of HEE cells and stromal cells in primary culture

To investigate the regulation of tight junctions in HEE cells, we isolated and cultured epithelial cells and stromal cells from human endometrium. The epithelial cells showed a polygonal pattern and expressed CK7 as an epithelial marker but not vimentin as a mesenchymal cell marker, whereas the stromal cells were spindle-shaped like fibroblasts and expressed vimentin but not CK7 (Fig. 2a–d). In RT-PCR, mRNAs of claudin-1, −3, −4 and −7 and occludin were detected in the epithelial cells but not the stromal cells, whereas mRNAs of ERα and PR were detected in both epithelial cells and stromal cells (Fig. 2)e. In Western blots, proteins claudin-1, −3, −4 and −7, occludin and E-cadherin were strongly expressed in the epithelial cells, whereas in the stromal cells, none of these proteins was expressed (Fig. 2f). Immunostaining of the epithelial cells revealed that claudin-1 and −7 and E-cadherin were distributed throughout the

Fig. 4 Transepithelial electrical resistance (TER) values (a) after plating of HEE cells in primary culture. TER values (b) and paracellular flux (c) by using fluorescein isothiocyanate (FITC)-dextran 4 kDa (FD4) in HEE cells treated with 100 μM E2 and/or 100 μM P4 for 24 h. *P<0.01. Fence function (d–f) examined by diffusion of labeled BODIPY-sphingomyelin in HEE cells treated with 100 μM E2 and/or 100 μM P4 for 24 h (cont control). Bars 20 μm. MTT assay (g) after 0.5–2 μg/ml Clostridium perfringens enterotoxin (CPE) for 2 h in HEE cells treated with 100 μM E2 and/or 100 μM P4 for 24 h

lateral membrane, whereas claudin-3 and −4 and occludin were localized at the apicalmost region (Fig. 2g–l).

Upregulation of tight junction proteins by P4 but not E2 in HEE cells

To investigate the changes of tight junction proteins induced by P4 and/or E2 in HEE cells, the epithelial cells were treated with 1–100 µM P4 and/or 100 µM E2 for 24 h. In Western blots, proteins claudin-1, −3, −4 and −7 and occludin were increased by treatment with 100 µM P4, whereas no change was observed after treatment with 100 µM E2 (Fig. 3a, b). Upregulation of the tight junction proteins by P4 was inhibited by E2 (Fig. 3a). In immunostaining, expression of claudin-1, −3, −4 and −7 and occludin was increased at the cell borders

Fig. 5 Immunocytochemical staining for bromodeoxyuridine (*BrdU*) and claudin-3 (**a**, **c**, **e**) in HEE cells treated with 100 µM E2 and/or 100 µM P4 for 24 h. 4,6-Diamidino- 2-phenylindole (*DAPI*) counterstaining of nuclei (**b**, **d**, **f**). *Bars* 100 µm (*CLDN* claudin). **g** Bar graph of the labeling index of BrdU. *$P < 0.01$

by treatment with 100 µM P4 (Fig. 3c–l). Furthermore, stress fibers indicated by F-actin staining were also increased by treatment with 100 µM P4 (Fig. 3m, n).

Changes of tight junction functions induced by P4 in HEE cells

To investigate the alterations of the barrier function of tight junctions induced by P4 and/or E2 in HEE cells, the epithelial cells were examined for TER and the paracellular flux of FITC-labeled dextran 4 kDa (FD4) after treatment with 100 µM P4 and/or 100 µM E2 for 24 h. The value of TER gradually increased after plating and reached 685 ± 90 ohm-cm^2 at 120 h after treatment (Fig. 4a). TER was decreased and FD4 was increased by treatment with 100 µM P4 for 24 h, whereas no change was induced by treatment with 100 µM E2 (Fig. 4b, c). The changes of TER and FD4 caused by treatment with P4 were not inhibited by treatment with E2 (Fig. 4b, c).

To investigate the changes of the fence function of tight junctions induced by P4 and/or E2 in HEE cells, BODIPY-sphingomyelin diffusion in the membrane was measured in the epithelial cells after treatment with 100 µM P4 and/or 100 µM E2 for 24 h. The BODIPY-sphingomyelin of control and P4-treated cells was effectively retained in the apical domain (Fig. 4d, e). Although the form of the cell surface was slightly changed in the cells after treatment with P4 and E2, treatment with P4 and E2 did not affect fence function (Fig. 4f).

To investigate the cytotoxicity of CPE via claudins of normal HEE cells and the effects of P4 and/or E2, the epithelial cells with or without 100 µM P4 and/or 100 µM E2 for 24 h were treated with 0.5–2 µg/ml CPE for 1 h and measured by using the MTT assay. Cytotoxicity was not observed in the epithelial cells at any concentration of CPE with or without P4 and E2 (Fig. 4g).

P4 inhibits cell proliferation in HEE cells

To investigate the effects of cell proliferation induced by P4 and/or E2 in HEE cells, the epithelial cells were treated with 100 µM P4 and/or 100 µM E2 for 24 h and then measured by the BrdU assay. The ratio of BrdU-positive cells was significantly reduced on treatment with P4 (Fig. 5a–d, g). However, the decrease of BrdU-positive cells induced by P4 was not reversed by E2 (Fig. 5e–g).

Upregulation of claudin-3 and −4 by E2 but not P4 in the human endometrial cancer cell line Sawano

In the human endometrial cancer cell line Sawano, claudin-1, −3, −4 and −7 and E-cadherin were observed throughout the lateral membrane, whereas occludin was localized at the apicalmost region (Fig. 6a–f).
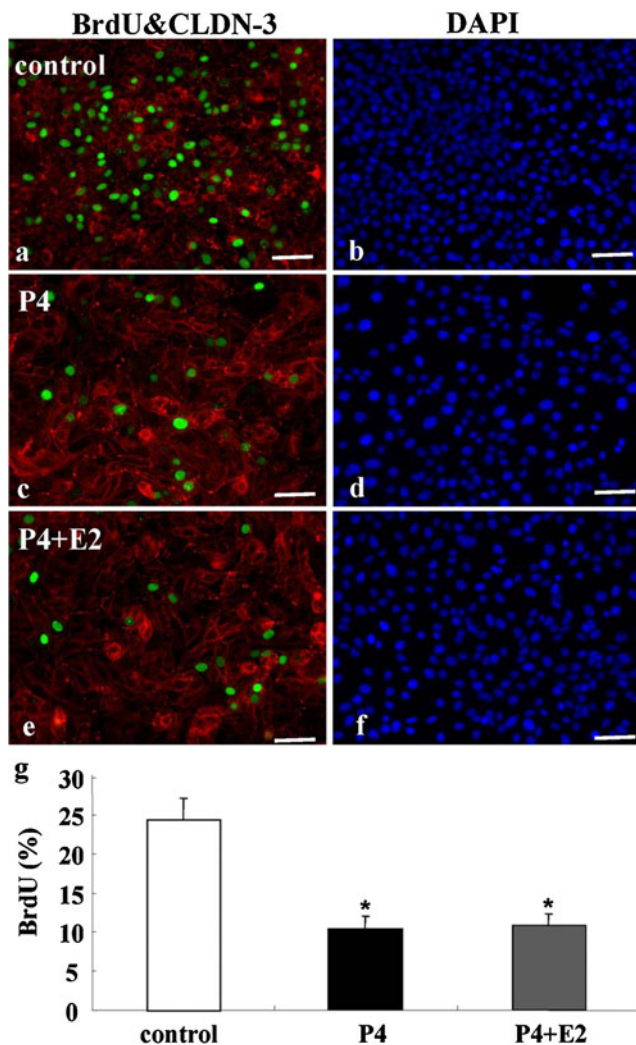
To investigate the changes of tight junction proteins induced by E2 and P4 in Sawano cells, the cells were treated with 1–100 μM E2 or 1–100 μM P4 for 24 h. In Western blots, proteins claudin-3 and −4, following 10 μM E2 treatment and occludin, following 1 μM E2 treatment, were increased, whereas proteins claudin-1 and −3 and occludin were decreased, following treatment with 100 μM E2 (Fig. 6g). In real-time PCR, mRNAs for claudin-3 and −4 were significantly increased on treatment with 10 μM E2 (Fig. 7a). In immunostaining, no change of claudin-3 and −4 was observed (Fig. 7b–e). In Sawano cells treated with P4, no change of any tight junction protein was observed (Fig. 6h).

Changes of tight junction functions induced by E2 in the human endometrial cancer cell line Sawano

To investigate the changes of the barrier and fence functions of tight junctions caused by P4 and/or E2 in Sawano cells, the cells were treated with 1–100 μM E2 for 24 h. The TER indicated that the barrier function was decreased by treatment with 100 μM E2 compared with the control (Fig. 7f). On treatment with 100 μM E2, BODIPY-sphingomyelin diffused through the tight junctions and labeled the basolateral surfaces, whereas in the control, the probe was retained in the apical domain (Fig. 7g–i).

To investigate the cytotoxicity of CPE via claudins on Sawano cells and the effects of E2, cells with and without

Fig. 6 Immunocytochemical staining for claudin-1 (**a**), claudin−3 (**b**), claudin−4 (**c**), claudin−7 (**d**), occludin (**e**) and E-cadherin (**f**) in Sawano cells. *Bars* 40 μm. Western blotting (**g**) for claudin-1, −3, −4, −7, occludin and actin in Sawano cells treated with 1–100 μM E2 for 24 h. Western blotting (**h**) for claudin-1, −3, −4, −7, occludin and actin in Sawano cells treated with 1–100 μM P4 for 24 h (*CLDN* claudin, *OCLN* occludin, *E-cad* E-cadherin)
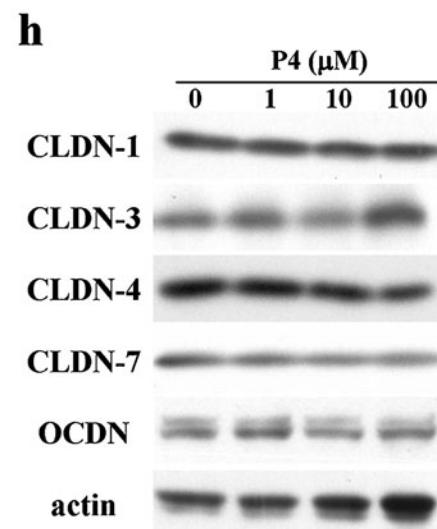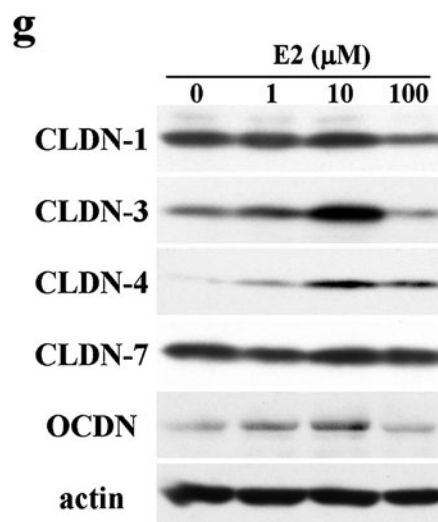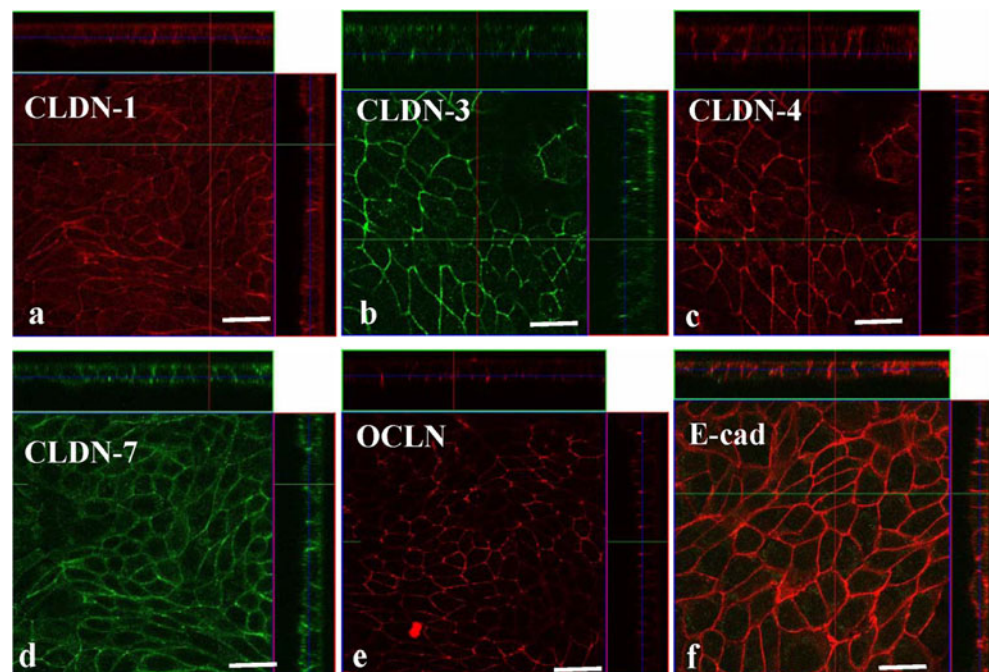
exposure to 10 and 100 μM E2 for 24 h were treated with 0.5–2 μg/ml CPE for 1 h and measured by using the MTT assay. Addition of CPE led to dose-dependent cytotoxic effects in the control Sawano cells and its cytotoxicity was enhanced from 10 μM E2 (Fig. 7j).

Changes of claudin-3 and −4 induced by P4 or E2 via distinct signal transduction pathways in HEE cells and the cancer cell line Sawano

When Sawano cells were pretreated with 100 μM P4 before treatment with 10 μM E2, upregulation of claudin-3 and −4 by E2 was inhibited by P4 (Fig. 7k).

We also investigated which signaling pathways were associated with the upregulation of claudin-3 and −4 by E2 in Sawano cells and compared them with the signal transduction pathways in the upregulation of claudin-3 and −4 by P4 in normal HEE cells. In Sawano cells, the upregulation of claudin-3 by 10 μM E2 was inhibited by the MAPK inhibitor U0126 and the upregulation of claudin-4 by 10 μM E2 was inhibited by U0126 and the hedgehog inhibitor cyclopamine (Fig. 8a). In the normal epithelial cells, the upregulation of claudin-4 but not of claudin-3, by 100 μM P4 was inhibited by U0126 (Fig. 8b). The upregulation of claudin-3 and −4 by sex hormones was not prevented by the PI3K inhibitor

Fig. 7 Real-time PCR (a) for claudin-3 and −4 in Sawano cells treated with 10 μM E2 for 24 h. *P<0.01. Immunocytochemical staining for claudin-3 (b, d) and claudin−4 (c, e) in Sawano cells treated with 10 μM E2 for 24 h. TER values (f) in Sawano cells treated with 10 and 100 μM E2 for 24 h. Fence function (g–i) examined by diffusion of labeled BODIPY-sphingomyelin in Sawano cells treated with 10 and 100 μM E2 for 24 h. Bars 20 μm. MTT assay (j) after 0.5–2 μg/ml Clostridium perfringens enterotoxin (CPE) for 2 h added to Sawano cells treated with 10 and 100 μM E2 for 24 h. Western blotting (k) for claudin-3 and −4 after treatment with 10 μM E2 and/or 100 μM P4 for 24 h (CLDN claudin)
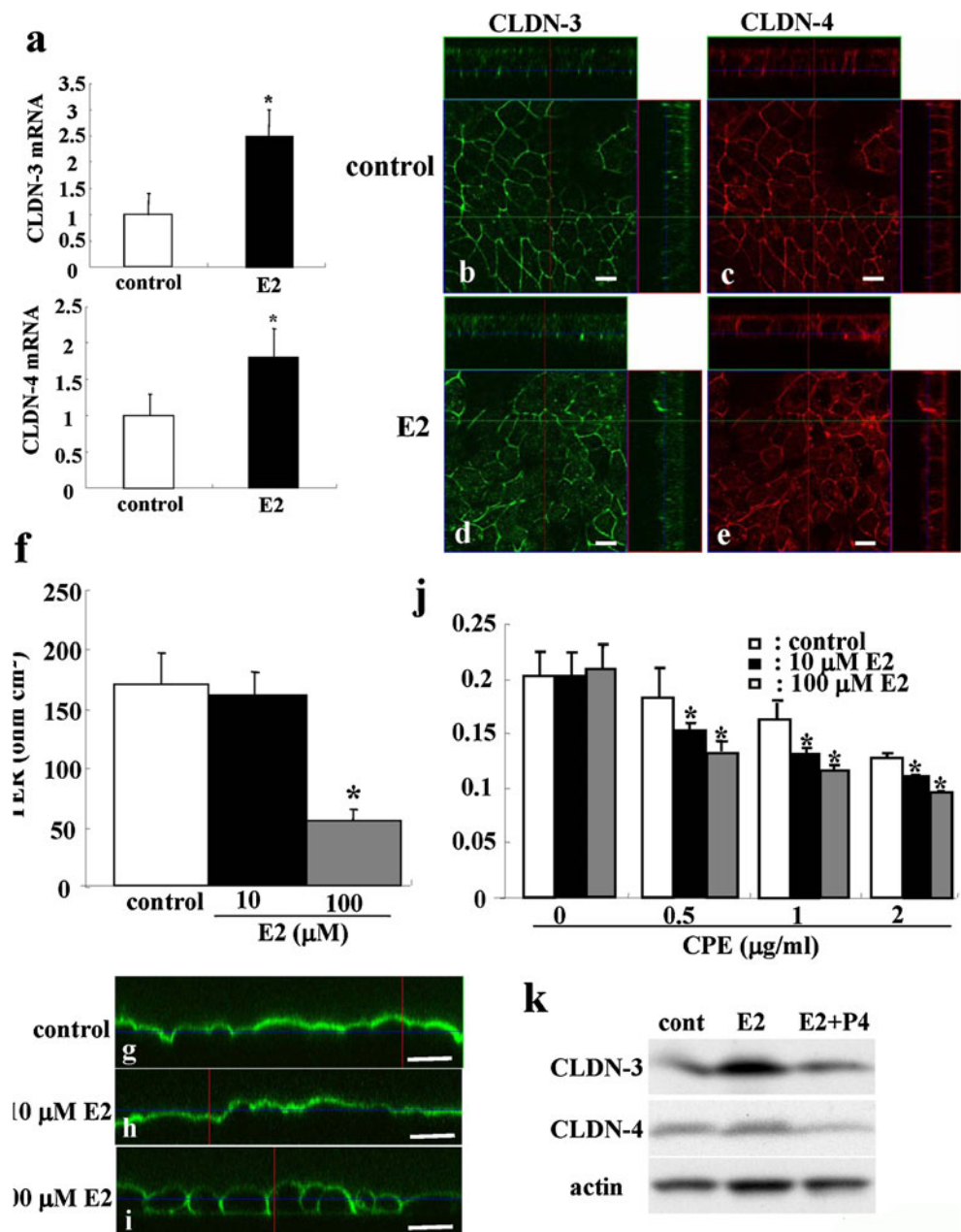
LY294002 or the GSK-3β inhibitor SB216763 in either cell type (Fig. 8a, b).

Discussion

In the present study of normal HEE cells and uterine endometrial cancer cells, we demonstrated the sex hormonal regulation of tight junctions, which are molecular targets for diagnosis and therapy in uterus cancers. In primary cultured HEE cells but not stromal cells, claudin-1, −3, −4 and −7 and occludin were detected together with ERα and PR. In immunostaining of the HEE cells, claudin-1 and −7 were distributed throughout the lateral membrane, as was the adherens junction protein E-cadherin, whereas claudin-3 and −4 and occludin were localized at the apicalmost region. The barrier function and fence function in primary cultured HEE cells were well maintained.

Tight junctional strands of HEE cells in vivo develop through the proliferative phase, form the most complex network in the early secretory phase and then turn into a disorderly formation in the late secretory phase (Iwanaga et al. 1985). In the present study, during the proliferative phase in HEE cells in vivo, claudin-1, −3 and −4 and occludin were localized in the subapical region of the cells, whereas claudin-7 was observed throughout the lateral membrane. During the secretory phase, claudin-1, −3 and −4 were

Fig. 8 a Western blotting for CLDN-3 and −4 in Sawano cells pretreated with inhibitors U0126, LY, CP and SB before treatment with 10 μM E2 for 24 h. b Western blotting for CLDN-3 and −4 in HEE cells in primary culture pretreated with inhibitors U0126, LY, CP and SB before treatment with 100 μM P4 for 24 h (CLDN claudin, LY LY294002, CP cyclopamine, SB SB216763)

redistributed to the lateral membrane, whereas occludin was maintained in the subapical region. These findings suggest that the distribution of tight junction proteins and their functions in primary cultured HEE cells are similar to those of HEE cells in the proliferative phase and early secretory phase.

Embryo implantation necessitates that the apical plasma membrane of uterine epithelial cells acquires adhesiveness and the maintenance of epithelial cell polarity, which is indicated as the fence function, might play a crucial role in embryo implantation (Thie et al. 1996). In the present study, in primary cultured HEE cells, all tight junction proteins, including claudin-3 and −4, were upregulated by P4 and the upregulation was inhibited by E2. The barrier function but not the fence function, in the HEE cells was downregulated by P4, which also inhibited the proliferation of the HEE cells. Regardless of the upregulation of tight junction proteins and inhibition of cell proliferation by P4, a downregulation of the barrier function was observed. We also found that the formation of stress fibers, demonstrated by F-actin staining, was induced by P4. P4 increases stress fiber formation via protease-activated receptor-1 (PAR1) and the alteration of the actin organization results in a decrease of the barrier function (Camussi et al. 1991; Diaz et al. 2012). The decrease of the barrier function caused by P4 in normal HEE cells might contribute to cytoskeletal remodeling. Furthermore, the finding that the fence function of normal HEE cells is not affected by the sex hormone might be associated with the maintenance of embryo implantation.

In the uterine cancer cell line Sawano, claudin-3 and −4 were upregulated by E2 and the upregulation was inhibited by P4. The barrier and fence functions in Sawano were downregulated by a high dose of E2. These results indicate the upregulation of claudin-3 and −4 without tight junction functions by E2 during uterus carcinogenesis.

CPE is thought to bind to the free second extracellular loop of claudins outside of the tight junctions, which leads to a cytotoxic effect (Winkler et al. 2009; Yamaguchi et al. 2011). In the present study, in primary cultured HEE cells, claudin-3 and −4 were localized to the apicalmost regions, which were tight junction areas and no cytotoxicity of CPE was observed. In the uterine cancer cell line Sawano, claudin-3 and −4 were found not only in the apicalmost regions but also at the basolateral membrane and CPE had a dose-dependent cytotoxic effect. Furthermore, the cytotoxicity of CPE in Sawano cells was enhanced via the induction of claudin-4 and the downregulation of the barrier function by E2. These findings suggest that, in uterine cancer cells, CPE binds to the free second extracellular loop of claudin-4 outside of the tight junctions, thereby producing a cytotoxic effect, whereas in normal HEE cells, it does not bind to this loop of claudin-4 in the tight junction areas and no cytotoxicity is observed at any concentration of CPE. E2 treatment
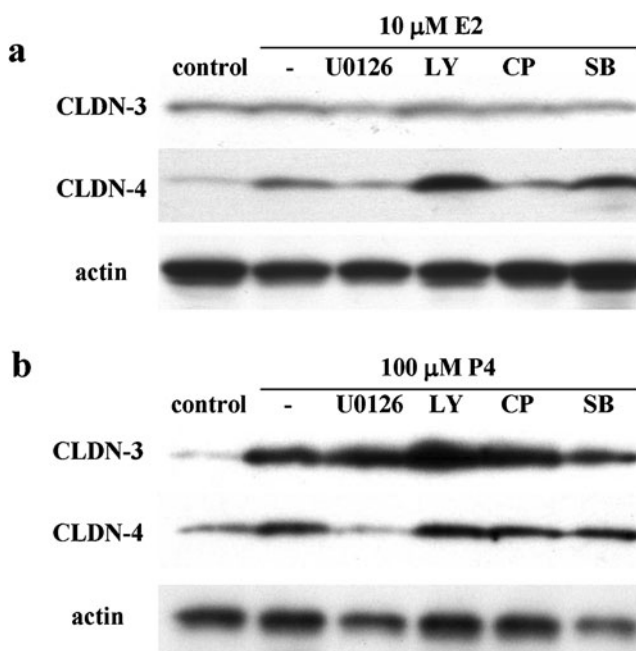
might therefore be useful as a tumor-targeted therapy together with CPE for uterine cancers.

Uterine endometrial epithelial cells are regulated via MAPK, PI3K/Akt and Wnt/β-catenin signaling pathways (Tulac et al. 2003; Villavicencio et al. 2010). In endometrial cancers, PI3K/AKT/mTOR and hedgehog signaling molecules are overexpressed (Zhou et al. 2003; Feng et al. 2007; Liao et al. 2009). Their signaling pathways, including Wnt/β-catenin and sex hormones, play a crucial role for endometrial carcinogenesis (Wang et al. 2010; Dellinger et al. 2012). On the other hand, claudins are also regulated by various cytokines and growth factors via distinct signal transduction pathways (Kojima et al. 2009; Ogawa et al. 2012). In addition, claudin-4 is controlled via the MAPK and PI3K/Akt signal pathways (Pinton et al. 2010; Ogawa et al. 2012). In the present study, when we used inhibitors of signal pathways of MAPK, PI3K/Akt, hedgehog and Wnt, the upregulation of claudin-4 by P4 in normal HEE cells was prevented by a MAPK inhibitor, whereas the upregulation of claudin-4 by E2 in the uterine cancer cells was prevented by inhibitors of MAPK and hedgehog. These findings suggest that the mechanisms of upregulation of claudin-4 by sex hormones are in part different between normal HEE cells and uterine cancer cells.

In conclusion, we have found sex hormonal regulation of tight junctions in normal HEE cells and uterine endometrial cancer cells. These findings not only indicate the physiological regulation of tight junctions in normal HEE cells during the menstrual cycle but also suggest the possibility of developing safer and more effective therapeutic methods targeting claudins in uterine endometrial cancer. Furthermore, the hedgehog signaling pathway, which can regulate claudin-4 in uterine cancer, might be a novel candidate for molecular targeting.

Conflicts of interest The authors declare no conflicts of interest.

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