Claudin-5 as a Novel Estrogen Target in Vascular Endothelium

Malgorzata Burek; Paula A. Arias-Loza; Norbert Roewer; Carola Y. Förster

Objective—Estrogens have multiple effects on vascular physiology and function. In the present study, we look for direct estrogen target genes within junctional proteins.

Methods and Results—We use murine endothelial cell lines of brain and heart origin, which express both subtypes of estrogen receptor, ERα and ERβ. Treatment of these cells with 17β-estradiol (E2) led to an increase in transendothelial electric resistance and a most prominent upregulation of the tight junction protein claudin-5 expression. A significant increase of claudin-5 promoter activity, mRNA, and protein levels was detected in cells from both vascular beds. In protein lysates and in immunoreactions on brain sections from ovariectomized E2-treated mice, we noticed an increase in claudin-5 protein and mRNA content. Treatment of cells with a specific ERβ agonist, diarylpropionitrile, revealed the same effect as E2 stimulation. Moreover, we detected significantly lower claudin-5 mRNA and protein content in ERβ knockout mice.

Conclusions—We describe claudin-5 as a novel estrogen target in vascular endothelium and show in vivo (brain endothelium) and in vitro (brain and heart endothelium) effects of estrogen on claudin-5 levels. The estrogen-induced increase in junctional protein levels may lead to an improvement in vascular structural integrity and barrier function of vascular endothelium. (Arterioscler Thromb Vasc Biol. 2010;30:298-304.)

Key Words: claudin-5 ■ endothelium ■ estrogen receptor ■ tight junction ■ vascular biology

Experimental and population-based studies indicate that women are protected from stroke and cardiovascular disease; however, this advantage is lost with menopause.1 The protective effects of female sex hormones, particularly estrogens, such as the natural endogenous estrogen 17β-estradiol (E2), were confirmed in in vitro experiments and in vivo in animal models. Studies with estrogen receptor (ER) knockout mice confirmed that the vasoprotective effects of estrogen are ER-dependent.2,3 Cellular signaling of estrogens is mediated through 2 ER subtypes, ERα and ERβ, which are expressed at differing levels in many tissues.4,5 The ER activate the transcription of genes directly by binding to estrogen response elements (ERE) in the promoter region of genes. ER-induced transcription may also be indirectly mediated by interaction with Sp1 and AP-1 transcription factors.6,7 Although both ER subtypes are expressed in endothelial and smooth muscle cells of blood vessels, most of E2 effects on the vasculature, such re-endothelialization8 and endothelial nitric oxide production,9 are ERα-mediated but not ERβ-mediated.

Intercellular junctions between endothelial cells are important for vascular integrity and function. Claudin-5 is an integral membrane tight junction protein that belongs to a family of transmembrane proteins with 4 transmembrane domains, of which, currently, 24 members have been described. Claudins play an essential role in the control of paracellular ion flux and in the maintenance of cell polarity.10 Claudin-5 protein was initially considered to be an endothelium-specific member of the claudin protein family.11 However, in recent years claudin-5 protein expression was demonstrated in numerous other cells and tissues, such as Schwann cells, olfactory epithelium, and in the lateral membranes of cardiomyocytes.12–14 Claudin-5 knockout mice are born alive but die within 1 day after birth without any morphological abnormalities. The integrity of the blood–brain barrier of these mice is severely affected, being selectively permeable to small molecules (<800-Da).15

After transient focal ischemia in animal models, tight junction proteins, such as claudin-5 and occludin, are degraded, leading to the opening of the blood–brain barrier, brain edema, and neuronal cell death.16 Thus, enhanced stability of these structural elements of the vessel lining may underlay the vasoprotective effects observed for estrogens.17 Therefore, in the present study, we searched for novel estrogen targets among proteins of the junctional complex. Herein, we describe for the first time to our knowledge the tight junction protein claudin-5 as a novel estrogen target in vascular endothelium.
Materials and Methods
Detailed explanation of experimental procedures is provided as supplementary material (available online at http://atvb.ahajournals.org).

Chemicals
The 17β-estradiol (E2) and propylpyrazole (PPT) were purchased from Sigma. ICI 182.780 and diarylpropionitrile (DPN) were purchased from Biotrend Chemicals AG.

Plasmids
Cloning of the murine claudin-5 promoter (GenBank Accession number EU623469) fragment –500/+111 in pGL3-basic (Promega) was described previously.18

Cell Cultures
Murine microvascular cerebral endothelial cell lines (cEND) and cerebellar endothelial cell lines (cerebEND), as well as the microvascular myocardial endothelial cell line (MyEND), were isolated and immortalized as described previously.19–23

Animal Treatment
The current study was approved by the institutional animal research committee. Twelve-week-old female C57BL/6 mice (Harlan, Eyrstrup, Germany) were sham-operated or ovariectomized and micro-osmotic pumps were implanted subcutaneously (model 1004; Alzet). Animals treated with estradiol received mini-pumps filled with cyclodextrin encapsulated estradiol (Sigma) at a dose of 2 µg/kg per day. Placebo-treated animals were implanted with micro-osmotic pumps filled with vehicle only (cyclodextrin 23 mg/mL water solution). Five animals per group were analyzed.

Transient Transfection and Reporter Gene Assay
Transient transfection of endothelial cell lines with claudin-5 promoter construct and reporter gene assay were performed as previously described.18

Western Blot Analysis
Protein extracts from cells or tissue were analyzed by Western blot. Antibodies against claudin-5 (Invitrogen), β-actin (Sigma), ERα, and anti-ERβ (Santa Cruz Biotechnology) were used. Densitometric analysis using Scion Image Beta 4.02 (Scion Corp) was performed for quantification.

Real-Time Polymerase Chain Reaction
RNA was isolated from tissue or cells and reverse-transcribed. Real-time polymerase chain reaction was performed using the TaqMan PCR Master Mix (Applied Biosystems). Assays for claudin-5, occludin, vascular endothelium-cadherin, and GAPDH were obtained from Applied Biosystems.

Transendothelial Electric Resistance
Transendothelial electric resistance of cell monolayer grown on transwell (0.4 µm pores; Falcon) was measured using an assembly containing current-passing and voltage-measuring electrodes (World Precision Instruments Inc). Resistances of blank filters were subtracted from those of filters with cells before final resistances (Ω cm²) were calculated. All experiments were repeated at least 3 times.

Immunostaining
Frozen brains from wild-type and ERβ knockout mice were a kind gift from T. Pelzer (University Würzburg, Department of Medicine, with the permission of Dr K.S. Korach, Receptor Biology Section, NIEHS, NIH, Research Triangle Park, NC).21 Brain sections were stained with antibodies against platelet/endothelial cell adhesion molecule-1 (1:100; BD Biosciences) and claudin-5 (1:100; Invitrogen). FITC-conjugated/carbocyanin 3-conjugated secondary antibodies were used. The slides were analyzed using a Leica TCS SP2 confocal microscope. Optical density measurements were performed on 10 randomly selected vessels from 5 sections each using Leica Confocal Software (LCS Lite; Leica Microsystems) to quantify fluorescence.

Analysis and Statistics
Throughout the experiments, averaged values were reported as means±SD. Mann-Whitney U-test was performed and P<0.05 was considered statistically significant.

Results
Claudin-5 Is a Target Gene for Estrogen Action in Microvascular Endothelium
In a first step, estrogen effects on endothelial barrier properties were tested on microvascular endothelial cells from murine cerebellum (cerebEND) by measuring changes in transendothelial electric resistance in response to treatment with 10⁻⁸ M E2 for 4, 8, and 24 hours. The cerebEND form a tight monolayer in vitro.20 Transendothelial electric resistance was increased by 150.6±20.8% after 4 hours, by 122.26±18.1% after 8 hours, and by 143.98±19.9% after 24 hours of treatment (Figure 1A). Next, we explored whether and which junctional proteins could be the molecular targets of estrogen action. We analyzed the expression of occludin, vascular endothelium-cadherin, and claudin-5 by real-time polymerase chain reaction (Figure 1B). We demonstrated that claudin-5 mRNA was increased up to 2.8±0.15-fold after 24 hours of treatment with E2. We also observed a small increase in occludin and vascular endothelium-cadherin mRNA expression.

The 2 ER subtypes, ERα and ERβ, are expressed differentially in diverse tissues.23 To control their expression levels in the 3 tested endothelial cell lines, we performed Western blot analysis (Figure 1C). We were able to detect strong protein expression of both ER subtypes in all endothelial cell lines tested.

The Claudin-5 Promoter Is Regulated by E2
To examine transcriptional regulation of claudin-5, we previously cloned the murine claudin-5 promoter.18 Analysis of the promoter sequence with a Genomatix MatInspector (http://www.genomatix.de) and TFSSEARCH (http://www.cbc.jp) revealed several potential transcription factor binding sites. Selected potential transcription factor binding sites are shown in a schematic diagram in Figure 2A. For the present investigation, we failed to identify a conventional ERE, 5’-AGGTCAANNTGACCT-3’.24 However, at positions −449 to −463, the murine claudin-5 promoter contains the sequence 5’-GGGTCATCCTGCCTAA-3’ (consensus nucleotides are written in italics). This sequence has the highest homology of the published ERE consensus sequence. We could also detect a putative Sp1 binding site (5’-GGGGCGAGGT-3’) at positions −45 to −55.

The claudin-5 promoter fragment −500/+111 was cloned into the pGL3-basic vector upstream of the luciferase gene and was then transfected into cEND, cerebEND, and MyEND cell lines. After transfection, the cells were stimulated with 10⁻⁸ M E2 for 24 hours. Afterward, lysates were prepared to evaluate induction of the luciferase enzyme activity that served as an indicator of promoter transactivation (Figure 2B). All cell lines showed an increased promoter activity in response to E2. In MyEND, promoter activity increased 1.36±0.03 fold,
in cerebEND promoter activity increased 1.5±0.11-fold, and in cEND promoter activity increased 3.7±0.34-fold.

Claudin-5 mRNA and Protein Induction by E2
To examine whether E2-dependent claudin-5 upregulation could be detected also on the protein and mRNA level, we first performed a dose–response curve in cerebEND cells (Figure 3A, B). We treated the cells with 10⁻⁶, 10⁻⁸, 10⁻¹⁰, and 10⁻¹² M of E2 for 24 hours and performed Western blots for claudin-5 and β-actin. Claudin-5 protein level was increased in comparison to the untreated sample 3.3±0.35-fold, 3.0±0.18-fold, 2.4±0.17-fold, and 2.2±0.16-fold in 10⁻⁶, 10⁻⁸, 10⁻¹⁰, and 10⁻¹² M samples, respectively (Figure 3A), as determined by densitometric analyses of Western blots.

Expression of claudin-5 mRNA increased in comparison to the untreated sample 1.3±0.16-fold, 1.5±0.27-fold, 1.3±0.11-fold, and 1.2±0.04 fold in 10⁻⁶, 10⁻⁸, 10⁻¹⁰, and 10⁻¹² M samples, respectively (Figure 3B). Next, we performed a time–response curve in cerebEND at constant physiological ligand concentration (10⁻⁸ M). We treated the cerebEND for 6, 12, 24, 48, and 72 hours with E2 (Figure 3C). The cells were then lysed and Western blots for claudin-5 and β-actin were performed. Claudin-5 protein levels were increased by 3.9±0.75-fold, 4.07±0.86-fold, and 5.23±1.28-fold after 6, 12, and 24 hours of E2 treatment, respectively, as determined by densitometric analyses of Western blots. The amount of protein in lysates after 48 hours and 72 hours of stimulation was similar to that of the untreated control. Accordingly, for further experiments we chose the 24-hour stimulation time.

Next, we evaluated whether other endothelial cell lines from different vascular beds, cEND and MyEND, display a similar claudin-5 protein expression pattern as cerebEND in response to E2 (Figure 3D). After 24 hours of E2 treatment, claudin-5 protein levels were increased by 2.8±0.16-fold in cEND and by 2.6±0.2-fold in MyEND in comparison to untreated controls. We also tested the mRNA expression of claudin-5 after 24 hours of treatment with E2 in all cell lines (Figure 3E). Similar to the protein levels, we observed significant increases in claudin-5 mRNA expression, with a 1.81±0.31-fold increase in cEND, a 1.46±0.13-fold increase in cerebEND, and a 1.61±0.3-fold increase in MyEND. The comparatively delayed induction of claudin-5 expression in response to E2 treatment indicated that the effect was caused by genomic action of estrogen. To examine this hypothesis, we included in the treatment solution the nonselective estro-
and Western blot with anti-claudin-5 and anti-β-actin antibody. D, Expression of claudin-5 protein in endothelial cell lines. The cEND and MyEND were treated with or without 10⁻⁸ M E2 for 24 hours and subjected to Western blot analysis with anti-claudin-5 and anti-β-actin antibody. E, Claudin-5 mRNA expression in endothelial cell lines after treatment with E2 or E2/ICI 182.780. The cEND, cerebEND, and MyEND were left untreated or were either treated with 10⁻⁸ M E2 or 10⁻⁸ M E2/1 μmol/L ICI 182.780 for 24 hours, followed by real-time polymerase chain reaction analysis. Data (means±SD) are expressed as fold over untreated control, which was set as 1. *P<0.05.

E2 Regulation of Claudin-5 Expression In Vivo in the Mouse

To investigate whether upregulation of claudin-5 expression also takes place in vivo, we sham-operated or ovariectomized mice and randomized them to receive either placebo or estradiol-filled osmotic mini-pumps. After 2 weeks of treatment after the operation, there were significant differences in control and E2-treated animals in weight and size of the uterus (sham-operated: 26.9±1 mg; ovariectomized: 15.2±0.3 mg; ovariectomized E2: 106.9±15 mg; P<0.05), which is an estrogen target organ, indicating applicability of the experimental set-up for studying generalized estrogen effects. We analyzed claudin-5 expression in the brains of sham-operated, E2-treated (ovariectomized E2), and placebo-treated ovariectomized (control) mice (Figure 4). The Western blot analysis showed in sham-operated animals a 0.84±0.05-fold decrease and in ovariectomized E2 brains a 1.53±0.11-fold increase of claudin-5 protein levels as compared to control ovariectomized brains (Figure 4A). Similarly, the mRNA level increased up to 1.6±0.1-fold in ovariectomized E2 brains, and in sham-operated animals the mRNA level was lowered to 0.92±0.13-fold as compared to ovariectomized mRNA sample, which was set as 1 (Figure 4B).

To further examine claudin-5 regulation in vivo, we performed immunofluorescence stainings with anti-claudin-5 and with anti-platelet/endothelial cell adhesion molecule-1 antibody, the latter serving as an endothelial marker protein, on brain sections from sham-operated, ovariectomized, and ovariectomized E2 mice (Figure 4C). Quantification of fluorescence signals revealed that in brains from E2-treated ovariectomized mice, claudin-5 staining was enhanced 2.3±0.55-fold (P<0.05) and in sham-operated mice claudin-5 staining was enhanced 1.5±0.18-fold (P<0.05) in comparison to ovariectomized controls, which confirmed claudin-5 upregulation by E2 treatment.
Figure 5. ERβ is a potential claudin-5 promoter regulator. A, Transactivation of the claudin-5 promoter in response to E2, DPN, and PPT. The cEND were transfected with the −500/+111 claudin-5 promoter construct. Cells were stimulated with 10⁻⁸ M E2, 10⁻⁸ M PPT, or 10⁻⁸ M DPN for 24 hours, lysed, and subjected to luciferase reporter gene assay. Data (means±SD) are expressed as fold over untreated control, which was arbitrarily set as 1. B. Western blot analysis of lysates from brain of ERβ⁻/⁻ and wild-type mice. Protein lysates from brain of ERβ⁻/⁻ and wild-type mice were prepared and subjected to Western blot analysis using anti-claudin-5 and anti-β-actin antibody. C, Claudin-5 mRNA expression in brain from ERβ⁻/⁻ and wild-type mice. Total RNA was isolated from brain tissue of ERβ⁻/⁻ and wild-type mice, reverse-transcribed, and analyzed in real-time polymerase chain reaction. Data (means±SD) are expressed as fold over sample from wild-type animals, which was set as 1. *P<0.05 D, Confocal fluorescence microscopy depicting localization of claudin-5 and platelet/endothelial cell adhesion molecule-1 in blood vessels from brain of ERβ⁻/⁻ and wild-type mice. Scale bar, 50 μm.

E2 Effects on Claudin-5 Expression Are Potentially Mediated Via ERβ

Western blot analysis had revealed the expression of both ER subtypes, ERα and ERβ, in cEND, cerebEND, and MyEND (Figure 1C). To test which of the ER subtypes could be mediating claudin-5 regulation, we transfected the claudin-5 promoter construct −500/+111 into cEND and treated the cells with different selective synthetic ER agonists, such as PPT (ERα agonist) and DPN (ERβ agonist), at a concentration of 10⁻⁸ M (Figure 5A). After stimulation, the cells were lysed and promoter activity assay was performed. We observed 1.69±0.27-fold promoter transactivation in the presence of E2 and 1.7±0.17-fold promoter transactivation attributable to DPN treatment. There was no transactivation of the claudin-5 promoter in cells stimulated with PPT. Next, we examined the level of claudin-5 expression in brains from ERβ knockout mice (Figure 5B–D). Western blot analysis of brains from wild-type and ERβ⁻/⁻ mice showed a 0.77±0.07-fold decrease in the level of claudin-5 protein (Figure 5B).

Similarly, the claudin-5 mRNA expression in ERβ⁻/⁻ mice was 0.6±0.12-fold lower as compared to the samples from wild-type animals (Figure 5C). Next, we performed immunofluorescence stainings with anti-claudin-5 and with anti-platelet/endothelial cell adhesion molecule-1 antibody on brain sections from wild-type and ERβ⁻/⁻ mice (Figure 5D). Quantification of fluorescence signals revealed that in brains from ERβ⁻/⁻ mice, claudin-5 staining was 0.6±0.04-fold (P<0.05) lower in comparison to wild-type animals.

Discussion

Protective effects of estrogens on the vasculature have been previously reported. However, the molecular mechanisms of these effects are not completely understood. Intercellular junctions are important structural elements that contribute to the structural and functional integrity of the vessel wall. Therefore, we hypothesized that influence on endothelial junction properties might underlay protective effects of estrogens on the vasculature. So, we designed experiments aiming at providing functional proof of this hypothesis by studying estrogen effects on endothelial permeability and, subsequently, identifying possible estrogen targets among endothelial intercellular junction proteins. To enable comparisons between endothelial cells derived from central nervous system and from non-neural vasculature, we included in the analyses immortalized endothelial cell lines of brain and heart origin, which have been previously proven to possess the typical endothelial properties. Estrogen effects on endothelial barrier functions were initially confirmed by the detection of an E2-mediated increase in transendothelial electric resistance of endothelial monolayers. Additionally, we detected increased expression of claudin-5, occludin, and vascular endothelium-cadherin after treatment with E2. Because E2-effects on claudin-5 expression were the most prominent, we proceeded to analyze in detail the E2-mediated regulation of claudin-5.

The analysis of the murine claudin-5 promoter revealed no canonical ERE in the promoter sequence. However, we identified an imperfect ERE and potential Sp1 transcription factor binding site. The regulation of the claudin-5 promoter could take place either directly by interaction of ER with the DNA via an imperfect ERE or indirectly by interaction with Sp1 protein. In the present study, we report for the first time to our knowledge an increased claudin-5 promoter activity by estradiol treatment of cEND, cerebEND, and MyEND cell lines. The exact molecular mechanism and promoter elements responsible for E2 regulation remain to be determined.

Claudin-5 is expressed ubiquitously in the vascular endothelium. Overexpression of claudin-5 improved blood-brain barrier function, reduced paracellular cation permeability in Madin-Darby canine kidney II cells, and narrowed paracellular cleft. Here, we demonstrate that with treatment with estrogen, claudin-5 protein levels significantly increased in cEND, cerebEND, and MyEND. The increase was time-dependent and dose-dependent, being strongest after 24 hours of 10⁻⁸ M E2 stimulation. E2 also mediated the induction of claudin-5 expression at the mRNA level, and this effect was blocked by co-administration of the nonselective ER antagonist ICI 182,780.

In humans, ERα and ERβ are expressed in vascular endothelial cells. We also detected expression of both ER subtypes in the murine endothelial cell lines used for this study. The presence of both ER subtypes raised the question of which of the ER could be responsible for the observed claudin-5 regulation. We demonstrate that in the presence of DPN, but not after the stimulation with PPT, claudin-5
promoter activation was increased, indicating that ERβ might be responsible for claudin-5 regulation. Both ER subtypes have been described to play a role in vascular biology and function. The ERα and ERβ knockout mice differ from each other in several ways. The ERα−/− mice present uterus atrophy and polycystic ovaries and both male and female mice are sterile, whereas the ERβ−/− mice exhibit different degrees of subfertility. However, this first ERα knockout mice study reported residual estradiol binding capacity in uterine tissue of 5% to 10%; a clear-cut leakage of E2 effect was attributed incorrectly to ERβ. These mice were subsequently shown to present a transcriptional leakage attributable to a non-natural alternative splicing of the ERα mRNA, resulting in the expression of a truncated 55-kDa isoform. In ERβ−/− mice, morphological changes have been demonstrated in prostatic epithelium and in the brain. Further abnormalities demonstrated in ERβ−/− were development of hypertension on aging, abnormal vascular and myocardial function, and reduced expression of cell adhesion molecules in various cell types; however, the present work is the first demonstration, to our knowledge, of a direct endothelial action of E2 through ERβ. Our experiments with the brains from ERβ−/− mice and in an ovariectomized mouse model showed that claudin-5 expression is E2-regulated not only in vitro but also in vivo. We showed a significant increase of claudin-5 mRNA and protein in brain tissue from ovariectomized mice treated with E2 and a significant decrease of claudin-5 mRNA and protein in brains from ERβ−/− mice.

In summary, this study describes a new target gene of estrogen action in vascular endothelium. Using a murine claudin-5 promoter construct and immortalized microvascular endothelial cell lines of brain and heart origin, we show E2-mediated claudin-5 upregulation on promoter, mRNA, and protein levels. Moreover, our observations could be confirmed for brain endothelium in experiments in ovariectomized E2-treated mice and in ERβ−/− mice. Whether a similar regulation of claudin-5 also occurs in other endothelia should be determined in future work.

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Disclosure

None.

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Materials and Methods

Chemicals
17β-estradiol (E2) and propylpyrazole (PPT) were purchased from Sigma (Sigma, Taufkirchen, Germany), ICI 182.780 (fulvestrant) and diarylpropionitrile (DPN) were purchased from Biotrend Chemicals AG (Zürich, Switzerland). Stock solution were prepared in dimethylsulphoxide (DMSO) and kept at -20 °C. Desired dilution was made before the experiment in cell culture medium. The final concentration of DMSO in the treatment medium was lower than 0.01% (v/v). To obtain the best receptor specificity, PPT and DPN were used at a concentration of $10^{-8}$ M.

Plasmids
Cloning of the murine claudin-5 promoter (GenBank Accession No.: EU623469) fragment -500/+111 in pGL3-basic (Promega, Mannheim, Germany) was described previously.

Cell Cultures
Murine microvascular cerebral (cEND) and cerebellar (cerebEND) endothelial cell lines as well as the microvascular myocardial endothelial cell line MyEND were isolated and immortalized as described previously. Endothelial cell lines were cultivated in Dulbecco's modified Eagle's medium (DMEM) with 10% Fetal Calf Serum (FCS). All cultures were supplemented with 100 i.u. ml$^{-1}$ penicillin and 100 mg ml$^{-1}$ streptomycin (1% PEST) (Sigma). Cells were maintained in an atmosphere of 5.0% CO$_2$–95% O$_2$ and at 37°C. Endothelial cell lines were grown on plates coated with collagen IV (Fluka, Taufkirchen, Germany).
Animal Treatment

The current study was approved by the institutional animal research committee (Tierschutzbeauftragter) and the animals were kept under conventional conditions in the animal facility. Twelve weeks old female C57BL/6 mice (Harlan, Germany), under an adequate depth anesthesia (tribromoethanol / amylen hydrate - “Avertin”; 2,5% WT/vol, 6 µl/g BW ip.), were sham operated or ovariectomized and micro-osmotic pumps were implanted subcutaneously (Alzet, model 1004). Animals treated with estradiol received minipumps filled with cyclodextrin encapsulated estradiol (Sigma) at a dosis of 2 µg/kg/day. This dosis was chosen to ensure normal physiological response to estradiol. Placebo treated animals were implanted micro-osmotic pumps filled with vehicle only (cyclodextrin 23mg/ml water solution). Five animals per group were analyzed. The animals used for the study were also employed to study the cardiovascular metabolic effects of estradiol (Arias-Loza P.A., unpublished data) therefore after 2 weeks of treatment the animals were fasted overnight, and were killed by decapitation under glucose stimulus (1,5 mg/g body weight).

Transient Transfection and Reporter Gene Assay

Endothelial cell lines were seeded on a collagen IV coated 6-well cell culture plate at a density of 3 x 10^5 cells per well. The cells were grown in DMEM medium with 10% FCS to confluence prior to transfection. Transient transfection experiments using the Effectene reagent (Qiagen, Hilden, Germany) were performed as suggested by the manufacturer in DMEM medium supplemented with 1% FCS, using 2 µg of claudin-5 promoter construct DNA and 1 µg of the internal control reporter pTRL-TK. After 24 hours the cells were washed and 2 ml fresh medium with or without 10^-8 M E2 was added. After 24 hours stimulation the cells were lysed and the cellular extracts were analyzed for luciferase activity. Measurement of both firefly and Renilla luciferase activity was performed with the Dual-
Luciferase assay kit (Promega) according to the manufacturers’ instructions. Protein concentration was estimated by standard Bradford protein assay. Cell lysate (20µl) was used for assaying the enzymatic activities, using a LB9507 luminometer with dual injector (Berthold, Bad Wildbad, Germany). Each lysate was measured twice. Promoter activities were expressed as relative light units (RLU) normalized for the protein content and the activity of Renilla luciferase in each extract. The data were calculated as the mean of three identical set-ups and expressed as fold over the untreated control, which was arbitrarily set as 1.

**Western Blot Analysis**

Cells were plated on collagen IV coated dishes at a density of $1.1 \times 10^5$ cells per 3.5 cm$^2$ and grown in DMEM medium with 10% FCS to confluence. At confluence, cells were maintained at 1% FCS for 48 hours followed by treatment with E2 as indicated in the figure legends. For Western blot analyses, cells or tissue samples were extracted in Laemmlli sample buffer. Protein contents were quantified by protein estimation directly from SDS-PAGE loading buffer using 0.1% (w/v) Amidoschwarz (AppliChem, Darmstadt, Germany) in 25% (v/v) methanol–5% (v/v) acetic acid, and equal protein amounts were loaded on SDS-polyacrylamide gels for Western blot analysis. For immunoblotting, proteins were transferred in Kyhse-Andersen transfer buffer to Hybond nitrocellulose membranes (Amersham, Braunschweig, Germany) which were blocked with 10% (w/v) low fat milk in PBS and incubated overnight at 4°C with the respective primary antibody (in PBS plus 10% low fat milk). Mouse monoclonal anti-mouse claudin-5 antibody was purchased from Invitrogen (CA, USA), anti-β-actin antibody was purchased from Sigma, anti-ERα and anti-ERβ antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). As secondary antibody, horseradish peroxidase-labelled goat anti-mouse IgG or goat anti-rabbit IgG (Jackson Immuno Res. Laboratory, West Grove, PA, USA) was used diluted 1: 3000 with
PBS/ 10% low fat milk. Bound immunoglobulins were visualized by the enhanced chemiluminescence technique (ECL, Amersham). Densitometric analysis using Scion Image Beta 4.02 (Scion Corp., MD, USA) was performed for quantification.

**Real-Time PCR**

For real-time PCR, RNA was isolated from tissue or cells treated or untreated with 10^-6, 10^-8, 10^-10 and 10^{-12} M E2 or 10^{-8} M E2/ 1 µM ICI 182.780 using RNeasyMini kit (Qiagen). One microgram of RNA was used for the cDNA synthesis using iSCRIPT cDNA synthesis kit (Bio-Rad, München, Germany). Real-time PCR was performed using the TaqMan PCR Master Mix (Applied Biosystems, Foster City, CA). Assays for claudin-5 (Mm00727012_s1), occludin (Mm00500912_m1), VE-cadherin (Mm00486938_m1) and GAPDH (Mm99999915_g1) were obtained from Applied Biosystems. All PCR reactions were performed in triplicate for each target. Data were acquired with the ABI PRISM 7300 system (Applied Biosystems). The ABI PRISM 7300 SDS software (relative quantification study) was used to determine the threshold cycle (Ct) for each reaction and gene expression was normalized to the expression of the endogenous housekeeping gene GAPDH based on the 2^-∆∆Ct method.

**Transendothelial Electrical Resistance**

Cells were plated in DMEM medium with 10% FCS on top of collagen IV-coated transwell chambers for six-well plates (0.4 µm pores; Falcon, Heidelberg, Germany) at densities of 1 x 10^4 cells per well. When they had reached confluence, the different experimental sets of cells were transferred to 1% FCS or were treated with 10^{-8} M E2 at 1% FCS, respectively. The control set was maintained in basal medium (DMEM, 1% FCS) for an additional 8 hours, at which point the permeability measurement was performed. Transendothelial electrical
resistance (TER) was measured using an assembly containing current-passing and voltage-measuring electrodes (World Precision Instruments Inc., New Haven, CT, USA). Resistances of blank filters were subtracted from those of filters with cells before final resistances (in $\Omega \times \text{cm}^2$) were calculated. All experiments were repeated at least three times.

**Immunostaining**

Frozen brains from wild type and ER$\beta$ knockout mice were a kind gift from T. Pelzer (University Würzburg, Department of Medicine, with the permission of Dr. K.S. Korach, Receptor Biology Section, NIEHS, NIH, Research Triangle Park, North Carolina)\(^9\). Brains from sham operated, OVX and OVX E2-treated mice were frozen in liquid nitrogen-cooled isopentane. Ten-µm-thick sections were cut in a cryostat and mounted on glass slides (Superfrost Plus, Menzel). Frozen sections were air dried for 30 min. After washing with ice-cold methanol and acetone, each for 3 min, sections were fixed for 10 min at room temperature in 4% formaldehyde prepared freshly from paraformaldehyde. All sections were treated with 0.1% Triton X-100 in PBS for 5 min, incubated with 10% normal goat serum in PBS for 30 min at 25°C to reduce unspecific binding of secondary antibodies, and exposed in sequence to primary antibodies: rat monoclonal antibody to mouse PECAM-1 (platelet/endothelial cell adhesion molecule 1 \(^{10}\)) (BD Biosciences, Heidelberg, Germany; 1:100), rabbit polyclonal anti-claudin-5 (Invitrogen, Camarillo, CA; 1:100) and FITC-/Carbocyanin 3 (Cy3)-conjugated goat anti-rat and goat anti-rabbit secondary antibodies, respectively. Incubation without primary antibody served as a background control. After incubation, coverslips were mounted onto slides using 60% (w/v) glycerol and 1.5% (w/v) $n$-propylgallate (Fluka) as antifading component. The slides were analysed using a Leica TCSSP confocal microscope. Optical density measurements were carried out on 10 randomly selected vessels from five sections each using Leica Confocal Software (LCS Lite) (Leica Microsystems, Heidelberg, Germany) to quantify fluorescence.
Analysis and Statistics

Throughout the experiments, averaged values were reported as means ± standard deviation (s.d.). Mann–Whitney \( U \)-test was performed and \( P<0.05 \) (*) was considered statistically significant.

Supplemental References: