Estrogen-dependent regulation of sodium/hydrogen exchanger-3 (NHE3) expression via estrogen receptor β in proximal colon of pregnant mice

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Running title: Up-regulation of NHE3 via ERβ in pregnant mouse colon

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Abstract

Although constipation is very common during pregnancy, the exact mechanism is unknown. We hypothesized the involvement of estrogen receptor (ER) in the regulation of electrolyte transporter in the colon leading to constipation. In this study, the intestines of normal female ICR mouse and pregnant mice were examined for the expression of ERα and ERβ by immunohistochemistry and *in situ* hybridization. ERβ, but not ERα, was expressed in surface epithelial cells of the proximal, but not distal, colon at pregnancy days 10, 15 and 18, but not day 5, and the number of ERβ-positive cells increased significantly during pregnancy. Expression of NHE3, the gene that harbors estrogen response element, examined by immunohistochemistry and western blotting, was localized in the surface epithelial cells of the proximal colon and increased in parallel with ERβ expression. In ovariectomized mice, NHE3 expression was only marginal and was up-regulated after treatment with 17β-estradiol (E_2), but not E_2+ICI 182,780 (estrogen receptor antagonist). Moreover, knock-down of ERβ expression by electroporetically transfected siRNA resulted in a significant reduction of NHE3 expression. These results indicate that ERβ regulates the expression of NHE3 in the proximal colon of pregnant mice through estrogen action, suggesting the involvement of increased sodium absorption by up-regulated NHE3 in constipation during pregnancy.

Keywords: estrogen receptor β, NHE3, proximal colon, pregnancy, constipation

Introduction

In pregnancy, blood volume increases about 50% to ensure adequate blood supply for normal fetal development and other organs (Curtis 2009). The large blood volume is maintained during pregnancy through up-regulation of sodium and water absorption (Parry et al. 1970). The increased sodium and water absorption is implicated in constipation in pregnant woman. In fact, the reported prevalence rate of constipation in pregnant women is up to 40% (Bradley et al. 2007, Cullen and O'Donoghue 2007). The marked increase in blood estrogen level during pregnancy is also considered a contributing factor to constipation (McCormack and Greenwald 1974, Arnaud 2003). However, the precise mechanism of estrogen action on constipation is still unclear.

Estrogen plays a critical role in maintaining the structure and function of various reproductive (Pelletier and El-Alfy 2000, Wang et al. 2000, Ulziibat et al. 2006) and non-reproductive organs (Nishihara et al. 2000, Tsurusaki et al. 2003, Kawano et al. 2004, Shukuwa et al. 2006). In general, the biological actions of estrogen are mediated through its binding to its receptors, estrogen receptor (ER)-α and ERβ, which then bind to the estrogen response element (ERE), which is harbored in the promoter region of various estrogen-dependent genes (Klinge 2001, Marzouk et al. 2008). In contrast, the ERβ plays a dominant role in mediating the action in non-reproductive tissues such as the cardiovascular system and colon, where it is expressed primarily in epithelial cells (Campbell-Thompson et al. 2001, Wada-Hiraike et al. 2006, Harris 2007). ERs are products of different genes and exhibit tissue and cell-type specific expression (Sar and Welsch 1999, Matthews and Gustafsson 2003). The presence of ERβ has been reported in rodent colon (Kawano et al. 2004, Wada-Hiraike et al. 2006), human colon cancer (Castiglione et al. 2008, Giroux et al. 2008) and cancer cell line (Campbell-Thompson et al. 2001, Martineti et al. 2005) and determined as a predominant

ER subtype in the colon. However, the functional role of ERβ in the colon is not fully understood.

The main functional role of the colon is absorption; 90% of ileal effluent is absorbed, passing through the ileocecal valve (Sandle 1998). The key determinant of colonic water absorption is the rate of sodium absorption. Evidence suggests that sodium transport processes are not distributed uniformly throughout the colon, such as greater absorption in the proximal colon than in the distal colon (Araki et al. 1996). Recent studies have shown that the electroneutral sodium absorbing molecule, NHE3 is abundantly expressed in the proximal colon but not in the distal colon in mice (Talbot and Lytle 2010). Moreover, estrogen mediated up-regulation of NHE3 was found in rodent reproductive tissue (Joseph et al. 2010, Zhou et al. 2001). However, the involvement of estrogen in the regulation of NHE3 in the colon remains to be clarified.

In the present study, we first investigated the pregnancy-dependent expression of ERα and ERβ in mice by immunohistochemistry, western blotting and *in situ* hybridization (ISH). Then, the spatial and temporal relationship between ERβ and NHE3 expression in the colon of pregnant mice was examined. Moreover, for a more direct investigation of the effect of estrogen on the expression of ERβ and NHE3 in the colon, we analyzed the effects of E_2 or pure estrogen receptor antagonist ICI 182,780 injected into overiectomized (OVX) mice. Finally, the ERβ-dependency of the expression of NHE3 in the proximal colon was examined by knock-down of ERβ gene with electroporated transfection of small interfering RNA (siRNA) siRNA for ERβ.

Materials and methods

Chemicals and biochemicals

Paraformaldehyde (PFA) was purchased from Merck (Darmstadt, Germany). Trizma base, 17β-estradiol, fulvestrant (ICI 182,780), phenylmethylsulfonyl fluoride (PMSF), sodium molybdate, bovine serum albumin (BSA), 2-mercaptoethanol, d-mannitol, 3-aminopropyl-triethoxysilane, Triton X-100, Brij 35, yeast transfer RNA, and salmon testis DNA were purchased from Sigma Chemical Co. (St Louis, MO, USA). Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) reagents and the molecular marker set were purchased from Daiichi Pure Chemicals (Tokyo, Japan). Polyvinylidene fluoride membrane (PVDF) was purchased from Millipore (Bedford, MA, USA). Lima bean trypsin inhibitor was purchased from Worthington Biochemical (Lakewood, NJ, USA). The protein assay kit and Coomassie brilliant blue were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Ponceau-S and deionized formamide were purchased from Nacalai Tesque (Kyoto, Japan). Digoxigenin-11-dUTP and terminal deoxynucleotidyl transferase (TdT) were from Roche Diagnostics (Mannheim, Germany). 3,3′-Diaminobenzidine–4 HCl (DAB) was purchased from Dojindo Chemicals (Kumamoto, Japan) and 4-Cl-1-naphthol was from Tokyo Kasei Kogyo (Tokyo, Japan). All other reagents used in this study were from Wako Pure Chemicals (Osaka, Japan) and were of high analytical grade.

Antibodies

A mouse monoclonal antibody against ERα (ER88; dilution 1:160) was purchased from BioGenex (San Ramon, CA, USA) and a rabbit polyclonal antibody against ERβ (PA1–310B; dilution 1:100) was purchased from Pierce Biotechnology (Rockford, IL, USA). Rabbit polyclonal antibody against NHE3 (AB3085; dilution 1:500) was purchased from

Millipore (Temecula, CA, USA). Mouse monoclonal antibody against β-actin (AC-15; dilution 1:12,800) and normal goat and sheep IgG were purchased from Sigma. Horseradish peroxidase (HRP)-goat anti-mouse IgG (dilution 1:100) and HRP-goat anti-rabbit IgG (dilution 1:200) were purchased from Millipore (Temecula, CA, USA). HRP-sheep anti-digoxigenin IgG (dilution 1:100) was purchased from Roche Diagnostics (Mannheim, Germany) and HRP-mouse monoclonal anti-thymine-thymine (T–T) IgG (dilution 1:80) was from Kyowa Medex (Tokyo, Japan). Normal mouse and rabbit IgG were purchased from DAKO (Glostrup, Denmark).

Animals

Adult female and pregnant ICR mice (8-12 weeks) weighing 27-65 g were used in the present study. Mice were fed normal chow and allowed to drink water *ad libitum*. The experimental protocol was approved by the Animal Ethics Review Committee of Nagasaki University (#1004010843). After coupling, female mice were examined every morning for the presence of a copulatory plug and the day of plug found was designated day 0 of pregnancy. The ICR pregnant mouse delivers on the evening of day 18. Therefore, we collected the intestinal tissues of pregnant mice in the morning of pregnancy days 5, 10, 15 and 18. Bilateral OVX was performed in all mice at 8 weeks of age and then randomly divided into three groups. OVX mice of the first group (n=3) were treated with E_2 dissolved in 100 µl of corn oil at a dose of 25 µg/kg of body weight. OVX mice of the second (n=3) and third (n=3) groups were treated with E_2+ICI 182,780 (0.3 mg/kg) dissolved in 100 µl of corn oil and vehicle only, respectively. After OVX, each mouse was injected subcutaneously with the above respective compounds on days 1, 5, 9 and 12, as described previously (Seinen et al. 1999). The E_2 injection regime was reported to induce significant changes in both the reproductive and non-reproductive tissues (Seinen et al. 1999). Nag and Mokha reported that after E_2 injection in rat,

serum estrogen level is significantly higher than normal level at 48 h and reached in normal level at day 7 (2006). E_2 level is expected to be high at 48 h after last injection of E_2. Therefore, 48 h after last injection of E_2, mice were sacrificed and intestinal tissues were sampled. For ERβ knock-down experiment, 24 h after last E_2 injection, electroporatic transfection of ERβ siRNA was conducted and mice were sacrificed 24 h after the electroporation.

Tissue preparation

After sacrifice, the intestinal tissues were removed, rinsed in ice-cold phosphate-buffered saline (PBS), opened through the mesenteric border and then divided into two pieces by longitudinal cutting. We defined here three segments of colon based on their distinctive patterns: proximal (0-20% length), middle (20-60% length), and distal (60-100% length) (Talbot and Lytle 2010). The first piece of the colon was snap frozen and kept at -80°C until used for western blotting. The second piece of the colon was fixed in 4% PFA in PBS (pH 7.4) at room temperature (RT) for about 20 h and then embedded in paraffin.

Immunohistochemistry for ERα, ERβ and NHE3

Paraffin-embedded tissues were cut into 5 μm thick sections and placed onto silane-coated slide glasses. The sections were deparaffinized with toluene, and rehydrated through graded ethanol series, and then autoclaved at 120 ºC for 15 min in 10 mM citrate buffer (pH 6.0) (Koji et al. 2008, Song et al. 2011). After inhibition of endogenous peroxidase activity with 0.3% H_2O_2 in methanol for 15 min, the sections were pre-incubated with 500 μg/ml normal goat IgG and 1% BSA in PBS for 1 h to block non-specific binding of antibodies. Unless otherwise specified, all reactions were conducted at RT. Then, the sections were reacted with the primary antibodies for 16 h. After washing with 0.075% Brij 35 in PBS, they were

reacted with HRP-goat anti-mouse IgG or HRP-goat anti-rabbit IgG for 1 h. After washing in 0.075% Brij 35 in PBS, the HRP sites were visualized with DAB, Ni, Co, and H_2O_2 according to the method of Adams (1981). As a negative control, normal mouse or rabbit IgG was used at the same concentration instead of the primary antibodies in every experiment.

Double-staining for ERβ and NHE3

For simultaneous detection of ERβ and NHE3, we performed double-staining, as described previously (Shirendeb et al. 2009, Shukuwa et al. 2006). Briefly, after antigen retrieval, the sections were stained with anti-NHE3 for overnight and HRP sites were visualized as *brown* products of DAB and H_2O_2. Next, the slides were immersed and stirred in 0.1 M glycine-HCl buffer (pH 2.2) to remove immuno-complexes. After washing with Milli-Q water once and PBS three times, the sections were reacted with anti-ERβ for overnight. HRP sites were visualized by *purple-blue* product of 4-Cl-1-naphthol and H_2O_2 solution.

Isolation of the apical membrane of colon surface epithelium

To examine the expression of NHE3 in mouse colon, the apical membrane of colon surface epithelium was isolated by the magnesium precipitation method as described previously. (Hoogerwerf et al. 1996). Briefly, the intestinal wall was cut along the mesenteric border, then gently washed in ice-cold PBS and stripped of the mucosal layer by blunt dissection using the edge of a glass slide on ice-cold plate. The obtained tissue was snap frozen on dry ice and kept in -80ºC until used for protein extraction. Tissues were thawed in ice-cold lysis solution containing 10 mM mannitol, 2 mM Tris/HCl (pH 7.1) and 50 µg/ml lima bean trypsin inhibitor. For isolation of apical membrane of colon surface epithelium, magnesium precipitation and differential speed centrifugation were performed according to the method of Booth and Kenny (1974). To analyze the expression levels ERβ protein in the colon, the

excised tissue was homogenized in a lysis buffer containing 50 mM Tris-HCl buffer (pH 7.5), 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM PMSF, 20 mM sodium molybdate and 50 µg/ml lima bean trypsin inhibitor, as described previously (Kawano et al. 2004, Shukuwa et al. 2006). Tissues were homogenized with a glass-teflon homogenizer. After centrifugation of the homogenate at 15,000 rpm for 30 min at 4ºC, the supernatant was collected and stored at -80ºC. The protein concentration in each preparation was determined using a kit from Bio-Rad Laboratories according to the method of Lowry et al (1951), with BSA as a standard.

Western blot analysis

The lysate containing 20 µg of protein was mixed with the loading solution [200 mM Tris-HCl (pH 8.0), 0.5 M sucrose, 5 mM EDTA, 0.01% bromophenol blue, 10% 2-mercaptoethanol, and 2.5% SDS], boiled 5 min, and separated by SDS-PAGE with 4-20% gradient gel according to the method of Laemmli (1970), and electrophoretically transferred onto PVDF membranes. The membranes were stained with Ponceau-S to verify equal loading and transfer, and then washed with double distilled water. The membranes were blocked with 10% nonfat milk in Tris-buffered saline (TBS; 20 mM Tris buffer, pH 7.6, and 150 mM NaCl) overnight at 4ºC and then incubated for 3 h with rabbit polyclonal antibody anti-ERβ and anti-NHE3, diluted 1:500 and 1:1,000 with TBS/0.05% Triton X-100 buffer, respectively. As a secondary antibody, HRP-goat anti-rabbit IgG was diluted with 10% nonfat milk in TBS buffer for 1 h and membranes were washed 6 times for 15 min each with TBS/0.05% Triton X-100 buffer. The bands were visualized with DAB, Ni, Co and H_2O_2.

Preparation of oligo-DNA probes

A 45-base sequences corresponding to ERβ mRNA, nucleotide no. 845-889, GenBank accession no. U81451 (Tremblay et al. 1997) and NHE3 mRNA, nucleotide no. 1-45, GenBank accession no. (NM_001081060.1) were selected. The sequence for ERβ antisense probe was 5′-ACCTCCATCCAGCAGCTTTCCAAGAGGCGGACTTGGTCCAACAGG-3′ and for ERβ sense probe was 5′-CCTGTTGGACCAAGTCCGCCTCTTGGAAAGGTGCTGGATGGAGGT-3′. The sequences for NHE3 antisense probe was 5′-GGCCAGCAGCAGCTTCCACCCTGGTCCCAGAGCCCGGTGCCACAT-3′ and for NHE3 sense probe was 5′-ATGTGGCACCGGGCTCTGGGACCAGGGTGGAAGCTGCTGCTGGCC-3′. We conducted a computer-assisted search using BLAST software (http://www.ncbi.nlm.nih.gov) for the above sequences and found 100% homology with mouse ERβ mRNA and NHE3 mRNA sequences, respectively. These oligo-DNAs were labeled at their 3′-end with Dig-11-dUTP by TdT, as described in detail previously (Koji and Brenner 1993).

In situ hybridization

The specificity and sensitivity of the DNA probes were confirmed by immunodetection and dot-blot hybridization (Yoshii et al. 1995, Koji and Nakane 1996). Immunohistochemical ISH was performed as described previously (Koji and Brenner 1993, Yamamoto-Fukuda et al. 2003, Tamaru et al. 2004). The sections were treated with 0.3% H_2O_2 in methanol for 15 min to inhibit endogenous peroxidase activity, followed by 0.2 N HCl for 20 min and 50 μg/ml of proteinase K at 37°C for 15 min. After post-fixation with 4% PFA in PBS, the sections were immersed in 2 mg/ml of glycine in PBS for 30 min and kept in 40% deionized formamide in 4 × standard saline citrate (SSC) (1 × SSC = 0.15 M sodium chloride and 0.015 M sodium citrate, pH 7.0) until used for hybridization. Hybridization was carried out for 15-17 h at

37°C with 1 μg/ml of ERβ Dig-labeled oligo-DNA probe in the hybridization medium. Then, the slides were washed twice with 2 × SSC/50% formamide/0.075% Brij 35, twice with 0.5 × SSC/50% formamide/0.075% Brij 35, once with 0.2 × SSC/50% formamide/0.075% Brij 35 at 37°C and finally followed by 2 × SSC at RT. The signals were detected immunohistochemically, as described previously (Koji and Brenner 1993, Ulziibat et al. 2006). To examine the level of hybridizable RNAs in the tissue sections, a 28S rRNA probe was used as a positive control (Yoshii et al. 1995).

Transfection of ERβ siRNA by electroporation

Custom-synthesized ERβ siRNA (Invitrogen) annealed duplexes, nucleotide no. 982-1006, GenBank accession no. U81451 (Tremblay et al. 1997) (antisense 5′-AAGAUUUCCAGAAUCCCUUCCACGC-3′, sense 5′-GCGUGGAAGGGAUUCUGGAAAUCUU-3′) were used for gene knock-down. Scrambled siRNA, with the same nucleotide content, but without sequence homology to ERβ gene, was used as a negative control. After OVX, E_2 was injected into mice on day 1, 5, 9 and 12. 24 h after the last E_2 injection, mice were anesthesized and the abdominal cavity was opened. Control and ERβ siRNA were injected into the colonic lumen of mice (n=3, each), while holding the two edges with forceps. Three electric pulses (poring pulse: 40 V, 30 ms of length with 50 ms interval, transfer pulse: 10 V, 50 ms of length with 50 ms interval) were delivered into the colon using Nepa21 electroporator (Nepa Gene, Chiba, Japan). Colon tissues were excised 24 h after the electroporation.

Quantitative analysis

For quantitative analysis, more than 2,000 cells were counted in random fields at ×400 magnification, and the number of ERβ-positive cells was expressed as the percentage of the

positive cells per total number of epithelial cells. For image analysis of ERβ mRNA, *red* color was assigned to the positive cells by a computer-assisted image analyzer (DAB analysis system; Carl Zeiss, Göttingen, Germany). Positive cells were evaluated based on the staining density over the level of staining with the sense probe. Densitometric analysis was performed for western blots using DAB analysis system. β-actin was used as an internal standard, for normalization of the target protein expression, in each lane.

Statistical analysis

All data were expressed as mean±SD. Statistical significance was assessed by Student's *t*-test or one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test for multiple comparisons where appropriate. $P<0.05$ was considered statistically significant. All analyses were performed with The Statistical Package for Social Sciences (version 11.5; Chicago, IL, USA).

Results

ERβ expression in pregnant mouse colon

First, we used immunohistochemistry to determine the expression of ERα and ERβ in the colon of normal female and pregnant mice at pregnancy day 18. Fig. 1a provides a macrophotography of the mouse colon. The proximal, middle and distal parts of the colon were stained with H&E (Fig. 1b-d) and immunohistochemistry. ERβ was expressed in the epithelium of the proximal colon of pregnant mice (Fig. 1e), but not in the middle and distal colon (Fig. 1f, g). Furthermore, ERβ was not detected in the colonic epithelial cells of non-pregnant female mice, irrespective of the stage of estrus cycle (Fig. 1h). Further analysis revealed lack of ERβ expression in the proximal colon at pregnancy day 1 (Fig. 1i). The control sections reacted with normal rabbit IgG were negative (Fig. 1j). In all mice, no expression of ERα was detected except in neuronal plexuses (data not shown).

Pregnancy-dependent ERβ expression in mouse proximal colon

The expression of ERβ protein in the proximal colon was analyzed by western blotting at various time-points of pregnancy. As shown in Fig. 2a, a single band corresponding to 55 kDa was detected in the proximal colon, and the amount of ERβ protein reached a maximum at pregnancy day 18. In this experiment, ovarian tissue was used as a positive control. The loading of equal amount of total protein was confirmed by β-actin (42 kDa). Densitometric analysis of ERβ bands using the DAB image analyzer system showed a significant increase in pregnancy day 18 (Fig. 2b). ERβ-positive cells appeared at day 10 and their number increased markedly at pregnancy days 15 and 18 (Fig. 2d-g). The ERβ signal was localized predominantly in the nuclei of the surface epithelial cells. Quantitative analysis showed a significant increase in the percentage of ERβ-positive cells at pregnancy days 15 and 18 compared to pregnancy days 5 and 10 (Fig. 2c). Moreover, in parallel with the expression of

ERβ protein, ERβ mRNA was detected in the proximal colon epithelial cells (Fig. 2h-k). No staining was detected when adjacent sections were reacted with the sense probe (Fig. 2l-o). In the ISH experiment, 28S rRNA was used as a positive control for hybridizable mRNA in the tissue section (Fig. 2p-s).

NHE3 expression in the proximal colon during pregnancy

Next, we examined NHE3 expression in the colon of pregnant mice by immunohistochemistry, *in situ* hybridization and western blotting. The expression of NHE3 was observed along the apical membrane of the epithelial cells in the proximal colon. The staining intensity for NHE3 was significantly increased on pregnancy days 15 and 18, compared to that of pregnancy days 5 and 10 mice (Fig. 3a-d). The distal colon was negative for NHE3, and no change in the expression level was evident during pregnancy (Fig. 3e). The control sections reacted with normal rabbit IgG at the same concentrations were negative (Fig. 3f). In accord, NHE3 mRNA was detected in the pregnancy day 18 mouse proximal colon surface epithelial cells (Fig. 3g). NHE3 mRNA and protein expressions were co-localized in the colon surface epithelium. No staining was detected when adjacent sections were reacted with the sense probe (Fig. 3h). 28S rRNA was used for control of hybridizable mRNA in colon tissue (Fig. 3i). The apical membrane of colon surface epithelium was isolated and used for western blotting to analyze the amount of NHE3 protein in proximal and distal colon segments. In western blot analysis, NHE3 (85 kDa) protein was abundant in the proximal colon at pregnancy day 15 and reached a maximum at pregnancy day 18 (Fig. 3j), while only a weak band was detected in the distal colon. Densitometry analysis revealed that the amounts of NHE3 protein at pregnancy days 15 and 18 were 2.4- and 2.7-fold that of day 5, respectively (Fig. 3k).

E_2 increases the expressions of ERβ and NHE3 in proximal colon of OVX mice

To evaluate whether estrogen up-regulates ERβ and NHE3 expression in the proximal colon, adult OVX mice were treated with E_2 or E_2+ICI 182,780, and the expression was examined by immunohistochemistry and western blotting. The colon of control OVX mice injected with vehicle only was negative for ERβ expression and revealed a normal distribution pattern of NHE3 in the epithelial cells (Fig. 4a). As expected, treatment with E_2 induced ERβ expression as well as increased the expression of NHE3 in the proximal colon (Fig. 4b, d). OVX Mice treated with E_2+ICI 182,780 were negative for ERβ, and the NHE3 signal was at basal level similar to that of the control mice (Fig. 4c). However, the expression of ERβ and NHE3 in the distal colon did not change in all the groups (data not shown). The results of immunohistochemistry were confirmed by western blot analysis, i.e., NHE3 was up-regulated by E_2, but no effect was found in vehicle alone or E_2+ICI 182,780 treated groups (Fig. 4d). Densitometric analysis showed 2.5-fold increase in the amount of NHE3 protein in E_2-treated mice, relative to that of vehicle alone mice (Fig. 4e).

Transfection of ERβ siRNA in E_2-treated OVX mice reduces NHE3 expression

To examine the ERβ dependency of NHE3 expression, we performed ERβ knock-down by electroporatic transfection of ERβ siRNA in colon of E_2-treated OVX mice. As shown in Fig. 5a, transfection of scrambled siRNA into the proximal colon did not change ERβ or NHE3 expression. However, in ERβ knock-down colon, complete loss of ERβ expression and marked reduction of NHE3 expression were observed (Fig. 5b). Both ERβ and NHE3 protein amounts were examined by western blot analysis. In ERβ knock-down colon, the expression of ERβ and NHE3 were decreased to the basal level (Fig. 5c). Densitometry analysis revealed that the amount of these proteins were decreased to 51% and 64% compared to the control, respectively (Fig. 5d, e).

Discussion

The major finding of this study was the estrogen-dependent expression of ERβ and the parallel increase in NHE3 expression in the surface epithelial cells of proximal colon of mice. Moreover, the increase in NHE3 expression seemed to depend on ERβ, but not ERα. These results indicate that up-regulation of NHE3 expression by estrogen via ERβ may be one of the reasons for constipation during pregnancy.

The expression of ERβ in surface epithelial cells of the proximal colon during late pregnancy suggests that high concentration of circulating estrogen during pregnancy induces such expression. Although the sex difference in ERβ mRNA expression in human and mouse colon suggests the estrogen dependency of ERβ expression in the colon, there is no direct evidence that estrogen regulates the expression of ERβ (Campbell-Thompson et al. 2001, Looijer-van Langen 2011). The expression of ERβ mRNA and protein were up-regulated by treatment with E_2 in OVX mice and this up-regulation was canceled when E_2 was combined with ER antagonist, ICI 182,780. This finding demonstrates that estrogen regulates the expression of ERβ in the mouse proximal colon. The functional role of ERβ in the colon was suggested in mice and rats. ERβ knocked-out mice showed higher proliferation rate, decrease in apoptosis and expression of cellular adhesion molecules in the colon epithelium compared with normal mice (Wada-Hiraike et al. 2006). Furthermore, estrus cycle-dependent variations in colonic paracellular permeability were reported in the rat colon, and estrogen dependency of this variation was confirmed by treatment with estrogen agonist and antagonist after OVX (Braniste et al. 2009). Moreover, the role of estrogen in proximal colon is also inferred by the lower incidence of proximal colon cancer in young women than men and old women (Koo et al. 2008). In these studies, however, it remains to be elucidated whether the effect of estrogen on the colon is directly mediated through ERβ expression in the colonic epithelium. In vivo electroporation of ERβ siRNA in E_2-treated mouse colon after OVX showed that ERβ

directly regulated the expression of sodium transporter, NHE3 in the mouse proximal colon. Absorption is the major function of colon and constipation is reported by up to 40% of pregnant women (Bradley et al. 2007). Therefore, there is a need to investigate the effect of estrogen in the regulation of sodium and water absorption.

ERβ, but not ERα, was detected in the colon surface epithelium. This finding indicates that ERβ is the predominant ER type in the colon, in agreement with previous reports (Campbell-Thompson et al. 2001, Papaxoinis et al. 2010, Wada-Hiraike et al. 2006). However, Wada-Hiraike et al. (2006) found ERβ expression throughout the colon of non-pregnant mouse, while we detected the expression only in the proximal colon of pregnant mouse. Though it is difficult to explain the inconsistency, in this study, the specific localization of ERβ mRNA and protein expressions in the proximal colon was confirmed repeatedly by *in situ* hybridization and immunohistochemistry, respectively, in the pregnant mice. Other studies have also described the function and expression of ERβ in non-pregnant rat and human (Braniste et al. 2009, Koo et al. 2008). Thus, the basal level of ERβ expression in the colonic epithelium seems to be species-dependent. Nevertheless, the important finding is that estrogen upregulates NHE3 expression through ERβ in the proximal colon. It is well known that down-regulation of ERβ correlates with progression of colon cancer (Campbell-Thompson et al 2001, Castiglione et al. 2008). During the reproductive age, the incidence of proximal colon cancer is lower in women compared with men, and the incidence rate increases progressively in women after menopause (Koo et al. 2008). This finding suggests a specific role for estrogen in the proximal colon in human. In this study, ERβ expression was detected at pregnancy day 5 by western blotting, while no signals were found in the colonic epithelium by immunohistochemistry. Previous study described ERβ expression in the intestinal nerve cells in mice, irrespective of the pregnancy day (Kawano et al. 2004). Thus, it is assumed that the basal expression level of ERβ at pregnancy day 5 was derived from

intestinal nerve cells, while the increased expression of ERβ at pregnancy days 10, 15 and 18 was from the proximal colon epithelium.

The signals for ERβ and NHE3 were co-localized in the surface epithelium of the proximal colon during pregnancy. It is well known that colonic surface epithelial cells are terminally differentiated and are most functionally active absorptive cells in the crypt (Kunzelmann and Mall 2002). Moreover, NHE3, which is a member of electroneutral NaCl absorption, functions in the surface epithelium. Following active sodium absorption, water absorption is supposed to be increased through osmotically driven paracellular movement (Naftalin and Pedley 1999). Moreover, it is known that the rat (Zhou et al. 2001) and mouse NHE3 promoter region harbors a palindromic ERE with only 1 base pair change from the consensus sequence (GtTCAgtcTGACC), also an additional consensus ERE half-site. Many natural EREs with imperfect palindromic sequences can bind to ERs (Driscoll et al. 1998, Klinge 2001) and also consensus ERE half-sites can bind to ERs (Charn et al. 2010, Marzouk et al. 2008). Therefore, it is not surprising that estrogen may directly regulate the expression of NHE3 in the colon epithelium, through ERβ, indicating the possible involvement of estrogen in water metabolism in the colon.

In the present study, ERβ knock-down reduced NHE3 expression in the proximal colon of E_2-treated OVX mice. The level of NHE3 expression was significantly decreased after 24 h of electroporaion. The half-life of NHE3 had been reported to be 14 h in intestinal epithelial cells (Cavet et al. 2001). Therefore, protein degradation could be explain the mild but significant decrease of NHE3 expression observed in western blotting. In addition, the membrane protein could internalize quickly from the apical membrane to sub-apical region, such as NHE3 and AQP2 could internalize within 1 h and 2 h, respectively (Clayburgh et al. 2006, Tajika et al. 2004). Thus, it is possible that NHE3 was internalized to sub-apical region after ERβ knock-down and signal in the immunostaining was clearly disappeared from the

apical membrane within 24 h. These results suggest that there are two patterns of NHE3 expression; one is estrogen-independent and involved in setting the basal level of NHE3, and the other is estrogen-dependent. The estrogen-dependent one may be specific to the proximal region. Various sodium transporters are differentially expressed in the colon, such as the β-subunit of epithelial sodium channel localized in the proximal colon and involved in sodium absorption (Greig et al. 2002). In addition, short chain fatty acid stimulates sodium absorption prominently in the proximal colon (Sandle 1998, Kiela et al. 2007). The specific expression of NHE3 in the proximal colon was consistent with the previous report (Talbot and Lytle 2010). Our findings suggest a new functional role for ERβ expression in the regulation of NHE3 in the proximal colon. In human, as in rodents, the proximal colon appears to have a great capacity for sodium and water absorption, compared with the distal colon (Sandle 1998, Talbot and Lytle 2010). The morphological features of the proximal colon provide various advantages for excellent absorption, such as 2.8 times more capillary layers and 2.2 times large area of the collecting venules in the human ascending colon compared to that of sigmoid colon (Araki et al. 1996). Therefore, the rate of sodium and water absorption in the proximal colon seems to influence constipation.

The causes of constipation in pregnancy appear to be multi-factorial, such as hormonal effects on intestinal transit time, increased water absorption, dietary and mechanical factors (Cullen and O'Donoghue 2007). In the present study, we found that the involvement of ERβ in the regulation of NHE3 in the proximal colon during pregnancy would be one of the possible influencing factors. Although our results did not provide direct evidence on the role of NHE3 in constipation, these findings could form the basis for the design of new therapies for constipation in pregnant women.

In conclusion, the present study demonstrated that estrogen regulates the expression of NHE3 via ERβ in the proximal colon during pregnancy, suggesting the involvement of NHE3 up-regulation in constipation through increased sodium absorption.

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Figure legends:

Fig. 1. ERβ expression in pregnant mouse colon

Immunohistochemical localization of ERβ in normal and pregnant mice colon sections. Paraffin-embedded sections of normal and pregnant mice colon were used for H&E staining and immunohistochemistry. (**A**) Macrophotography of mouse colon and schematic representation of proximal, middle and distal colon. H&E staining of pregnancy day 18 mouse proximal (**B**), middle (**C**), distal (**D**) part of colon. Immunohistochemistry for ERβ in pregnancy day 18 mouse proximal (**E**), middle (**F**), distal (**G**) part of colon. (**H**) Non-pregnant (estrus stage) and pregnancy day 1 mouse proximal colon (**I**). (**J**) Pregnancy day 18 mouse proximal colon section was incubated with normal rabbit IgG as a negative control. Magnification (**B-D**) ×100; (**E-J**) ×400. Scale bar (**B-D**) 120 µm, (**E-J**) 30 µm.

Fig. 2 Pregnancy-dependent ERβ expression in proximal colon.

Western blot analysis of ERβ in pregnancy day 5-18 mice proximal colon. Twenty micrograms of extracts from proximal colon were subjected to SDS-PAGE. The loading of equal amount of total protein was confirmed by β-actin. (**A**) ERβ in pregnancy day 5-18 mice proximal colon. Arrows are indicating ERβ (55 kDa) and β-actin (42 kDa). (**B**) Densitometry analysis of western blot. ERβ protein expression was normalized by β-actin, in each lane. Data represent the mean ± SD of three independent experiments. (**C**) Quantitative analysis of ERβ-positive cells on proximal colon of pregnancy day 5-18 mice (n=3). ERβ protein and mRNA expressions in adjacent sections of pregnant mouse proximal colon. Adjacent sections of paraffin-embedded mouse colon were used for immunohistochemistry and *in situ* hybridization. Immunohistochemical localization of ERβ in proximal colon of pregnancy day 5, 10, 15 and 18 mice (**D-G**). ERβ-antisense digoxigenin-labeled oligo-DNA probe in proximal colon of pregnancy day 5-18 mice (**H-K**). The *red* color represents positive cells as

638 determined by DAB image analyzer. Antisense and sense slides were analyzed in same
639 condition and positive cells were evaluated based on the staining density over the level of
640 staining with the sense probe. ERβ-sense digoxigenin-labeled oligo-DNA probe in proximal
641 colon of pregnancy day 5-18 mice (**L-O**). 28S rRNA complementary thymine-thymine (T-T)
642 dimerized oligo-DNA probe (**P-S**). *P<0.05, **P<0.01, Magnification ×400. Scale bar = 30
643 μm.
644
645 **Fig. 3** NHE3 expression in proximal colon during pregnancy.
646 Immunohistochemical localization of NHE3 in paraffin-embedded sections of mouse colon.
647 (**A-D**) Localization of NHE3 in proximal colon of pregnancy day 5, 10, 15 and 18 mice.
648 Arrows are indicating increased expression of NHE3 in proximal colon surface epithelium.
649 Cellular nuclei were counterstained with hematoxylin. (**E**) Immunostaining for NHE3 in
650 distal colon of pregnancy day 18 mouse. (**F**) Pregnancy day 18 mouse proximal colon section
651 was incubated with normal rabbit IgG as a negative control. NHE3 mRNA expression in
652 pregnancy day 18 mouse colon. Adjacent sections were hybridized with antisense (G), sense
653 (H) and 28S rRNA (I) probes. Positive cells (*red*) were evaluated based on the staining
654 density over the level of staining with the sense probe. (**J**) Western blot analysis of NHE3 in
655 pregnancy day 5-18 mice proximal and distal colon. The apical membrane of colon surface
656 epithelium was isolated from proximal and distal colon as described in detail in “Materials
657 and methods”. Twenty micrograms of isolated preparations were subjected to SDS-PAGE.
658 Arrows are indicating NHE3 (85 kDa) and β-actin (42 kDa). (**K**) Densitometry analysis of
659 western blots. NHE3 protein expression was normalized by β-actin. Data represent the mean
660 ± SD of three independent experiments. *P<0.05, Magnification ×400. Scale bar = 30 μm.
661

Fig. 4 Up-regulated ERβ and NHE3 expression in proximal colon of E_2-treated mouse. Double-staining for ERβ and NHE3 in paraffin embedded sections of mouse proximal colon. Paraffin embedded sections were first processed for NHE3 immunostaining, followed by ERβ immunostaining. Corn oil (**A**), E_2 (**B**) and E_2+ICI 182,780 (**C**) treated ovariectomized mice proximal colon. Negative control of both antibodies was shown in inset (**A**). Arrows (ERβ) and arrowheads (NHE3) are indicating positive cells. NHE3-positive cells were stained *brown* (DAB), whereas ERβ-positive cells were stained *purple-blue* (4-Cl-1-Naphtol). (**D**) Western blot analysis of NHE3 in proximal colon. The apical membrane of colon surface epithelium was isolated from E_2, E_2+ICI and corn oil treated OVX mice and twenty micrograms of lysates were subjected to SDS-PAGE. Arrows are indicating NHE3 (85 kDa) and β-actin (42 kDa). (**E**) Densitometry analysis of western blot. NHE3 protein expression was normalized by β-actin. Data represent the mean ± SD of three independent experiments. *$P<0.05$, Magnification ×400. Scale bar = 30 μm.

Fig. 5 ERβ silencing reduces NHE3 expression in proximal colon

Double-staining for ERβ and NHE3 in paraffin embedded sections of ERβ siRNA and scrambled siRNA electroporated mouse proximal colon. Paraffin-embedded sections of proximal colon were first processed for NHE3 immunostaining and positive cells were stained *brown* (DAB), followed by ERβ immunostaining and positive cells were stained *purple-blue* (4-Cl-1-Naphtol). (**A**) Scrambled siRNA electroporated mouse proximal colon. (**B**) ERβ siRNA electroporated mouse proximal colon. Magnification ×400. Scale bar = 30 μm. (**C**) Western blot analysis of ERβ and NHE3 in proximal colon. Proteins were extracted from scrambled and ERβ siRNA electroporated mouse proximal colon and twenty micrograms of sample lysates were subjected to SDS-PAGE. Arrows indicate bands for ERβ (55 kDa), NHE3 (85 kDa) and β-actin (42 kDa). Densitometry analysis of western blot ERβ

687 (**D**) and NHE3 (**E**) protein expression were normalized by β-actin. Data represent the mean ±

688 SD of three independent experiments. *P<0.05.

Fig. 1

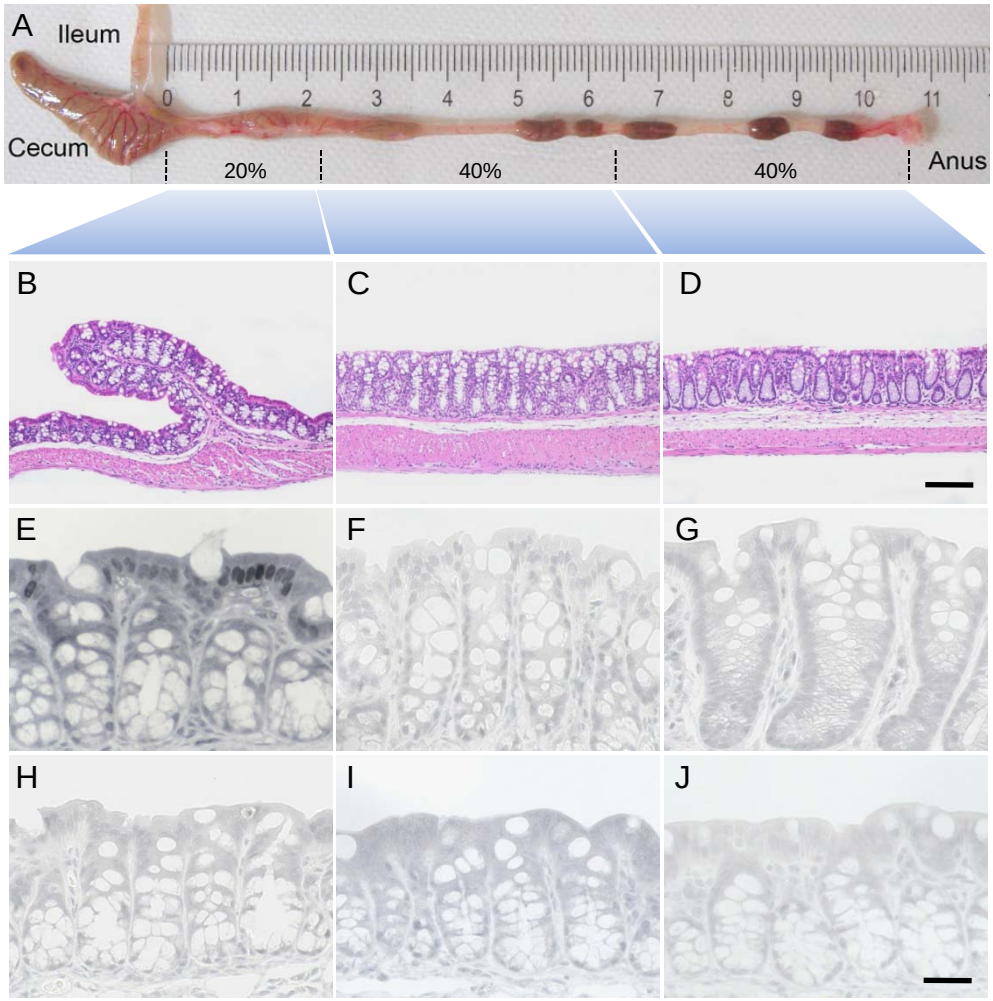


Fig. 2

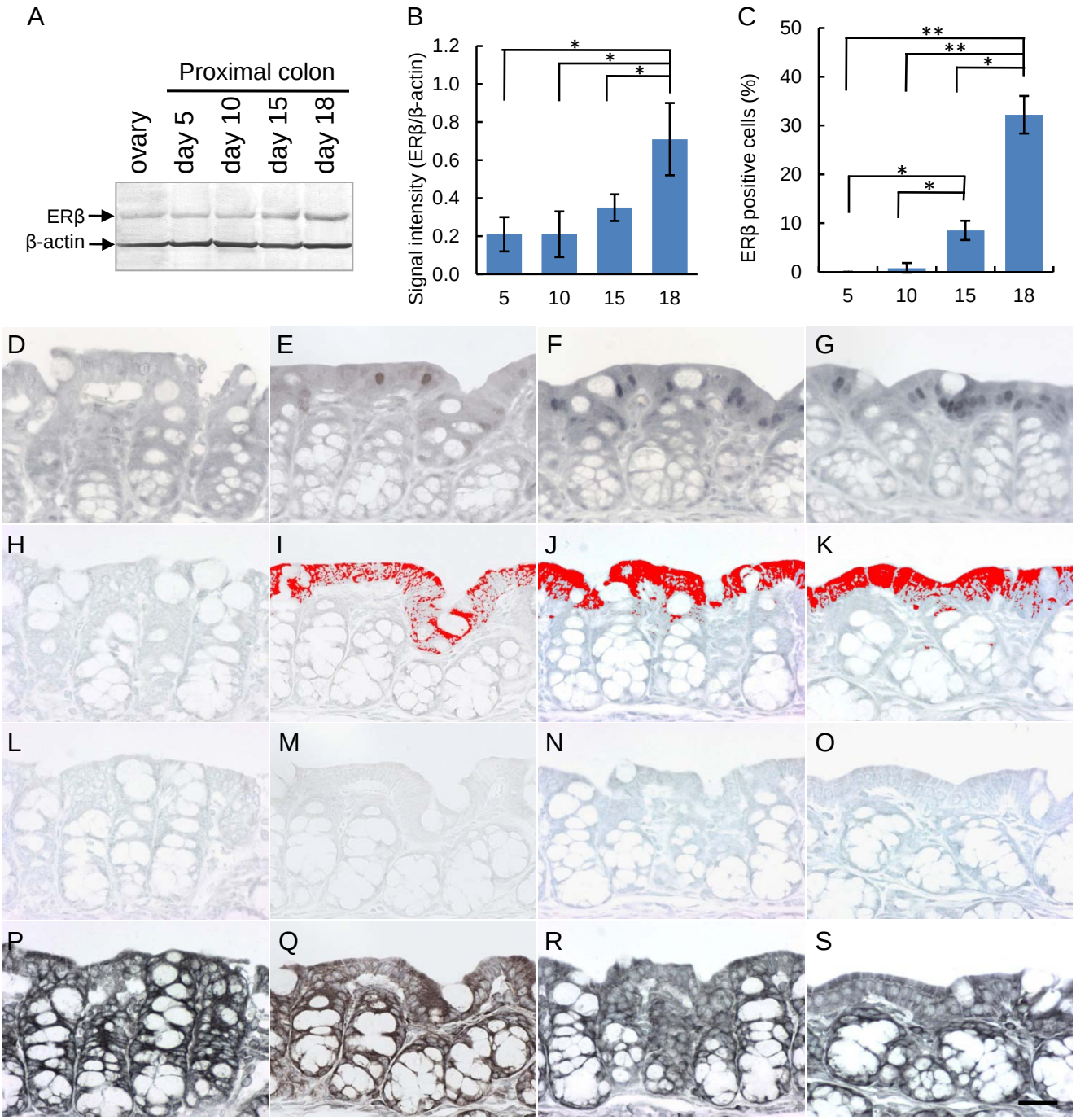
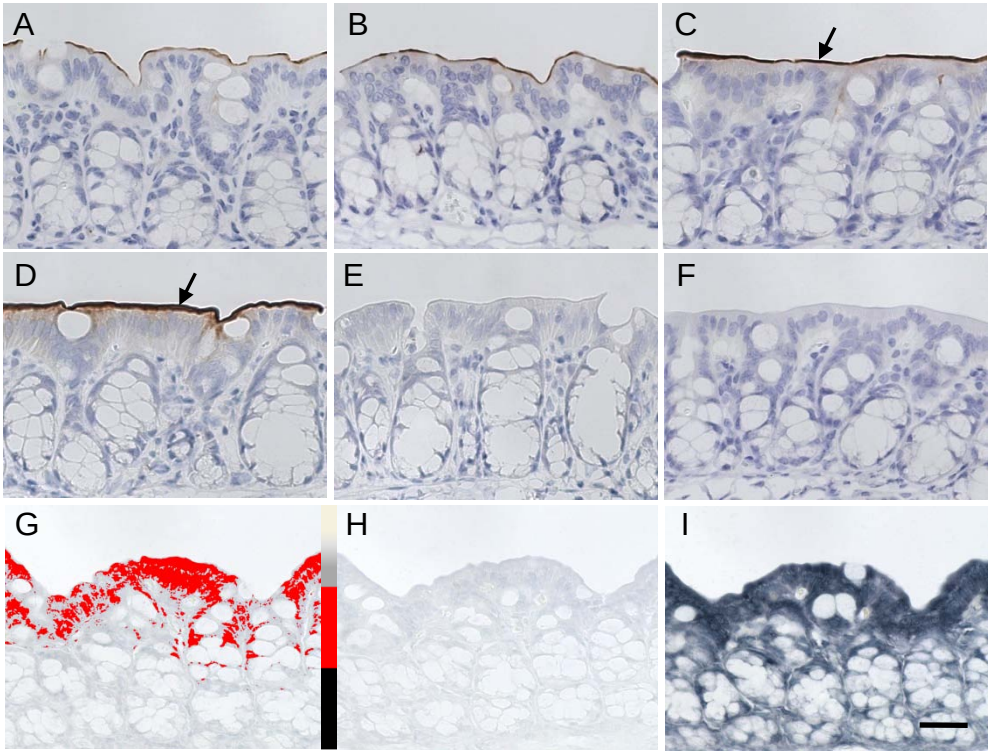
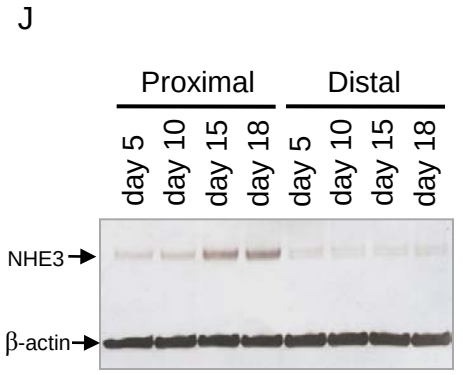

A
Proximal colon
ovary
day 5
day 10
day 15
day 18
ERβ
β-actin
B
Signal intensity (ERβ/β-actin)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
5
10
15
18
C
ERβ positive cells (%)
50
40
30
20
10
0
5
10
15
18
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S

Fig. 3


A
B
C
D
E
F
G
H
I


J
Proximal
Distal
day 5
day 10
day 15
day 18
day 5
day 10
day 15
day 18
NHE3
β-actin

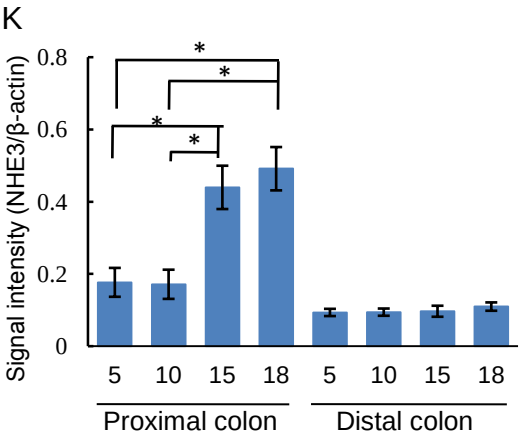

K
Signal intensity (NHE3/β-actin)
0.8
0.6
0.4
0.2
0
*
*
*
*
5
10
15
18
5
10
15
18
Proximal colon
Distal colon

Fig. 4

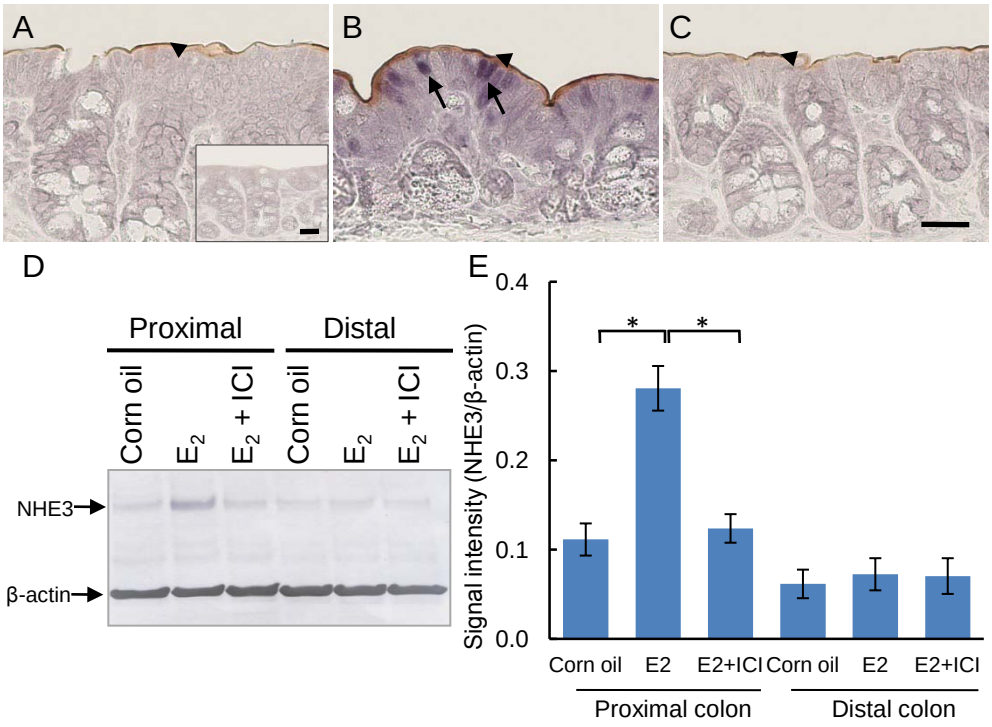
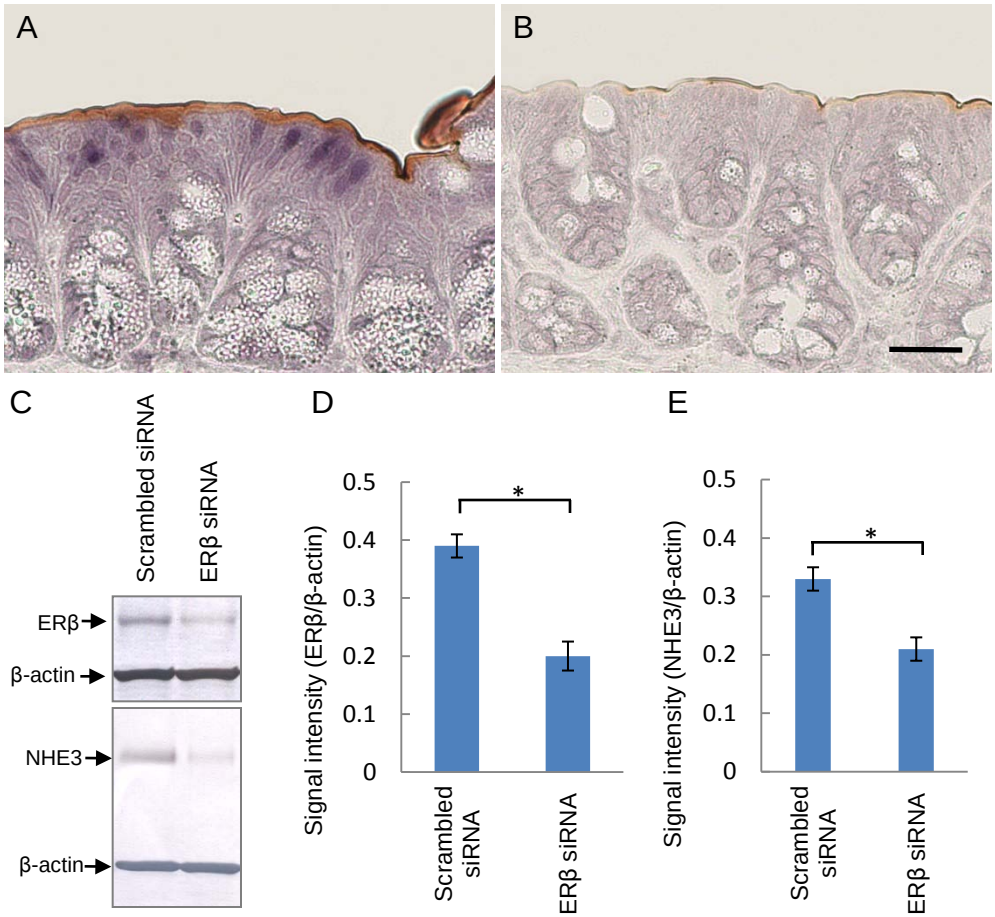

A
B
C
D
Proximal
Distal
Corn oil
E2
E2 + ICI
Corn oil
E2
E2 + ICI
NHE3
β-actin
E
Signal intensity (NHE3/β-actin)
0.4
0.3
0.2
0.1
0.0
*
*
Corn oil
E2
E2+ICI
Corn oil
E2
E2+ICI
Proximal colon
Distal colon

Fig. 5


A
B
C
Scrambled siRNA
ERβ siRNA
ERβ
β-actin
NHE3
β-actin
D
Signal intensity (ERβ/β-actin)
0.5
0.4
0.3
0.2
0.1
0
*
Scrambled siRNA
ERβ siRNA
E
Signal intensity (NHE3/β-actin)
0.5
0.4
0.3
0.2
0.1
0
*
Scrambled siRNA
ERβ siRNA