Progesterone and heparin-binding epidermal growth factor-like growth factor regulate the expression of tight junction protein Claudin-3 during early pregnancy

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Objective: To determine Claudin-3 expression and its regulatory factors during embryo implantation.
Design: Experimental mouse models and cell culture.
Setting: University research laboratory.
Animal(s): Sexually mature female CD-1 strain mice.
Intervention(s): Ovariectomy and treatments.
Main Outcome Measure(s): In situ hybridization and immunohistochemistry for detecting Claudin-3 messenger RNA and protein expression in mouse uterus, respectively; Western blot for detecting protein levels; immunofluorescence for detecting Claudin-3 protein in cultured cells.
Result(s): Claudin-3 is strongly expressed in the uterine luminal epithelium on days 3 and 4 of pregnancy, and diminished at day 5 implantation sites. Then it is expressed at secondary decidual zone on day 8. Pseudopregnant uteri have a similar expression pattern as pregnant uteri from days 1–5. Claudin-3 expression is down-regulated after delayed implantation is activated by estrogen (E) treatment. Meanwhile Claudin-3 expression is stimulated by artificial decidualization. In ovariectomized mice, P induces Claudin-3 expression in the luminal epithelium, which is abrogated by P receptor antagonist RU486. Heparin-binding–epidermal growth factor (HB-EGF) down-regulates Claudin-3 expression, but enhances transcription factor Snail expression. In human endometrial epithelial ECC-1 cells, both E and P could stimulate Claudin-3 expression, whereas HB-EGF decreases Claudin-3 and increases Snail expression.
Conclusion(s): Claudin-3 expression in uterine luminal epithelium is stimulated by P and suppressed by HB-EGF in mice and humans. (Fertil Steril® 2013;100:1410–8. ©2013 by American Society for Reproductive Medicine.)
Key Words: Uterus, embryo implantation, Claudin-3

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Embryo implantation is a dynamic interaction between activated blastocyst and receptive uterus. However, the molecular basis of the interaction between blastocyst and maternal uterus remains largely unknown (1, 2). During mouse early pregnancy, the uterus creates a permeability barrier to the diffusion of solutes trafficking toward the developing fetus. The barrier is initially accomplished by the receptive luminal epithelium around hatched blastocysts, and then replaced by differentiated decidualized stromal cells (3). Ovarian estrogen (E) and progesterone (P) directing epithelial cell proliferation and stromal cell differentiation are vital for successful

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implantation during early pregnancy (4, 5). Heparin-binding–epidermal growth factor (HB-EGF), a member of epidermal growth factor (EGF) family, has pivotal effects on blastocyst apposition (6, 7) and the induction of stromal cell differentiation (8–10). But the function of HB-EGF in regulating uterine epithelial junctions is still unclear.

Dynamic modification of tight junctions is responsible for the formation of the permeability barrier in polarized epithelial cells and facilitates substance transportation. Claudin-3 (*Cldn3*) is a transmembrane protein that belongs to a large family of 24 closely related members (11). Claudin family functions through homophilic and heterophilic interactions, and is essential for the formation, integrity, and function of tight junctions, epithelial permeability barrier, and epithelial polarization (11). Claudins hold cells together and participate in processes that pattern cells into tissue for their normal physiology including organogenesis, tissue organization, embryo development, and cancer progression (12–18). Claudin-3 is mainly expressed in the luminal epithelium in rat uterus during the estrous cycle (14). In human endometrium, Claudin-3 expression is predominantly localized to the glandular epithelial cell membrane. Furthermore, Claudin-3 is strongly expressed in atypical hyperplasia and endometrioid adenocarcinoma, but less frequently in normal endometrium (19).

Our preliminary microarray data showed that a strong expression of *Cldn3* on days 3 and 4 of pregnancy in mice, suggesting that Claudin-3 may be involved in blastocyst implantation. In the present study, we showed that Claudin-3 is strongly expressed in the luminal epithelium on days 3 and 4 of pregnancy and regulated by P and HB-EGF during embryo implantation.

MATERIALS AND METHODS

Animals and Treatments

Adult CD-1 female mice were mated with fertile or vasectomized males of the same strain to induce pregnancy or pseudopregnancy (day 1 = day of vaginal plug). Implantation sites were identified as previously described (20). All animal procedures were approved by the Institutional Animal Care and Use Committee of Shantou University.

To induce delayed implantation, pregnant mice were ovariectomized under anesthesia between 8:30 and 9:00 AM on day 4 of pregnancy. Progesterone (1 mg/mouse; Sigma) was injected to maintain delayed implantation from days 5–7. Estradiol-17β (25 ng/mouse; Sigma) was administrated to P-primed delayed implantation mice to terminate delayed implantation. The mice were sacrificed to collect uteri 24 hours after E treatment. Delayed implantation was confirmed by flushing blastocysts from one horn of the uterus.

Artificial decidualization was induced by intraluminally infusing 10 μL of sesame oil into one uterine horn on the day 4 morning of pseudopregnancy. The contralateral horn served as a control. The uteri were collected on day 8.

Two weeks after ovariectomy, mice were treated with SC injections of E_2-17β (100 ng/mouse), P (1 mg/mouse), or a combination of the same doses of E_2-17β and P dissolved in sesame oil. For RU486 treatment, pregnant mice were injected with RU486 twice at 9 PM on day 2 and at 9 AM on day 3. The uteri were collected at 9 AM on day 4.

In Situ Hybridization

Total RNAs from mouse uteri on day 4 of pregnancy were reverse transcribed and amplified with the corresponding primers (5′-CCTTCATCGGCAGCAGCATC and 5′-GGCAG GAGCAACACAGCAAG). The amplified fragment was cloned into pGEM-T plasmid (Promega) and then amplified with the primers for T7 and SP6 to prepare the templates for labeling antisense probe. Digoxigenin-labeled antisense complementary RNA probe was transcribed in vitro using digoxigenin RNA labeling kit (Roche Diagnostics).

In situ hybridization was performed as previously described (20). Briefly, liquid nitrogen-frozen uteri were cut into 10-μm frozen sections, mounted on 3-aminopropyltriethoxy-silane (Sigma)-coated slides, and fixed in 4% paraformaldehyde (Sigma) solution. All of the sections were counterstained with 1% methyl green and the positive signal was visualized as dark brown.

Immunohistochemistry

Immunohistochemistry was performed with paraffin-embeded sections. Briefly, endogenous horseradish peroxidase activity of the sections was blocked in 3% hydrogen peroxide for 15 minutes. The sections were incubated with rabbit anti-Claudin-3 antibody (Invitrogen) or rabbit anti-green fluorescence protein antibody (Santa Cruz Biotechnology) in 10% horse serum overnight at 4°C and followed by biotinylated secondary antibody and streptavidin-horseradish peroxidase complex. The positive signals were visualized using DAB Horseradish Peroxidase Color Development Kit according to the manufacturer's protocol (Zhongshan Golden Bridge Bio-technology) as a reddish-brown color.

Isolation of Uterine Luminal Epithelial Sheets

Luminal epithelium was isolated by mild enzymatic digestion and mechanical methods from pseudopregnant mice at 4 PM on day 4, as described previously (21, 22). Briefly, mouse uteri were isolated, washed, and cut into short fragments. After a digestion in the fresh medium (Hanks' balanced salt solution [HBSS] with antibiotics; Sigma) containing 1.2 mg/mL dispase (Roche) and 10 mg/mL trypsin (Sigma) at 4°C for 90 minutes and at room temperature for 30 minutes, the tissues were washed with HBSS and incubated in HBSS containing 1% fetal bovine serum (Bovogen) for 30 minutes. After brief shaking in fresh medium, luminal epithelial sheets were collected from the supernatants with a mouth-controlled pipette under a stereomicroscope. Only the pieces in a single layer were collected to avoid the contamination of glandular epithelium and suspended cells. Then the supernatants were centrifuged for 5 minutes at 200 × g to remove any attached uterine stromal cells. These sheets were washed with HBSS and cultured in suspension with Opti-medium (Invitrogen) for 1 hour, and then were treated with HB-EGF (100 ng/mL; Sigma) in Opti-medium.

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