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Placental claudin expression and its regulation by endogenous sex steroid hormones

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ABSTRACT

Tight junctions (TJs) form continuous intercellular contacts controlling the paracellular transportation across the cell-to-cell junction. TJ components include the peripheral protein zonula occludens-1 (ZO-1), junctional adhesion molecules (JAMs), and integral proteins such as occludin and claudins. Among the junction proteins, claudins play a major role in regulation of paracellular electrolyte transportation. This study explores the expression and distribution of tight junctions and their regulation during pregnancy. To study the regulation of claudin family, we examined expression of mouse placental tight junction proteins, including claudin-1 to -24, with real-time PCR and Western blotting and distribution of tight junction proteins with immunohistochemistry. Pregnant C57/BL6 mice were used in this study. The pregnant mice were divided into three groups depending on pregnant day (on days 12, 16, and 20 of gestation). Regarding the transcription levels, claudin-1, claudin-2, claudin-4, and claudin-5 expression levels were relatively high compared to other claudin family in all periods of pregnancy. Claudin-4 and 5 expressions, which reduce ion permeability, were increased over a period of time. However, claudin-2 expression, that is the responsive protein for a decrease in paracellular conductance, was decreased. Following this modulation of expression during mid-term pregnancy, we identified endogenous hormonal modulation of claudin family using estrogen receptor antagonist ICI 182,780 and progesterone receptor antagonist RU-486. After administration of ICI and RU-486, expression of claudin-4 mRNA and protein was increased. In addition, immunohistochemistry was performed to identify their localization for inferring permeability in placenta. Due to the function of claudins as effectors of ion transport at the end of regulatory pathways, they must be transducing proteins that modulate the function of claudins and thus link the physiologic inputs to the final effectors. This study will provide the claudin expressions and their localization in the mouse placenta, and their regulation by endogenous hormones. Taken together, the results of this study may contribute to assuming the roles and regulatory mechanism of these tight junction genes regarding maternal-fetal ion transportation in the placenta.

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1. Introduction

Placenta is essential tissue for development of embryo and fetus. It plays an important role in adequate delivery of oxygen, nutrients from dam to fetus. It also acts as a front-line barrier against hazardous materials or pathogens from maternal circulation. Placenta regulates the transfer of materials between the mother and the developing fetus. Survival and growth of the fetus are critically dependent on the placenta [1]. Development of the placenta depends on the differentiation of trophoblast cells, the epithelial cells of the placenta. Mouse has a fully developed placenta composed of three major layers. Histologically, the outer maternal layer includes decidual cells of the uterus as well as the maternal vasculature, which brings blood to/from the implantation site. A middle "junctional" region attaches the fetal placenta to the uterus and contains feto-placental (trophoblast) cells that invade the uterine wall and maternal vessels, and an inner layer is composed of highly branched villi designed for efficient nutrient exchange [2]. Continuous endothelium or epithelium has two different routes of cell junction pathway: One is transcellular, by means of transcytosis, and the other is paracellular, via intercellular junctions, such as tight junctions [3]. The transepithelial electric resistance of epithelium from different tissues varies by two orders, which is thought to reflect variation in the permeability of tight junctions [4]. Tight junctions show charge and size selectivity in their permeability. Ionic transport across tight junctions is mainly cation-selective. However, in a few cases, some tight

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junctions are anion-selective [5]. Non-charged matter, such as water or glucose, also moves across tight junctions [6,7].

Calcium is transported across epithelia by those two transport mechanisms. The transportation is regulated by a complex constituent mediated by hormonal, developmental and physiological factors [8,9]. Expression of calcium-associated junctional proteins, transient receptor potential, vanilloid, 6(TRPV6), transient receptor potential, vanilloid, 5 (TRPV5), and Sodium–calcium exchanger 1 (NCX1) were induced in both maternal and fetal placenta, and these genes regulate placental calcium binding proteins such as s100g (also known as Calbindin-$_D$9k, CaBP-9k) [10]. These findings suggest that calcium may be transported by transcellular pathway in the placenta. Paracellular transport is also responsible for calcium transportation in placenta. For example, claudin-1 and -2 are responsible for the paracellular water channel in the placenta [11,12], and claudin 2, 4, and 7 are powerful effectors of paracellular permeation. Knockdown of claudin-2 resulted in depressed ion permeation, and loss of claudin-4 and -7 increase permeation and epithelial resistance [13]. Claudins have been studied to show ion selectivity to the paracellular pathway. Claudin-4, -5, -8, -11, and -14 showed selective depression in the permeability of cations through tight junctions, while the permeation of anion is largely unchanged [14–17]. The primary role of claudin family is regulation of paracellular selectivity for ions. Claudins build functionally equivalent-charge selective pores for promoting selective ion permeation. Interestingly, claudin-4 increases transepithelial resistance (TER), although other claudins generally increase rather than restrict paracellular permeation [13,18,19]. To date, more than 24 claudins have been identified. Claudins are 20–27 kDa tetra-transmembrane proteins, which have cytoplasm-oriented N- and C-termini and two extracellular loop domains [20,21]. However, currently, there are only a few claudin regulation mechanisms. Kobayashi et al. showed that progesterone maintains amniotic tight junctions during mid-pregnancy in mice [22], and Burek et al. demonstrated that claudin-5 is regulated by estrogen in vascular endothelium.

In this study, we aimed to determine which claudins influence mid-term pregnancy in mice placenta, and how these claudins are regulated by physiological factors during pregnancy. First, we identified the presence of claudins in GD12, 16, 20 mouse placentas. An estrogen receptor (ER) antagonist ICI 182,780 and a progesterone receptor (PR) antagonist RU-486 were administered to pregnant mice to block endogenous ER/PR pathway in mouse placenta *in vivo*. The effects of these endogenous hormonal factors on placental tight junctions, particularly in claudins were also investigated with Realtime PCR and Western blotting.

2. Materials and methods

2.1. Materials

ICI 182,780 was purchased from Tocris (Avonmouth, UK). RU-486 was purchased from Sigma–aldrich Corp (St. Louis, MO).

2.2. Animal experiments

In Experiment I, mature (>6 weeks old) female ICR mice were obtained from Koatech (Pyungtaek, Gyeonggi, Korea). All animals were housed in polycarbonate cages and acclimatized in an environmentally controlled room (temperature: 23 ± 2 °C, relative humidity: 50 ± 10%, frequent ventilation, and 12:12-h light–dark cycle) before use. Mature mice (>6 weeks old) were mated with Mature male mice (>8 weeks old) overnight. The following morning the presence of vaginal plug was examined, and the day was designated as day 0 pregnancy (P0). Mice were sacrificed on GD12, 16 and 20 ($n = 7$ mice per group). Whole placenta was collected for total RNA, protein isolation and tissue fixation.

In Experiment II, female mice were mated as described above and three groups of five animals were injected (s.c) with 10 and 2.5 μg/mouse of RU486 or 2 μg/mouse of ICI 182,780 on pregnancy day 15 (P15) prior to maximum level of hormone, respectively. Dose of steroid antagonists was selected according to the previous studies that reported a significant amount for anti-steroidal effect and not occurring abortion. One group of five animals was administered with 2.5 μg/mouse of RU486 and ICI 182,780 simultaneously. All chemicals were purchased from Sigma–Aldrich Co. (St. Louis, MO) and dissolved in ethanol. Mice injected with vehicle (ethanol) were served as a negative control. The mice were sacrificed at 24 and 48 h after a single injection of steroid antagonists. Institutional Animal Care and Use Committee (ACCUC) of Chungbuk National University approved all animal experimental procedures (Approval N. CBNUA699-14-02).

2.3. Total RNA extraction and quantitative real-time PCR

Mice were sacrificed by cervical dislocation, and their placenta were rapidly excised. After fetus were completely removed, the whole placenta were dissected at different pregnancy and lactation days and washed in cold sterile 0.9% NaCl solution. Total RNA was extracted with Trizol (Life Tech., Rockville, MD) according to manufacturer's suggested procedure, and the concentration of RNA was determined by the absorbance at 260 nm. Total RNA (1 mg)was reverse transcribed into first-strand cDNAs using Moloney murine leukemia virus (MMLV) reverse-transcriptase (iNtRON Bio, Sungnam, Gyeonggi, Korea) and random primers (9-mers; TaKaRa Bio., Inc., Otsu, Shiga, Japan). 2 μl of cDNA template was added to 10 μl of 2SYBR Premix Ex Taq (TaKaRa Bio) and 10 pmol of each specific primer. The reactions were carried out for 40 cycles according to the following parameters: denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 30 s. Fluorescence intensity was measured at the end of the extension phase of each cycle. The threshold value for the fluorescence intensity of all samples was set manually. The reaction cycle at which PCR products exceeded this fluorescence intensity threshold was identified as the threshold cycle in the exponential phase of the PCR amplification. The expressions of claudin family were quantified against that of β-actin. Relative quantification was based on the comparison of CT at a constant fluorescent intensity. The amount of transcript is inversely related to the observed CT, and for every twofold dilution in the transcript, CT is expected to increase by 1. Relative expression was calculated using the equation $R = 2^{-(\Delta CTsample - \Delta CTcontrol)}$.

2.4. Western blot analysis

The placenta of euthanized mice were rapidly excised and washed in cold sterile 0.9% NaCl solution. Protein was extracted with Pro-prep (InTron., Inc., Seoul, Korea) according to the manufacturer's instructions. Total protein (50 mg per lane) was separated on a 10% SDS–polyacrylamide gel electrophoresis (PAGE) and transferred to a polyvinylidene fluoride transfer membrane (Perkin Elmer Co., Wellesley, MA) in a TransBlot Cell (TE-22, Hoefer Co., CA, USA) according to the manufacturer's protocol. The resulting blot was blocked in TBS (Tris-buffered saline) containing 5% skim milk for 60 min, then incubated in primary antibody for 4 h, or β-actin (rabbit-monoclonal, 1:2000, Assay Design, Inc., CA, USA) for 60 min at room temperature. After washing with TBS-T buffer, the membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies (anti-rabbit, 1:2000, Santa Cruz, or anti-mouse, 1:5000, Santa Cruz) for 1 h at room temperature (RT). After washing, the blots were

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