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Changes of Gap and Tight Junctions during Differentiation of Human Nasal Epithelial Cells Using Primary Human Nasal Epithelial Cells and Primary Human Nasal Fibroblast Cells in a Noncontact Coculture System

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Abstract The epithelium of upper respiratory tissues such as nasal mucosa forms a continuous barrier to a wide variety of exogenous antigens. The epithelial barrier function is regulated in large part by the intercellular junctions, referred to as gap and tight junctions. However, changes of gap and tight junctions during differentiation of human nasal epithelial (HNE) cells are still unclear. In the present study, to investigate changes of gap and tight junctions during differentiation of HNE cells *in vitro*, we used primary human HNE cells cocultured with primary human nasal fibroblast (HNF) cells in a noncontact system. In HNE cells cocultured with HNF cells for 2 weeks, numerous elongated cilia-like structures were observed compared to those without HNF cells. In the coculture, downregulation of Cx26 and upregulation of Cx30.3 and Cx31 were observed together with extensive gap junctional intercellular communication. Furthermore, expression of the tight junction proteins claudin-1, claudin-4, occludin and ZO-2 was increased. These results suggest that switching in expression of connexins and induction of tight junction proteins may be closely associated with differentiation of HNE cells *in vitro* and that differentiation of HNE cells requires unknown soluble factors secreted from HNF cells.

Keywords Gap junction · Tight junction · Human nasal epithelial cell · Human nasal fibroblast cell · Coculture

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Introduction

Epithelial-mesenchymal interactions promote normal epithelial differentiation via mediators including various growth factors (Kumar et al., 2005; Rubin, 2007). In lung development and repair, epithelial-mesenchymal interactions have an instructive role (Wartburton & Lee, 1999; Whitsett, 2002). In human nasal epithelial (HNE) cells *in vitro*, human fibroblasts affect the pseudostratified organization (Wiszniewski et al., 2006).

The epithelial barrier of the upper respiratory tract, which is the first site of exposure to inhaled antigens, plays a crucial role in host defense in terms of innate immunity. The epithelium of the upper respiratory tract, such as that of the nasal mucosa, forms a continuous barrier against a wide variety of exogenous antigens (Herard et al., 1996; van Kempen, Rijkers & Van Cauwenberge, 2000). The epithelial barrier is regulated in large part by intercellular junctions, referred to as gap and tight junctions.

Gap junction channels, composed of connexins, mediate reciprocal exchange of ions and small molecules of less than 1 kDa, including the second messengers cyclic adenosine monophosphate, inositol 1,4,5-trisphosphate and Ca^{2+}, between adjacent cells (Saez et al., 1986; Kumar & Gilula, 1996; Kojima et al., 2001). Gap junctional intercellular communication (GJIC) is thought to play a crucial role in development, cell growth and cell differentiation

(Loewenstein, 1979; Yamasaki & Naus, 1996; Trosko & Ruch, 1998). In recent studies, several connexins have been shown to interact with the PDZ-containing tight junction protein ZO-1 (Giepmans, 2004). It is also thought that gap junctions may be closely associated with tight junctions (Kojima et al., 2003; Morita et al., 2004; Go et al., 2006).

Tight junctions, the most apical component of intercellular junctional complexes, separate the apical from the basolateral cell surface domains to establish cell polarity (performing the function of a fence). Tight junctions also possess a barrier function, inhibiting the flow of solutes and water through the paracellular space (Schneeberger & Lynch, 1992; Gumbiner, 1993). They form a particular netlike meshwork of fibrils created by the integral membrane proteins occludin and claudin as well as members of the immunoglobulin superfamilies JAM and CAR (Tsukita, Furuse & Itoh, 2001; Sawada et al., 2003; Schneeberger & Lynch 2004). Several peripheral membrane proteins, ZO-1, ZO-2, ZO-3, 7H6 antigen, cingulin, symplekin, Rab3B, Ras target AF-6 and ASIP (an atypical protein kinase C-interacting protein), have been reported (Tsukita et al., 2001; Sawada et al., 2003; Schneeberger & Lynch 2004). The claudin family, consisting of 24 members, is solely responsible for forming tight junction strands (Tsukita et al., 2001). In the epithelium of human nasal mucosa *in vivo*, claudins 1, 4, 7, 8, 12, 13 and 14, occludin, JAM-1, and ZO-1 were detected together with continuous tight junction strands that formed well-developed networks (Takano et al., 2005).

In the present study, to investigate changes of gap and tight junctions during differentiation of HNE cells *in vitro*, we used primary HNE cells cocultured with primary human nasal fibroblast (HNF) cells in a noncontact system. In HNE cells cocultured with HNF cells, numerous elongated cilia-like structures, switching in expression of connexins and induction of tight junction proteins, were observed.

Materials and Methods

Antibodies

Rabbit polyclonal anti-Cx26, anti-Cx30.3, anti-Cx31, anti-Cx43, anti-claudin-1, anti-claudin-7, anti-occludin, anti-JAM-1 and anti-ZO-2 and mouse monoclonal anti-claudin-4 antibodies were obtained from Zymed Laboratories (San Francisco, CA).

Isolation and Cell Culture

For primary cultured HNE cells, human nasal mucosa tissues were minced into pieces 2–3 mm^3 in volume and washed with phosphate-buffered saline (PBS) containing antibiotics four times. Human nasal specimens were obtained during surgery from inferior turbinate mucosa of patients with hypertrophic rhinitis and chronic sinusitis. Informed consent was obtained from all patients, and the study was approved by the ethics committee of Sapporo Medical University. These tissue specimens were suspended in 10 ml of dispersing solution with 0.5 μg/ml DNase I (Sigma, St. Louis, MO) and 0.08 mg/ml Liberase Blenzyme 3 (Roche, Basel, Switzerland) in PBS and then incubated at 37°C for 20 min. The dissociated specimens were subsequently filtered with 300 μm mesh followed by filtration with 40 μm mesh. After centrifugation at 1,000 × *g* for 4 min, the cells were cultured in serum-free bronchial epithelial basal medium (BEBM; Clonetics, San Diego, CA) supplemented with 0.5 μg/ml hydrocortisone, 5 μg/ml insulin, 10 μg/ml transferrin, 0.5 μg/ml epinephrine, 6.5 μg/ml triiodothyronine, 50 μg/ml gentamycin, 50 μg/ml amphotericin B, 0.1 ng/ml retinoic acid, 0.5 ng/ml epidermal growth factor (Clonetics), bovine pituitary extract (1% vol/vol; Pel-Freez Biologicals, Rogers, AR), 100 U/ml penicillin and 100 μg/ml streptomycin. Isolated HNE cells were plated on 35-mm culture dishes (Corning, Corning, NY), which were coated with rat tail collagen (500 μg dried tendon/ml 0.1% acetic acid). For primary HNF cells, the supernatant was centrifuged at 1,400 × *g* for 4 min and the pellet suspended in modified BEBM with 10% fetal bovine serum (FBS) and then plated on 60-mm dishes. The cells were placed in a humidified, 5% CO_2:95% air incubator at 37°C.

Coculture System

HNE and HNF cells were replated on inner chambers of 12-mm Transwells with a 0.4-μm pore size filter or outer chambers in the modified BEBM with 10% FBS for 1 week. Some inner chambers plated with HNE cells were inserted into outer chambers plated with HNF cells and then incubated for 2 weeks (noncontact system, Fig. 1a).

Scanning Electron Microscopy

Cells grown on inner chambers were fixed with 2.5% glutaraldehyde/0.1 M PBS (pH 7.3) overnight at 4°C. After several rinses with PBS, the cells were postfixed in 1% osmium tetroxide at 4°C for 3 h and then rinsed thoroughly with distilled water, dehydrated by graded ethanol and freeze-dried. The specimens were sputter-coated with platinum and observed with a scanning electron microscope (S-4300; Hitachi, Tokyo, Japan) operating at 10 kV.

RNA Isolation and Reverse Transcription-Polymerase Chain Reaction Analysis

Total RNA was extracted and purified using TRIzol reagent (GIBCO BRL, Gaithersburg, MD), and reverse transcription-polymerase chain reaction (RT-PCR) was

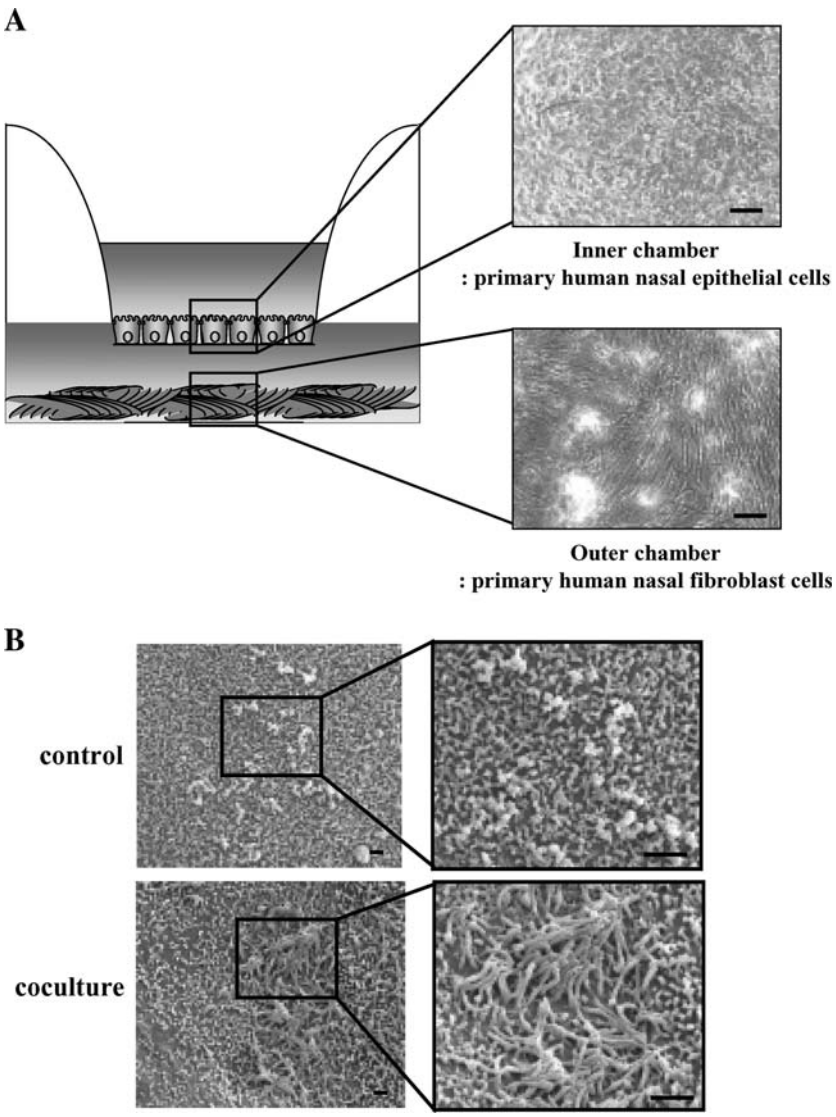

Fig. 1 (**A**) Phase-contrast images of primary HNE cells cocultured with primary HNF cells in a noncontact system. (**B**) Scanning electron microscopic images of primary HNE cells cocultured with or without primary HNF cells. Numerous elongated cilia-like structures are observed in the coculture system compared to the control. Bars: **a** = 100 μm, **b** = 1 μm

performed as previously described (Go et al., 2006). The PCR primers for mouse cDNAs were as follows: Cx30 (sense 5′-CCCTGGGTGTTGAAATGTGG-3′ and antisense 5′-CAAGTCATTGGGAGTGAAGACA-3′), Cx30.3 (sense 5′-CAAGTCATTGGGAGTGAAGACA-3′ and antisense 5′-AAATGCCCAGTTCATGCTG-3′), Cx31 (sense 5′-CGACAACGCAGGCAAGAA-3′ and antisense 5′-GTAGGTCGGGCAATGTAGCAGT-3′), Cx31.1 (sense 5′-TGCTCCACTCATTCTACCCCAAAT-3′ and antisense 5′-GGCATCTCTTGCTCACCAGGTA-3′) and ZO-2 (sense 5′-GCCAAAACCCAGAACAAAGA-3′ and antisense 5′-ACTGCTCTCTCCCACCTCCT-3′).

Western Blot Analysis

Cells grown on inner chambers were scraped in 200 μl of buffer (1 mM $NaHCO_3$ and 2 mM phenylmethylsulfonyl fluoride), collected in microcentrifuge tubes and then sonicated for 10 s. The protein concentration of the samples was determined using the BCA Protein Assay Reagent Kit (Pierce, Rockford, IL). Aliquots of 15 μg protein/lane for each sample were separated by electrophoresis in 4/20% sodium dodecyl sulfate polyacrylamide gels (Daiichi Pure Chemicals, Tokyo, Japan). After electrophoretic transfer to a nitrocellulose membrane (Immobilon; Millipore, Bedford, MA), the membrane was saturated for 30 min at room temperature with blocking buffer (25 mM Tris [pH 8.0], 125 mM NaCl, 0.1% Tween 20 and 4% skim milk) and incubated with anti-Cx26, anti-Cx30.3, anti-Cx31, anti-Cx43, anti-claudin-1, anti-claudin-4, anti-claudin-7, anti-occludin, anti-JAM-A, anti-ZO-2 and anti-actin antibodies at room temperature for 1 h. The membrane was incubated with horseradish peroxidase-conjugated anti-rabbit or mouse immunoglobulin G (IgG; Dako, Copenhagen, Denmark) at room temperature for 1 h. The immunoreactive bands were detected using an enhanced

chemiluminescence Western blotting system (Amersham, Aylesbury, UK).

Immunocytochemistry

Cells grown on inner chambers were fixed with cold absolute acetone or a 1:1 ethanol and acetone mixture at -20°C for 10 min. The cells were stained with anti-Cx26, anti-Cx31 and anti-Cx43 antibodies overnight at 4°C. Alexa 488 (green)-conjugated anti-rabbit IgG and Alexa 592 (red)-conjugated anti-mouse IgG (Molecular Probes, Eugene, OR) were used as the second antibodies. The specimens were examined and photographed with an Axioskop 2 plus microscope (Carl Zeiss, Oberkochen, Germany) and a confocal laser scanning microscope (MRC 1024; Bio-Rad, Hercules, CA). Phase-contrast photomicrographs were taken with a Zeiss Axiovert 200 inverted microscope.

Measurement of GJIC

Lucifer yellow (5% wt/vol, Sigma) was injected into individual cells through sharp microelectrodes (Sterile Femtotips II; Eppendorf, Hamburg, Germany). Photographs documenting the extent of dye coupling were taken 3 min after the end of the injection using an inverted fluorescence microscope equipped with an appropriate filter (Olympus, Tokyo, Japan).

Measurement of Transepithelial Electrical Resistance

Cells were cultured to confluence on inner chambers of 12-mm Transwell inserts with 0.4-μm pore size filters. Transepithelial electrical resistance (TER) was measured using an EVOM voltameter with an ENDOHM-12 (World Precision Instruments, New Haven, CT) on a heating plate (Fine, Tokyo, Japan) adjusted to 37°C. The values are expressed in standard units of ohms per square centimeter and presented as the mean ± standard deviation (SD). For calculation, the resistance of blank filters was subtracted from that of filters covered with cells.

Data Analysis

Signals were quantified using Scion Image Beta 4.02 Win (Scion, Frederick, MD). Each set of results shown is representative of three separate experiments.

Results and Discussion

Epithelial-mesenchymal interactions promote normal epithelial differentiation via mediators including various growth factors (Kumar et al., 2005; Rubin, 2007). To investigate the effect of HNF cells on differentiation of HNE cells, we examined changes of cilia formation, which is one of the differentiation markers, in HNE cells cocultured with HNF cells in a noncontact system (Fig. 1b). In HNE cells cocultured with HNF cells for 2 weeks, numerous elongated cilia-like structures were observed compared to the control (Fig. 1b). In human bronchial epithelial cells cocultured with lung fibroblast cells for 2 weeks, ciliated cells were increased (Myerburg et al., 2007). Hepatocyte growth factor secreted from lung fibroblast cells is important for differentiation of human bronchial epithelial cells (Myerburg et al., 2007). In this experiment using a noncontact system, unknown soluble factors secreted from HNF cells may be important for differentiation of HNE cells.

GJIC may modulate the metachronal ciliary beating of respiratory mucosa (Yeh et al., 2003). It is thought that GJIC may also be important in nasal mucosa. We investigated expression of connexins in control HNE cells by RT-PCR. Expression of mRNAs of Cx26, Cx32, Cx43, Cx30, Cx30.3, Cx31 and Cx31.1 was detected (Fig. 2a). Changes in the expression pattern of connexins are observed during cell differentiation (Brissette et al., 1994; Zhang & Thorgeirsson, 1994). In addition, during calcium-induced keratinocyte differentiation *in vitro*, expression of Cx26 and Cx43 is downregulated whereas that of Cx31 and Cx31.1 is induced (Brissette et al., 1994). Furthermore, in well-differentiated human airway epithelial cells *in vitro*, downregulation of Cx26 and Cx43 and maintenance of Cx31 are observed together with GJIC (Wiszniewski et al., 2007). In this experiment, during differentiation of HNE cells cocultured with HNF cells in a noncontact system, we investigated the changes in expression pattern of connexins by Western blotting. Downregulation of Cx26 and upregulation of Cx30.3 and Cx31 were observed in the coculture compared to the control, whereas no change of Cx43 was observed (Fig. 2b). In immunostaining, Cx26-positive spots were markedly reduced and Cx31-positive spots were slightly increased at cell borders in the coculture compared to the control (Fig. 2c). Furthermore, extensive GJIC measured by lucifer yellow dye transfer was observed in the coculture compared to the control (Fig. 2d). The switch in gap junction protein expression during differentiation of nasal epithelial cells *in vitro* enhanced GJIC and might play an important role in the control of the differentiation process.

Tight junction proteins play an important role in the establishment and maintenance of epithelial cell polarity, which is closely associated with epithelial differentiation (Tsukita et al., 2001). We previously reported that in the *in vivo* epithelium of human nasal mucosa from patients with allergic rhinitis, claudins 1, 4, 7, 8, 12, 13 and 14; occludin; JAM-1; and ZO-1 were detected together with

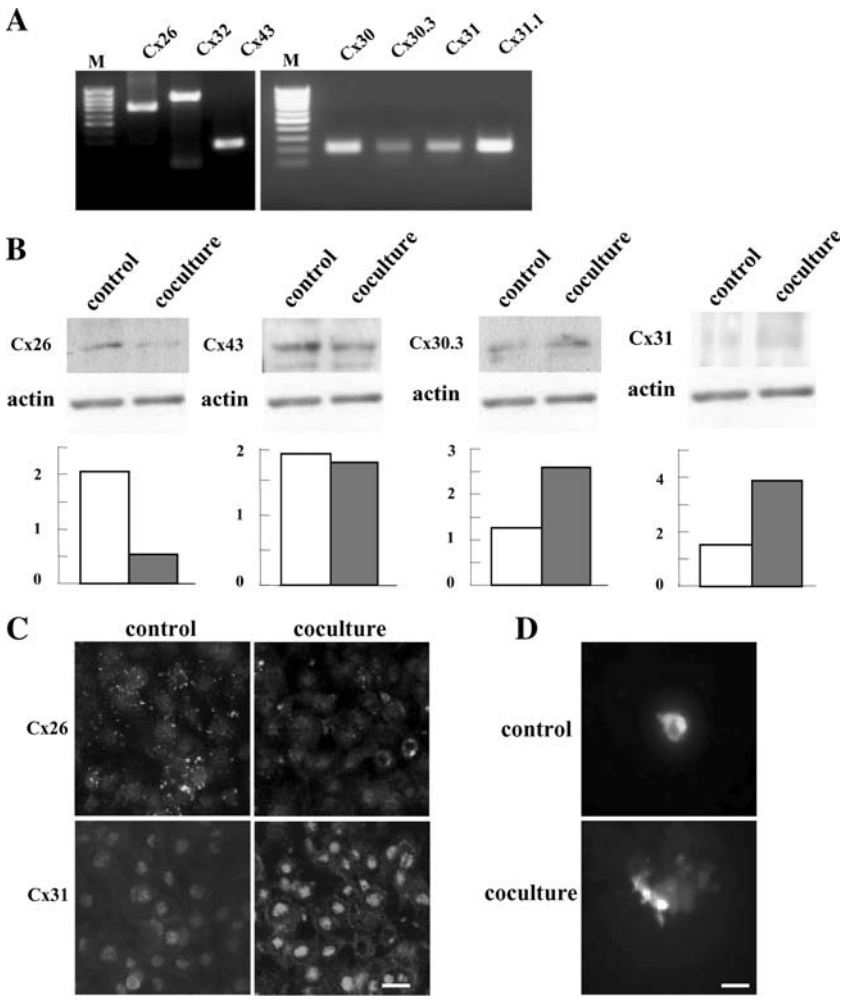

Fig. 2 (**A**) RT-PCR of mRNAs of connexins in primary HNE cells. Cx26, Cx32, Cx43, Cx30, Cx30.3, Cx31 and Cx31.1 are detected. (**B**) Western blots for Cx26, Cx43, Cx30.3 and Cx31 in primary HNE cells cocultured with or without primary HNF cells. Downregulation of Cx26 and upregulation of Cx30.3 and Cx31 are observed in cocultured HNE cells compared to control. (**C**) Immunostaining of Cx26 and Cx31 in primary HNE cells cocultured with or without primary HNF cells. Downregulation of Cx26 and upregulation of Cx31 are observed at cell borders of cocultured HNE cells compared to the control. (**D**) GJIC by lucifer yellow dye in primary HNE cells cocultured with or without primary HNF cells. Bars = 20 μm

continuous tight junction strands that formed well-developed networks (Takano et al., 2005). In this experiment, we investigated expression of tight junction proteins in primary HNE cells by RT-PCR. Expression of mRNAs of claudins 1, 2, 4, 5, 6, 7, 8, 9 and 12; occludin; JAM-1; and ZO-2 was detected (Fig. 3a). During differentiation of HNE cells cocultured with HNF cells in a noncontact system, we investigated the changes in expression of tight junction proteins by Western blotting. Upregulation of claudin-1, claudin-4, occludin and ZO-2 was observed in the coculture compared to the control, whereas no changes of claudin-7 and JAM-1 were observed (Fig. 3b). When we examined barrier function measured as TER in both the coculture and control, a slight increase of TER was observed in the coculture compared to control, but it was not significant (Fig. 3c).

In airway epithelial cells, the relationship between ciliogenesis and the barrier function of tight junctions is still unclear. In HNE cells using liquid-covered culture (LCC) or air-interfaced culture (AIC), upregulation of ciliogenesis and barrier function measured as TER was observed in the cells grown under AIC compared to those grown under LCC (Lee et al., 2005). However, in human bronchial epithelial Calu-3 cells using LCC and AIC, downregulation of TER and ZO-1 was observed in the cells grown under AIC compared to those grown under LCC (Grainger et al., 2006). Since the nose and bronchi have a different embryological origin (Bousquet et al., 2004), the epithelium of nasal mucosa and bronchial mucosa presented some differences in expression and regulation of tight junctions but both had well-developed tight junctions.

The interactions of connexins with tight junction proteins may be cell type-specific, and tight junction function may in part be controlled by connexins via both GJIC-dependent and -independent mechanisms in epithelial cells (Kojima et al., 2003; Morita et al., 2004; Go et al., 2006). In Calu-3 cells, Cx26 overexpression prevented disruption of tight junctions after ouabain treatment (Go et al., 2006). In our HNE cells, downregulation of Cx26 and upregulation of Cx30.3, Cx31, claudin-1, claudin-4,

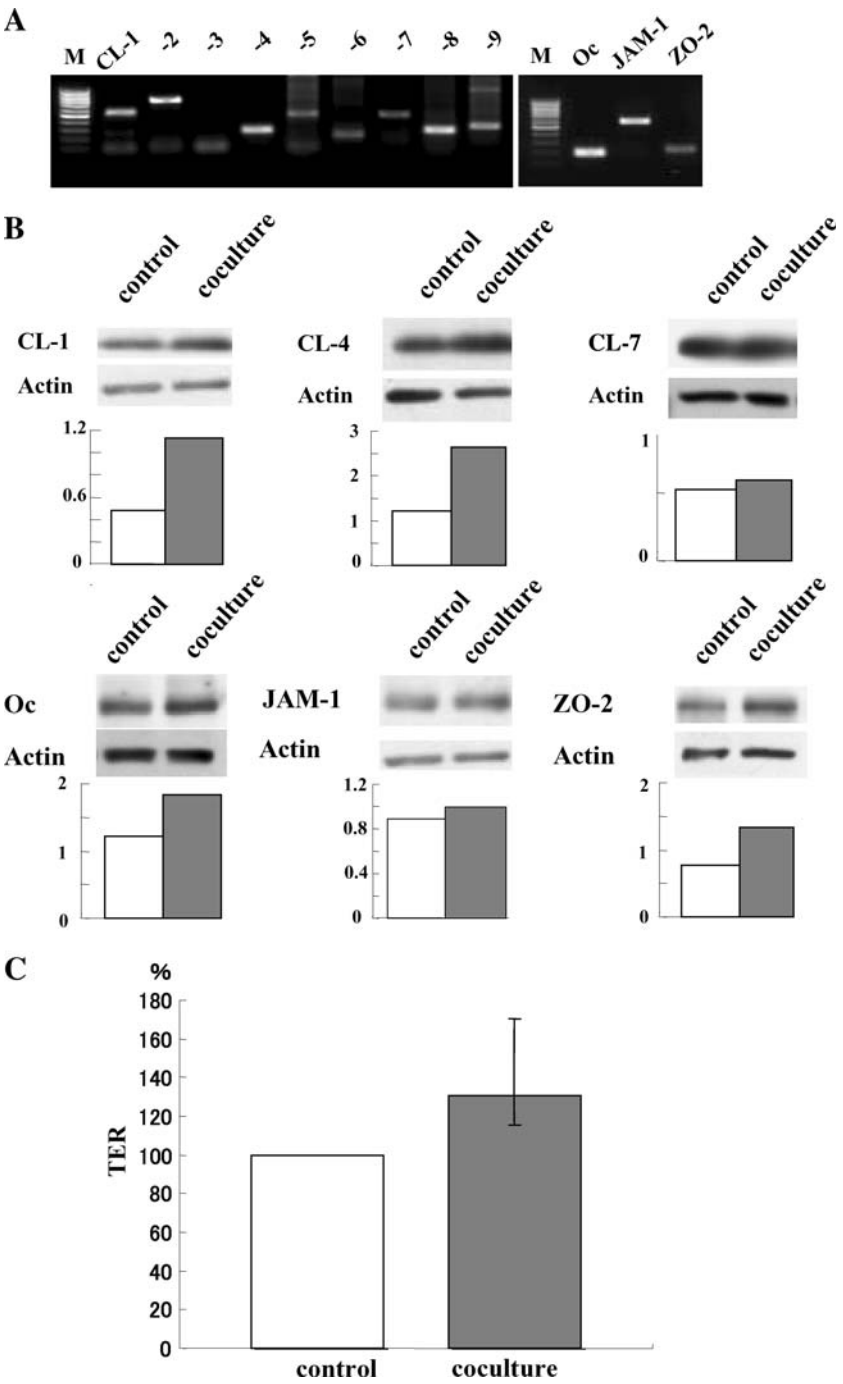

Fig. 3 (**A**) RT-PCR of mRNAs of tight junction molecules in primary HNE cells. Claudins (CL) 1, 2, 4, 5, 6, 7, 8 and 9; occludin (Oc); JAM-1; and ZO-2 are detected. (**B**) Western blots for CL-1, CL-4, CL-7, Oc, JAM-1 and ZO-2 in primary HNE cells cocultured with or without primary HNF cells. Upregulation of CL-1, CL-4, Oc and ZO-2 are observed in cocultured HNE cells compared to the control. (**C**) Barrier function measured as TER in primary HNE cells cocultured with or without primary HNF cells

occludin and ZO-2 were observed in the coculture. In this experiment, the interactions of connexins with tight junction proteins were unclear during differentiation of HNE cells.

In summary, during differentiation of primary HNE cells cocultured with primary HNF cells in a noncontact system, elongated cilia-like structures, switching in expression of connexins and induction of tight junction proteins, were observed, suggesting that unknown soluble factors secreted from HNF cells may affect not only ciliogenesis but also expression of gap and tight junctions in HNE cells.

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