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Developmental Changes in the Expression of Tight Junction Protein Claudins in Murine Metanephroi and Embryonic Kidneys

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ABSTRACT. Claudins are the major constituents of tight junction (TJ) strands and participate in the cell-cell adhesion and permeability barrier in epithelial cell layers. To investigate the suitability of metanephroi for analysis of the function of the TJ protein claudins in renal tubular formation, mouse metanephroi from embryos at day 12 of gestation were cultured and expression of claudins was compared with that in embryonic kidneys. During *in vitro* culture for 8 days, the metanephroi showed expression patterns very similar to those observed in embryonic kidneys in reverse transcription-polymerase chain reaction for the claudins examined, including claudins 1–4, 8, 10, 11, and 16, and the TJ proteins occludin and ZO-1. Immunofluorescence microscopy for claudins 1–4, 8, 10, and 16 showed localization of these claudins at the TJ with occludin and ZO-1 in some restricted tubular segments. These findings indicate that the metanephroi show developmental changes in the expression of the TJ protein claudins, representing those in embryonic kidneys, and thus suggest that the mouse metanephros is suitable to examine the functions of specific claudins in the kidney.
KEY WORDS: claudin, metanephroi, mouse, renal tubule, tight junction.

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The tight junction (TJ) is located at the apicalmost region of the lateral membrane of the epithelial cell, and plays a critical role in sealing the intercellular space in epithelial cellular sheets. The TJ behaves as the primary barrier to the diffusion of solutes and water through the paracellular pathway and maintains cell polarity as a boundary between the apical and basolateral plasma membrane domains [6, 16, 19, 20]. In freeze-fracture replicas of epithelial cells, TJs appear as a continuous anastomosing network of intramembranous particle strands, i.e., TJ strands. The major components of TJ strands are the integral membrane proteins occludin and claudins, and some cytoplasmic scaffolding proteins such as ZO-1 which anchor occludin and claudins to actin filaments [20]. The claudins are a family of more than 20 homologous subtypes which share the same membrane topology and have various tissue-specific and segment-specific distribution patterns [19, 20]. Heterogenous claudins form individual TJ strands as heteropolymers to adhere to each other at the cell-cell interface [4] to generate consequential variations in the tightness of individual TJ strands [19].

Pathological phenotypes of humans and animals with mutations in genes of specific claudins, including claudins 11 [5], 14 [2, 22], and 16 [7, 11, 17], demonstrate the importance of these TJ proteins in the formation and maintenance of epithelial architecture. However, the precise mechanisms for degeneration of cells and tissues in these diseases remain unknown. *CLDN16* gene mutations cause familial hypomagnesemia with hypercalciuria and nephrocalcinosis in the human [17] and renal tubular dysplasia with interstitial nephritis in cattle [7, 11]. The expression of claudin-16 is exclusively restricted to the thick ascending limb (TAL) of Henle's loop, where reabsorption of Mg^{2+} and Ca^{2+} occurs via the paracellular pathway, in humans [17], mice [8], and cattle (H. Ohta *et al.*, unpublished data), indicating that claudin-16 forms aqueous pores selective to these divalent cations [17]. Interestingly, kidneys from affected animals [12, 15] and human patients [21] share some pathological findings such as tubular atrophy and interstitial fibrosis, suggesting that claudin-16 has some pivotal roles in the differentiation and formation of renal tubules in addition to the function in paracelluar transport of Mg^{2+} and Ca^{2+}. This putative function of claudin-16 appears to be indispensable and not compensated for by the presence of or interactions between claudins 3, 10, and 11, which have been shown to colocalize with claudin-16 at the TJ of the TAL segment in the mouse [8] and bovine (H. Ohta *et al.*, unpublished data) nephron. To examine these hypotheses, experimental systems in which expression of specific claudin genes can be regulated are desirable. Organotypic culture of metanephric explants seem to be an adequate model for this purpose since various studies have shown that mouse metanephroi removed from embryos continue to develop *in vitro* and display many of the processes of nephron induction and differentiation that are observed *in vivo* [1, 9, 10] and the expression of specific genes can be inhibited in the presence of antisense oligonucleotides [14] and/or interfering RNAs [3]. In the present study, we examined the expression of several different claudin genes in metanephroi and compared them with those in embryos to elucidate if metanephroi actually have normal expression levels and patterns for the claudins.

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MATERIALS AND METHODS

Antibodies: A rabbit anti-bovine claudin-16 polyclonal antibody (pAb) was kindly provided by Drs. Akiko Takasuga and Yoshikazu Sugimoto (Shirakawa Institute of Animal Genetics, Fukushima, Japan). Rabbit pAbs to claudins 1–3, 8, and 10, and ZO-1, and mouse monoclonal antibodies (mAb) to claudin-4 and occludin were purchased from Zymed Laboratories (South San Francisco, CA, U.S.A.).

Animals: Pregnant ICR mice purchased from Japan SLC (Hamamatsu, Japan) were housed in our own animal facility under conventional conditions, and the appearance of a vaginal plug was designated as day 0 of gestation. All experimental procedures met with the approval of the Laboratory Animal Experimentation Committee, Gaduate School of Veterinary Medicine, Hokkaido University.

Developmental analysis of the tight junction proteins in mouse embryonic kidneys: Embryos were removed from anesthetized pregnant ICR mice on embryonic days (E) 12, 14, 16, and 18 of the pregnancy and were dissected to obtain metanephoi. Total RNAs were prepared, reverse-transcribed into cDNAs as described previously [18], and amplified by polymerase chain reaction (PCR) for claudins 1–4, 8, 10, 11, and 16, occludin, ZO-1, and glyceraldehyde 3-phosphate dehydrogenase. The amplified cDNA fragments were confirmed by sequencing on a CEQ8800 DNA analysis system (Beckman Coulter, Fullerton, CA, U.S.A.). Primers used for PCR amplification are listed in Table 1.

Organ cultures: Metanephroi culture was carried out essentially as described previously [10, 14]. Metanephroi isolated from E12 embryos were placed on a Millicell®-CM cell culture plate insert (Millipore, Bedford, MA, U.S.A.) and cultured. A 25-mm diameter organ culture insert allowed placement of a minimum of six metanephroi in an interface in the presence of medium consisting of a 1:1 mixture of Dulbecco's modified Eagle's medium and F12 supplemented with 10% fetal bovine serum FBS and a 5-fold concentration of MITO serum extender (BD Biosciences, Bedford, MA, U.S.A.).

Cultured metanephroi were frozen in liquid nitrogen with or without OCT compound (Sakura Fine Technical Co., Tokyo, Japan) for immunohistochemistry and RNA analyses, respectively. Total RNAs were prepared from cultured metanephroi using an RNeasy Micro kit (QIAGEN GmbH, Hilden, Germany) and reverse-transcribed into cDNAs for reverse transcription (RT)-PCR analysis as described above. Cryostat sections of cultured metanephroi were prepared for histological and immunohistochemical analyses. Cryostat sections of post-explant day 2 (p.e.2), p.e.4, p.e.6, and p.e.8 metanephroi were fixed in 10% neutral buffered formalin and stained with hematoxylin and eosin.

Immunofluorescence microscopy: Cryostat sections of p.e.8 metanephroi were fixed with 95% ethanol at 4°C for 30 min, followed by 100% acetone at room temperature for 1 min. Sections were incubated with 1% bovine serum albumin in phosphate-buffered saline (PBS) for 20 min. Sections were then incubated with the appropriate anti-claudin antibodies and the anti-occludin or anti-ZO-1 antibody. Sections were then washed three times with PBS, followed by incubation for 30 min with secondary antibodies conju-

Table 1. Primers used for RT-PCR analysis of the TJ protein mRNAs

Proteins	Sequence	Accession number
Claudin-1	Forward, 5'-GCATCCTGCTGGGGCTGATCG-3'	
	Reverse, 5'-GGCTTGGGATAAGGCCGTGGTG-3'	AF072127
Claudin-2	Forward, 5'-CGTCCAGTGCAATGTCCTCGCTG-3'	
	Reverse, 5'-AGCCACTACCCCACCCTACCC-3'	BC015252
Claudin-3	Forward, 5'-AGCCGGTTCAAGTCCAGCAGC-3'	
	Reverse, 5'-CCTTGCGGTCGTAGGCGGTG-3'	NM_009902
Claudin-4	Forward, 5'-AGCTGGTGCATCGGACTCAGC-3'	
	Reverse, 5'-TCCCCAGCAAGCAGTTAGTGGC-3'	NM_009903
Claudin-8	Forward, 5'-TGTGCTGCGTCCGTCTTGGC-3'	
	Reverse, 5'-CGGCGTGGAAACTCCGTTGAGTG-3'	BC003868
Claudin-10	Forward, 5'-CCGGTGTCGCCAACTGCAAG-3'	
	Reverse, 5'-GTCCGAGAAGACATGACAGACGTGG-3'	NM_021386
Claudin-11	Forward, 5'-CCGCCATCTTGCTGCTGTTGAC-3'	
	Reverse, 5'-GGCAGGGAAGTGGGCTTCTCC-3'	BC021659
Claudin-16	Forward, 5'-CCCATGTGTCCCTTCCCAACAGG-3'	
	Reverse, 5'-GTCTCTGTCCGAGGGGCCTTG-3'	NM_053241
Occludin	Forward, 5'-GACTGGGTCAGGGAATATCCACC-3'	
	Reverse, 5'-AGCAGCAGCCATGTACTCTTCAC-3'	NM_008756
ZO-1	Forward, 5'-CCACCAAGGTCACACTGGTG-3'	
	Reverse, 5'-CGAGCGACCTGAATGGTCTG-3'	NM_009386
GAPDH	Forward, 5'-GAAGGTCGGTGTGAACGGATT-3'	
	Reverse, 5'-GAAGACACCAGTAGACTCCACGACATA-3'	NM_008084

Sequences of the primer pairs for PCR amplification of claudins, occludin, ZO-1, and glyceraldehydes 3-phosphate dehydrogenase (GAPDH) cDNAs and the GenBank accession numbers for the genes or clones containing the primer sequences are presented.

gated with AlexaFluor 488 or 568 (Molecular Probes, Inc., Eugene, OR, U.S.A.). After washing with PBS, sections were embedded in Prolong antifade reagent (Molecular Probes, Inc.) and examined under an ECLIPSE E800 microscope equipped with a deconvolution apparatus (Nikon, Tokyo, Japan).

The specificities of immunofluorescent signals for some claudins, including claudins 1–4 and 16 were examined using the glutathione S-transferase (GST)-tagged recombinant proteins containing the C-terminal region of each claudin used for immunization. These GST fusion proteins were prepared based on the nucleotide sequences of cDNA for bovine claudins 1–4 which we cloned previously (GenBank accession numbers AB178476, AB115779, AB115781, and AB185928, respectively) and claudin-16 [7]. The antibodies to claudins 1–4 and 16 used in this study specifically recognized the corresponding GST fusion proteins in immunoblotting, and immunofluorescent signals with these antibodies in mouse kidney sections disappeared when each GST-fused protein was included in the reaction (data not shown), indicating that the antibodies to claudins 1–4 and 16 used in this study immunospecifically recognized each claudin from cattle and mice. In addition, anti-claudin-8 and anti-claudin-10 antibodies, both raised against their C-terminal peptide regions, did not react with GST fusion proteins described above in immunoblotting (data not shown).

While we could identify renal tubular segments in the kidney from adult mice using the antibodies to several segment-specific marker proteins as reported by previous investigators [8], frozen sections of p.e.8 metanephroi showed no or very faint staining for some of these markers including the Tamm-Horsfall glycoprotein, a marker of the TAL segment. Thus, we have not defined the specific tubular segments in developing metanephroi in the present study.

RESULTS

Expression of claudins, occludin, and ZO-1 in mouse embryonic kidneys: We first examined the expression of claudins, occludin and ZO-1 in mouse developing kidneys by RT-PCR. Constant expression of ZO-1, a peripheral membrane protein, was detected throughout the period examined. Likewise, claudins 1, 3, 4, 8, and 11, and occludin as well, were observed in the metanephroi at E12, and most of them reached increased levels afterward till E18, corresponding to the levels in the adult kidney (Fig. 1A). The only exception was claudin-11, which presented a high level of expression throughout E12 to E18 and then appeared to be reduced to the low level observed in adult

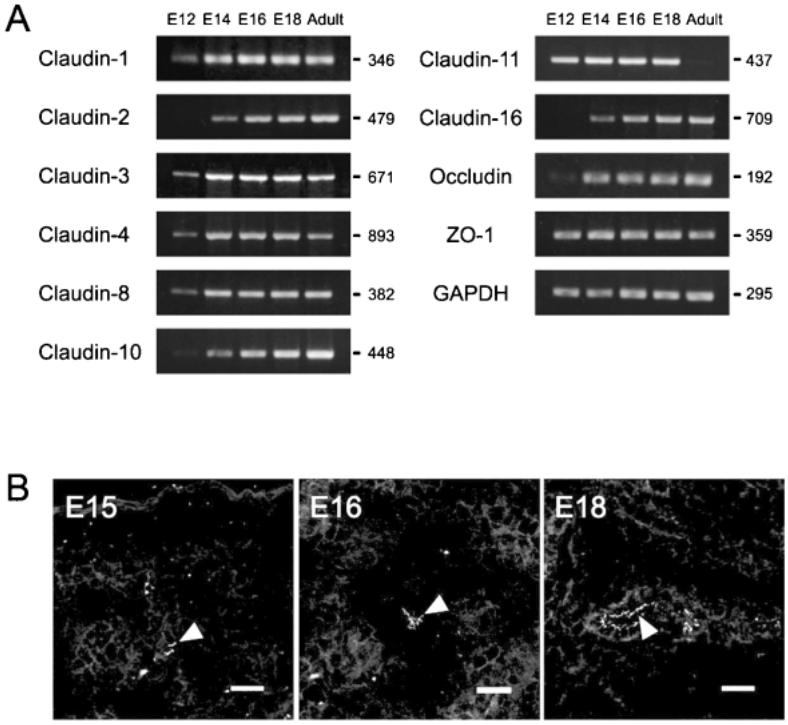

Fig. 1. Expression of claudins, occludin, and ZO-1 in developing and adult mouse kidneys. *A*, the expression of mRNAs for claudins (claudins 1–4, 8, 10, 11, and 16), occludin, and ZO-1 in mouse embryonic kidneys at day 12, 14, 16, and 18 of gestation (E12-E18) and adult mouse kidneys (Adult) was analyzed by RT-PCR followed by electrophoresis on agarose gels and the representative results of two separate experiments are shown. The expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is included as a control. The sizes of PCR products are indicated in bp. *B*, immunofluorescent detection of claudin-16 in embryonic kidneys. Frozen sections of the kidneys from mouse embryos at E15, E16, and E18 were reacted with the anti-claudin-16 pAb followed by detection with a secondary antibody labeled with AlexaFluor 488 (arrowheads). Bars = 20 μm.

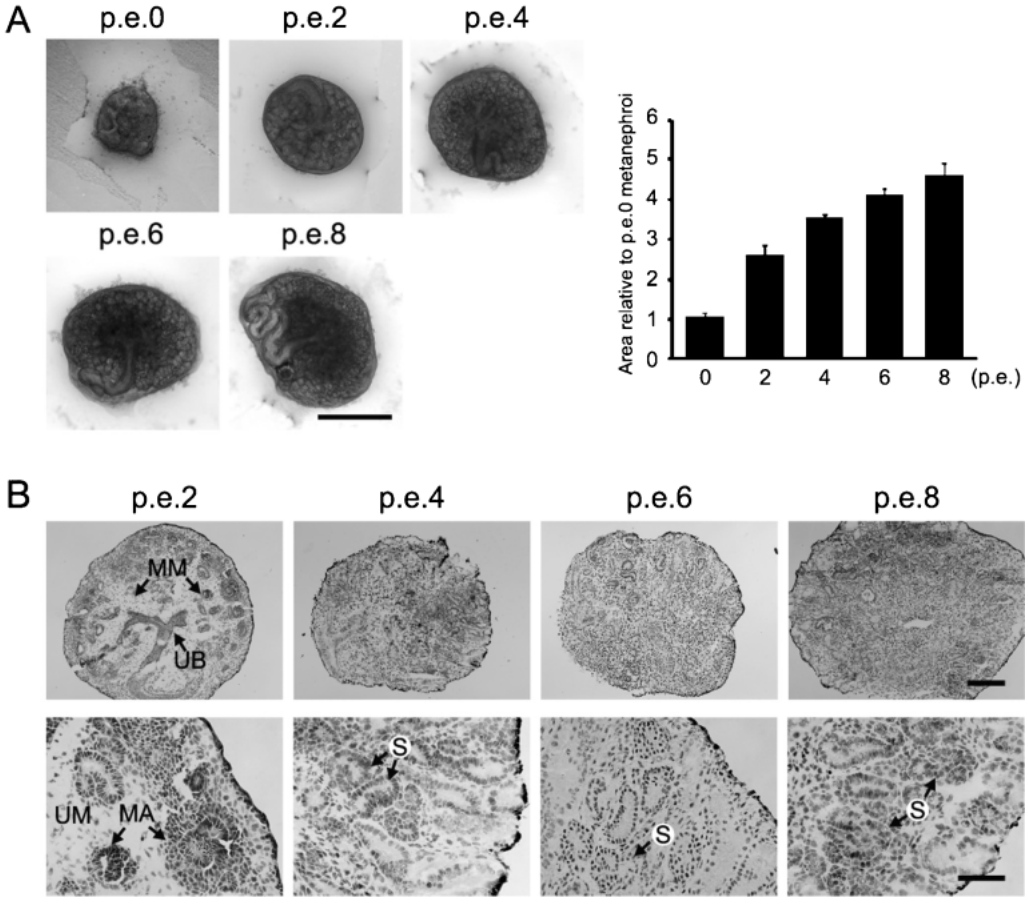

Fig. 2. Growth of mouse metanephroi in culture. *A*, metanephroi isolated from E12 (= p.e.0) mouse embryos were cultured on membrane filters for 2 (p.e.2), 4 (p.e.4), 6 (p.e.6), and 8 (p.e.8) days and were photographed under a bright-field microscope (left). The mean sizes of metanephric explants at days p.e.0 through p.e.8 were estimated as the areas of the organs in photos using NIH Image software. The ratios of areas relative to those of p.e.0 metanephroi are presented as the mean ± S.D. (n = 6) (right). Bar = 1 mm. *B*, histological changes of cultured mentanephroi. Cryostat sections of cultured metanephroi stained with hematoxylin and eosin are presented with low-power views (upper panels, original magnification, × 40) and high-power views (lower panels, original magnification, × 200). The branched ureteric bud (UB), metanephric mesenchyme (MM), mesenchymal aggregates (MA), and uninduced mesenchyme (UM) are indicated with arrows. Comma- and/or S-shaped metanephric tubules (S) are also indicated. Bars = 200 μm (upper panels) and 50 μm (lower panels).

animals.

In contrast, signals for claudins 2, 10, and 16 were not obvious at E12, they appeared clear on E14 and then increased to the adult levels (Fig. 1A). Consistent with this observation, the immunofluorescent signals for claudin-16 were demonstrated as early as E15 at the apical end regions of adjacent epithelial cells and appeared abundant from E16 onward during in utero development (Fig. 1B).

Organ culture and in vitro expression of TJ proteins: Metanephroi from E12 embryos continued to grow *in vitro* till post-explant day 8 (p.e.8) and showed increases in tubular branching with formation of comma-shaped or S-shaped metanephric tubules (Figs. 2A and 2B), consistent with previous observations [1, 10]. Histological features of metanephroi shown in Fig. 2B were principally the same as those observed in embryonic kidneys at corresponding days after gestation (data not shown), indicating that the process of tubular development in metanephroi represented that in developing kidneys *in vivo* as shown in previous studies [9, 10]. These metanephroi exhibited expression patterns of the TJ protein genes that were principally the same as those obtained for kidneys from embryos (Fig. 3A). PCR-amplified fragments of claudins 1, 3, 4, 8, 10, and 11 and ZO-1 were found throughout the incubation period. Apparent increases in signal intensities of claudin-1 and -10 and the expression of claudin-11 at high and constant levels during the incubation were comparable to those observed in embryonic kidneys (Fig. 1A). Moreover, the signals for claudin-16 and occludin were detected from p.e.2 onward (Fig. 3A). Occasionally, very weak but demonstrable signals were

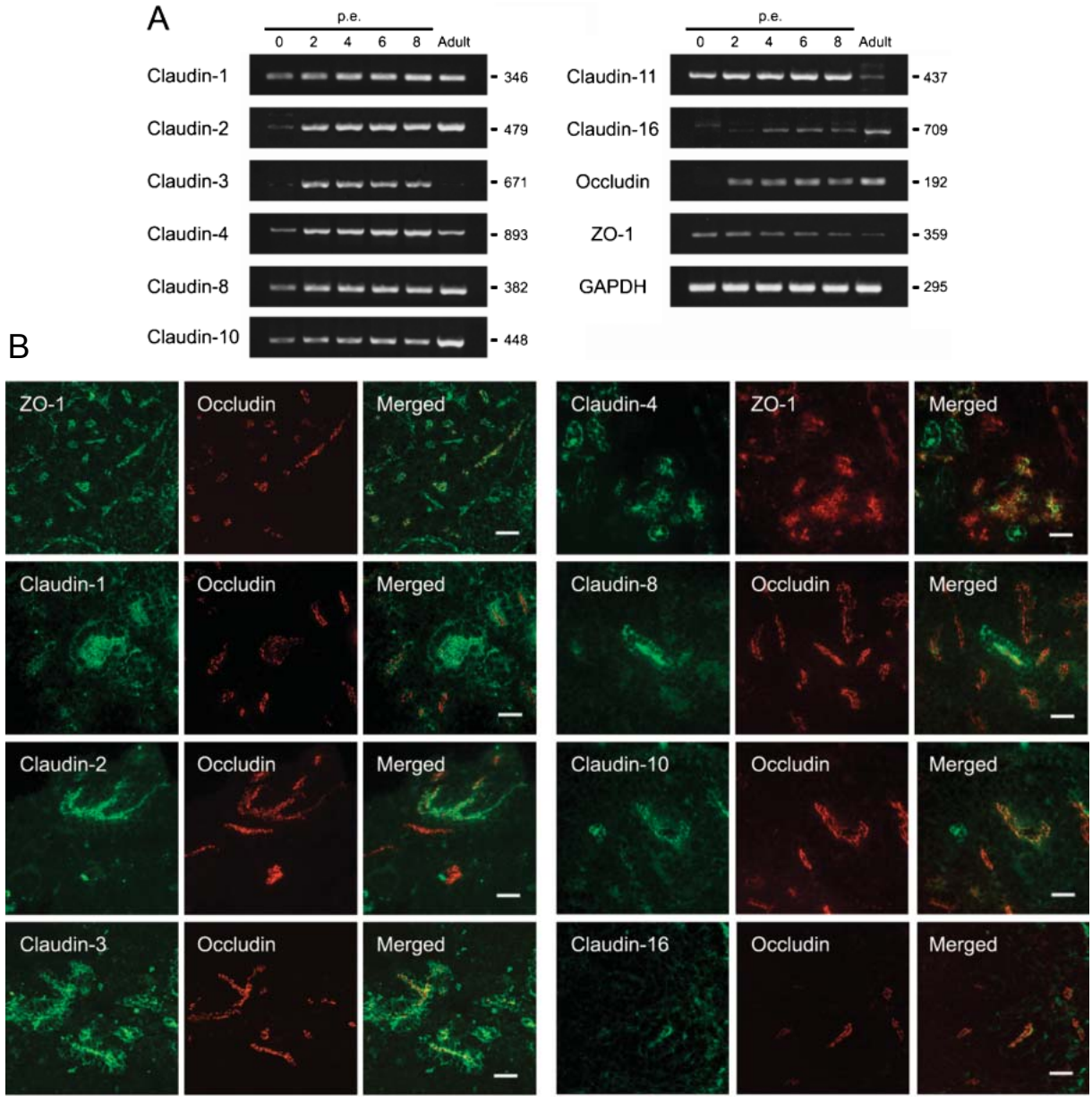

Fig. 3. Expression of the TJ proteins in cultured metanephroi. *A*, RT-PCR analyses for claudins (claudins 1–4, 8, 10, 11, and 16), occludin, and ZO-1 in metanephroi at p.e.0 through p.e.8 were performed as described in the legend for Fig. 1. PCR products for GAPDH are also shown. Product sizes are indicated in bp. A representative result of two independent experiments is shown. *B*, immunofluorescence microscopy for TJ proteins. Claudins 1–3, 8, 10, and 16, and ZO-1 were detected with pAbs specific to each protein and counterstained with the anti-occludin mAb. Claudin-4 was reacted with anti-claudin-4 mAb and then stained with anti-ZO-1 pAb. Specificities of antibodies to claudins 1–4 and 16 were confirmed by disappearance of immunofluorescent signals when the recombinant C-terminal peptide of each claudin tagged with glutathione S-transferase was included in the reactions as described previously (H. Ohta *et al.*, unpublished data). Bars = 20 μm.

found for claudin-2 and -3 in metanephroi on the day of dissection, p.e.0 as shown in Fig. 3A, presumably due to low levels of expression of these claudins around E12.

Immunofluorescence microscopy of occludin and ZO-1 in the p.e. 8 metanephroi showed that these proteins principally colocalized at the apicalmost regions of lateral membranes in tubular epithelial cells, indicating the formation of TJ in these regions (Fig. 3B). Fluorescent signals for occludin in some individual tubular segments were not obvious as compared to signal intensities of ZO-1 in the corresponding areas, possibly reflecting low levels of the relative abundance of occludin.

Immunofluorescence microscopy also showed that various claudin species, including claudins 1–4, 8, 10, and 16, were present in the p.e.8 metanephroi (Fig. 3B). A series of combined images for the claudins and occludin, or ZO-1, demonstrated that the fluorescent signals for individual claudins were merged with some but not all of those for occludin and ZO-1 in the corresponding areas of the tissue sections. This indicated that these claudins were present at the TJ and that distribution of individual claudins was restricted to one or several specific tubular segments. Unfortunately, however, we could not identify each tubule segment to distinguish the tubular localization of each claudin, because the tubules in the metanephroi, both grown in culture and in utero, lacked staining or had very weak and equivocal staining with several different markers specific to each segment such as the Tamm-Horsfall glycoprotein, which specifically discriminates the TAL from other tubular segments in adult kidneys (data not shown and ref. 8).

DISCUSSION

The present study demonstrated that mouse E12 metanephroi could grow up to 8 days with the expression of claudin proteins and occludin and ZO-1 as well at the TJ in tubular epithelial cells. The most characteristic finding was that claudins 2, 10, and 16 were abundant at p.e.2, followed by increases during the incubation period, preceded by the expression of other claudins such as claudins 3, 4, and 8 (Figs. 1 and 3). These differences in expression patterns among claudin subtypes seem to be representative of the spatial and periodical processes of renal branching morphogenesis, the formation of branched epithelial tubules, at later stages of kidney development [13] that leads to the segment-specific distributions of claudins found in the mouse nephron [8]. That is, claudins 3, 4, and 8 are abundant in collecting tubules in the developed kidney, while the others distribute to proximal tubules (claudins 2 and 10) or the TAL of Henle's loop (claudins 16 and 10) formed by branching of the ureteric bud and its daughter collecting duct and the concomitant metanephric tubule formation [13]. These findings indicate that the expression patterns of specific claudin genes in cultured metanephroi are comparable to the developmental changes in the expression of the TJ proteins, including various claudin subtypes in normal embryonic kidneys.

Mutations and targeted disruptions of some specific claudin genes are known to cause pathological phenotypes in humans and animals. Disruption of the claudin-11 gene causes a neurological deficit due to the absence of TJs in central nervous system myelin in mice [5]. Human patients with *CLDN14* mutations [22] and claudin-14 knockout mice [2] demonstrate inherited deafness due to cochlear hair cell degeneration. Various mutations of the *CLDN16* gene are responsible for familial hypomagnesemia with hypercalciuria and nephrocalcinosis in the human [17] and renal tubular dysplasia with interstitial nephritis in cattle [7, 11]. While altered ionic permeability of the paracellular barrier of the reticular lamina due to the absence of claudin-14 is suspected to cause prolonged depolarization and eventual death of outer hairy cells leading to deafness [2], pathogeneses in other diseases, including renal tubular dysplasia in cattle, are unknown. However, pathological findings including tubular atrophy and extensive interstitial nephritis in cattle at ages as young as 2–3 months [12, 15] suggest that tubular dysplasia is the primary change, and thus claudin-16 would have some role in developmental formation of renal tubules in addition to selective paracellular transport of divalent cations. Therefore, various approaches are desirable to clarify the roles of claudin-16 in developing kidneys.

One approach used by several groups to study the function of claudins has been to express individual claudins in monolayers of Madin-Darby canine kidney cells. A major limitation of this approach is that the cells already express a background of multiple claudins. Since heterogeneous claudins form individual TJ strands as heteropolymers and claudin molecules adhere to each other in both homotypic and heterotypic manner, at the cell-cell interface [4], one cannot define a specific unit for an individual claudin in the function for intercellular adhesion and/or permeability barrier [20]. The data presented above demonstrate that organotypic cultures of mouse metanephric explants have the ability to develop normally with regard to the expression of claudins, and thus the metanephroi are suitable to investigate the physiological and pathological roles of claudins in renal tubular formation. Further studies will involve applications of antisense oligonucleotides or interfering RNAs to the metanephroi as reported previously [3, 14] to repress specific claudin genes, including the *CLDN16* gene. The only disadvantage of this organ culture is the difficulty in identification of developing tubular segments with generally utilized marker proteins as described above.

In conclusion, our present study demonstrates developmental changes in the expression of the TJ protein claudins in cultured metanephroi representing those in embryonic kidneys, and thus suggests that the mouse metanephros is suitable to examine the functions of claudin-16 and other claudin species expressed in the kidney.

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