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Claudin 5 Is Transiently Expressed During the Development of the Retinal Pigment Epithelium

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Abstract. During the development of chick retinal pigment epithelium (RPE), the permeability and selectivity of the epithelium's tight junctions are continuously modulated. Overall paracellular permeability decreases, but selectivity increases. Because the claudin family of transmembrane proteins appears to provide the structural basis for selectivity, we examined the expression of claudins as a function of development in chick RPE. Degenerate primers were used with the reverse transcriptase-polymerase chain reaction (RT-PCR) to obtain complete sequences of chick claudins 3 and 5. Northern blotting and semiquantitative RT-PCR demonstrated that claudin 5 was expressed in RPE, but claudin 3 was expressed only in the choroid layer of the eye. Northern blotting, semiquantitative RT-PCR and immunoblotting demonstrated that the expression of claudin 5 was transient, with peak levels of expression between embryonic days 10 and 14. Primary cultures were used to demonstrate that factors secreted by the neural retina induced the expression of claudin 5 nearly 3-fold if RPE was isolated from embryonic day 7 embryos. There was little effect if RPE was isolated from embryonic day 14. The upregulation of claudin 5 correlates with permeability changes that occur during the intermediate stage of RPE development. Interestingly, claudin 5 must be replaced during the late stage of development when the number and complexity of tight junctional strands increases. This would imply more changes in selectivity.

Key words: Tight junctions — Retinal pigment epithelium — Blood-brain barrier — Retina — Junctions — Epithelia

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Introduction

The tight junction, or zonula occludens, is an occluding seal that encircles the endothelial or epithelial cell, binding it to each neighbor in the monolayer. Tight junctions retard diffusion across the monolayer through the spaces between cells (Mitic, Van Itallie & Anderson, 2000; Cereijido & Anderson, 2001). Viewed in the plane of the lateral membrane by freeze-fracture electron microscopy, tight junctions appear as a beltway of anastomosing strands. Tight junctions are often considered a static barrier, but physiologists have long recognized that these are dynamic structures whose permeability can be regulated. Tight junctions vary considerably in both permeability and selectivity (Frömter & Diamond, 1972; Powell, 1981). Various agents increase permeability, but neural tissue secretes factors that decrease the permeability of endothelia (Rubin & Staddon, 1999; Cereijido & Anderson, 2001; Wilt & Rizzolo, 2001). We extended this observation to show that the neural retina secretes factors that decrease permeability of the retinal pigment epithelium (RPE) during development (Rizzolo & Li, 1993; Ban & Rizzolo, 1997; Ban & Rizzolo, 2000a; Ban, Wilt & Rizzolo, 2000).

The zonula occludens proteins, ZO-1, ZO-2 and ZO-3, form a scaffold that assembles other junctional proteins into a complex, but it is the claudin protein family that forms the strands observed by freeze fracture electron microscopy (Furuse et al., 1998; Balda & Matter, 2000). Several lines of evidence suggest claudins lend specificity to the pores that are postulated to be in the strands. Thus far, claudins are the only proteins that shows tissue and regional specificity (Morita et al., 1999a; Rahner, Mitic & Anderson, 2001). Every epithelium and endothelium expresses a subset of the 20 known claudins. Claudin 16 (paracellin 1) regulates the permeability of the kidney tubules to magnesium (Simon et al., 1999).

Overexpression of claudin 2 and claudin 4 had contrasting effects. Overexpressed claudin 2 decreased the transepithelial electrical resistance, TER (Furuse et al., 2001). Overexpressed claudin 4 increased the TER by selectively decreasing permeability to alkali metals without affecting permeability to chloride ions (Van Itallie, Rahner & Anderson, 2001).

An attractive model to study claudins is the RPE of chicken embryos, because during development the permeability of their tight junctions is progressively decreased. Further, these events can be modeled in primary cell culture (Rizzolo, 1997; Wilt & Rizzolo, 2001). Besides decreased permeability, there are changes in selectivity (Ban & Rizzolo, 2000a). The development of RPE tight junctions can be divided into early, intermediate and late stages. In the early stage, key proteins are present, but the junctional complexes are rudimentary and leaky. The intermediate stage is characterized by changes in the isoforms and levels of expression of certain tight junction proteins, decreases in junction permeability and alterations of junction selectivity (Williams & Rizzolo, 1997; Ban & Rizzolo, 2000a; Wilt & Rizzolo, 2001). Of particular note is the observation that the number and branching of tight junctional strands are constant during the intermediate stage. This means that changes in permeability result from changes in the quality of the strands rather than the quantity of strands. The late stage is characterized by an increase in the number and branching of tight junctional strands, which likely decreases permeability further (Claude, 1978; Kniesel & Wolburg, 1993).

We developed a novel culture system that models the intermediate stage where strand morphology is constant. RPE was isolated from chick embryos near the end of the early stage (embryonic day 7, E7) and near the beginning (E10) and end (E14) of the intermediate stage. The cells were grown on filters in a chamber such that the RPE monolayer formed a barrier between the growth medium on the monolayer's apical and basal sides. The barrier properties of the cultures reflected the age of the embryo from which they were isolated (Rizzolo & Li, 1993; Ban & Rizzolo, 1997; Ban & Rizzolo, 2000a). These properties were modulated by medium that was conditioned by E14 neural retinas, which are retinas with the RPE layer removed (Rizzolo, 1997; Ban & Rizzolo, 2000a; Wilt & Rizzolo, 2001). Therefore, by varying age and the presence of retinal conditioned medium, six functional states of the junctions were demonstrated.

The current study demonstrates that claudin 5 is upregulated during the intermediate stage, but that it must be replaced in the late stage by a claudin other than claudin 3. Further, the upregulation of claudin 5 can be induced by diffusible factors secreted by the neural retina, but only in cells isolated near the beginning of the intermediate stage.

Materials and Methods

Tissue Isolation, Cell Culture and Statistical Analysis

RPE and choroid were isolated from white Leghorn chicken embryos of the indicated embryonic age, as described previously (Ban & Rizzolo, 1997; Ban & Rizzolo, 2000a). For primary cell culture, RPE was cultured on 12-mm Transwell filters (Corning Costar, Cambridge, MA) that were freshly coated with laminin (10 $\mu g/cm^2$) (UBI, Lake Placid, NY), as described previously (Ban & Rizzolo, 1997; Ban & Rizzolo, 2000a). Cultures were maintained in serum-free medium (SF2). For some cultures, the medium in the apical medium chamber was replaced with SF2 that was conditioned by E14 neural retinas (Ban & Rizzolo, 1997). Tests of significance were made with the Student's *t*-test.

RNA Isolation

For northern blotting, total RNA was isolated using Trizol reagent according to the manufacturer's instructions (Life Technologies, Rockville, MD). RNA was isolated from the RPE of E7, E10, E14 and E18 embryos. For semiquantitative PCR, total RNA was isolated using the GlassMAX microisolation spin cartridge system (Life Technologies), as described earlier (Ban & Rizzolo, 2000b). The GlassMAX system was also used to isolate RNA from cultured RPE. For positive controls RNA was also isolated from E14 liver and lung.

Cloning

To clone cDNAs of chick claudins, degenerate primers were designed from amino-acid sequences that were conserved among mouse claudins 1–8 (Morita et al., 1999a). Amino-acid sequences 48–55 and 144–151 were selected, and codons were selected to favor chick codon usage (*see* Fig. 1 and Table 1). PCR products of expected size (312bp) were cloned and sequenced by the Keck Center (Yale University). Only claudins 3 and 5 were identified. From these data, the primers indicated in Table 1 were used for 5-prime and 3-prime RACE reactions, according to the manufacturer's protocols (Life Technologies). The cDNAs from three independent experiments were cloned, sequenced in both directions. DNA sequences and amino-acid sequences inferred from the cDNA sequence were analyzed using BLAST software from the National Institutes of Health (http://www.ncbi.nlm.nih.gov/BLAST/).

Northern Blots

Northern blots were hybridized using ExpressHyb hybridization solution according to the manufacturer's instructions (Clontech, Palo Alto, CA). Probes for claudins were prepared from cloned cDNAs that correspond to positions 962–1194 (claudin 3) or 1378–1720 (claudin 5) and radiolabeled by random priming using the Megaprime kit according to the manufacturer's instructions (Amersham, Arlington Heights, IL). Probes for 18S rRNA were prepared from a cDNA that was cloned from chick rRNA using the Classic QuantumRNA 18S Internal Standards (Ambion, Austin, TX). The 18S probes were radiolabeled by diluting the specific activity of the radiolabel 6000-fold. Hybridizations were performed at 79°C (claudins) or 68°C (18S), and final washes were performed with 0.1% SDS in 0.1× SSC at 65°C (claudins) or 50°C (18S).

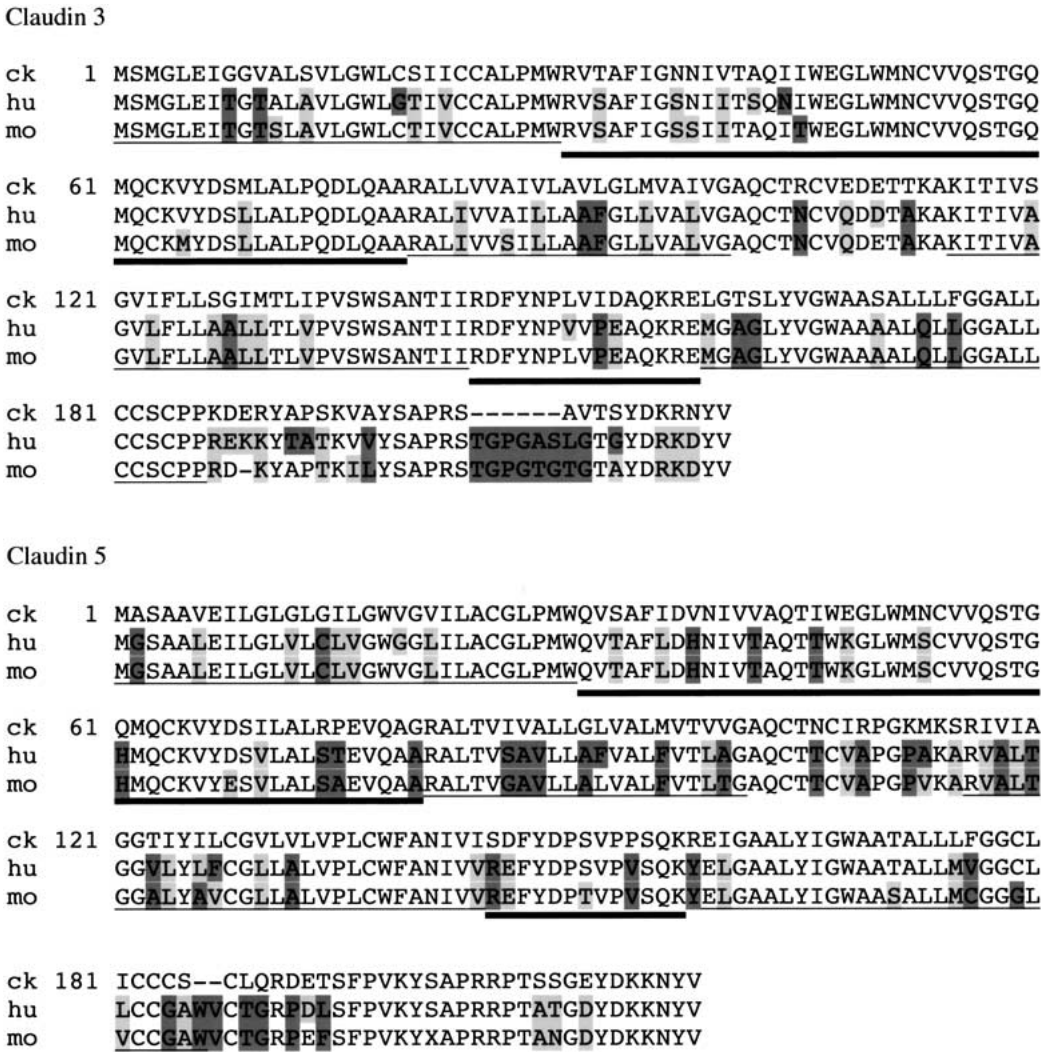

Fig. 1. Comparison of chick, human and mouse claudins 3 and 5. Sequences were inferred from the cDNA sequences that were deposited in GenBank. Amino-acid differences from chick are highlighted in dark gray (non-conservative substitutions) or light gray (conservative substitutions). Putative transmembrane domains are underlined by a thin bar. The putative extracellular domains are underlined by a thick bar. GenBank accession numbers: Chick claudin 3: AF334677, claudin 5: AF334678; Human claudin 3: NM 001306, claudin 5: XM 009839; Mouse claudin 3: NM 009902, claudin 5: NM 013805.

Semiquantitative PCR

The reverse transcriptase-polymerase chain reaction (RT-PCR) was performed using reagents supplied by Life Technologies. The primers used to amplify claudins 3 and 5 were designed using Primer3 software (Whitehead Institute/MIT Center for Genome Research, http://www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) and are described in Table 1. The RT-PCR reaction products were verified by cloning them into the pCR-TOPO vector (Invitrogen, Carlsbad, CA) and sequencing the DNA. The 18S rRNA primers and competing primers (competimers) of the QuantumRNA 18S Internal Standards Kit were supplied by Ambion, and were used according to the manufacturer's instructions. Comparison with the internal standard allows relative changes in the amount of mRNA to be quantified reproducibly. Briefly, RT-PCR was performed for multiple cycles to determine the linear range of amplification for each claudin mRNA. Using the minimal number of cycles in which claudin amplification products were detected, the optimal ratio of 18S primers and competimers was determined. The ratio was adjusted until the amount of 18S product was approximately the same as the claudin product. Because the claudin/18S ratio was very sensitive to the 18S primer/competimer ratio, a master mix of primers was prepared and used in all experiments contributing to a statistical analysis. Products were resolved on 7% polyacrylamide gels and detected using the SilverSNAP kit (Pierce, Rockford, IL). The linear range of the staining procedure was determined using an *Hae* III digest of *PhiX*174 (New England BioLabs, Beverly, MA). Images of the stained gels were acquired with a PowerLook 1100 scanner (UMAX Technologies, Fremont, CA) and analyzed using NIH Image 1.60 software.

Immunoblots

Antibodies raised against the conserved C-terminal domain of mouse claudin 5 were obtained from ZYMED (South San Francisco, CA). A monoclonal antibody to actin (JLA20) was obtained from the Developmental Studies Hybridoma Bank

Table 1. Primers for PCR

	5-prime→3-prime	Position in Sequence*
Degenerate Primers		
Upstream	A(AG)GG(AGC)CT(GC)TGGATGAA(CT)TGCGT(GC)G	239–262
Downstream	ACCAG(AGT)GG(AG)TTGTAGAA(AG)TC(CT)C	530–551
5′-RACE: Claudin 3	ACGATGGCCACCATC	1061–1047
Nested Sequences	GCAGGGCCAGCATGGAGT	982–965
	GATGATGCTGCACAGCCAG	861–813
3′-RACE: Claudin 3	CGTCTCCGGCGTCATCTTCCT	1119–1139
Nested Sequences	CTGGTCGGCCAACACCATCATC	1173–1194
5′-RACE: Claudin 5	ACCACGGTGACCATGA	395–380
Nested Sequences	GCAGCGCCAGGATGGAAT	316–299
	CGTACACCTTGCACTGCATCT	298–278
3′-RACE: Claudin 5	CGCCGGAGGGACCATCTACAT	453–473
Nested Sequences	CTGGTTCGCCAACATCGTCATC	507–528
Claudin 3 PCR		
Upstream	ACGACTCCATGCTGGCCCT	962–980
Downstream	GATGATGGTGTTGGCCGACC	1175–1194
Claudin 5 PCR		
Upstream	AGCCATTATTCCAGGTTCTCC	1378–1398
Downstream	AAGGCAAGTGCATGTTACCG	1701–1720

*Position refers to the GenBank sequence for claudin 3 (AF334677) or claudin 5 (AF334678).
The position for the degenerate primers is indicated for claudin 5.

(Iowa City, IA). Protein extracts were prepared from tissues (Williams & Rizzolo, 1997) and cultures (Ban & Rizzolo, 1997) as described. Samples (10 μg in vivo; 20 μg in vitro) were resolved on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted using the SuperSignal West Femto Maximum Sensitivity substrate for detection of the protein by chemiluminescence (Pierce, Rockville, IL). The chemiluminescence was captured on preflashed film to insure the signal lay in the linear range of the film, and was quantified as described above for the silver-stained gels. The level of actin staining was used as an internal standard to normalize the data. The level of actin per cell was assumed to be two-fold greater in E18 RPE (Nachmias et al., 1992).

Results

Partial sequences of claudins 3 and 5 (312 bp) were amplified from E7 chick RNA using degenerate primers (Table 1). Other claudins were not isolated with this primer set. These sequences were extended in both directions using RACE techniques to obtain overlapping cDNAs that cover the full length of each mRNA. The sequences were deposited in GenBank (claudin 3, #AF334677; claudin 5, #AF334678), and the inferred amino-acid sequences compared with human and mouse claudins 3 and 5 (Fig. 1). The claudin 3's exhibited 73% sequence identity and 87% sequence similarity with mammalian orthologs, if conservative amino-acid substitutions are allowed. The claudin 5's exhibited 70% sequence identity and 82% similarity. Dissimilar amino acids were distributed throughout the sequences. For claudin 5, notable differences were observed in the extracellular domains that potentially lend specificity to tight junctional pores. The mammalian sequences were more positively charged. For claudin 3, the extracellular domains were more highly conserved, but there was an insertion in the C-terminal cytoplasmic domain. The C-terminal domains of claudins bind the PDZ1 domains of ZO-1, ZO-2 and ZO-3 (Itoh et al., 1999). Notably, chick claudin 3 lacked six amino acids adjacent to the putative PDZ binding site.

Northern blots were prepared from embryonic chick RNA. When the blot was probed for claudin 5, two bands were observed at 2.7 and 3.8 kb (Fig. 2). There was a 2- to 3- fold increase in mRNA between E7 and E10 ($p < 0.05$). A slight decrease was consistently observed between E10 and E14, but this did not reach statistical significance ($p < 0.085$). Surprisingly, the amount of mRNA decreased between E14 and E18 to the level of E7 ($p < 0.01$). No signal above background was detected for claudin 3 (*data not shown*).

These results were confirmed by immunoblotting claudin 5. Antibodies raised against a C-terminal peptide of mouse claudin 5 cross-reacted with chick claudin 5 (Fig. 3). A protein was observed in E10 RPE that was the same size as claudin 5 isolated from rat lung. In addition, immunoreactive proteins migrated in the position of dimers and trimers. The peptide used to generate the antibodies blocked the labeling of these proteins. Further, the antibody only labeled tight junctions, as observed by immunofluorescence. When RPE was isolated from various aged embryos, claudin 5 was barely detected on E5 or E7 (Fig. 4). Notably, there was a statistically significant

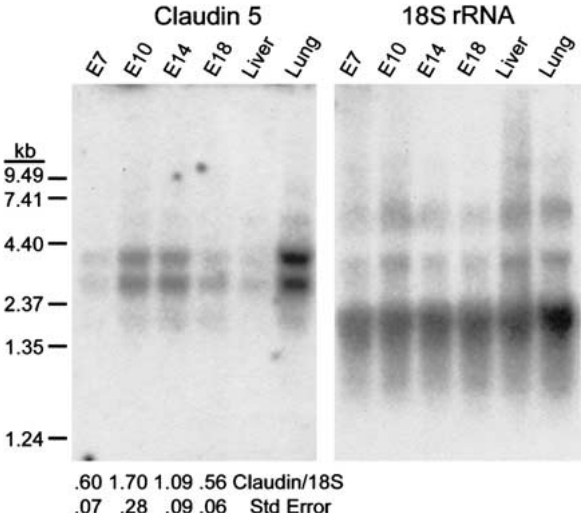

Fig. 2. Northern blots show that claudin 5 mRNA is transiently expressed during development. 10 μg of total RNA were isolated from RPE at the indicated embryonic age or from E14 liver and lung, and resolved on 1% agarose-formamide gels. Blots were prepared and probed for claudin 5, stripped and reprobed for 18S rRNA. A representative blot is shown. The claudin 5/18S ratio and standard error were determined from three independent blots. Size standards in kilobases are indicated on the left.

decrease in steady-state claudin 5 levels between E10 and E14. There was a slight, but detectable level of claudin 5 on E18.

The northern analysis was further confirmed using semiquantitative RT-PCR (Fig. 5). Products of the expected size were obtained and confirmed by cloning and sequencing. The results from the blotting and PCR techniques were quantitatively the same. There was a 3- to 4-fold increase in mRNA between E7 or E18 and E10 or E14 ($p < 0.02$), and a slight, but statistically insignificant decrease between E10 and E14. Although RT-PCR could detect claudin 3 in the liver and choroid layer of the eye, it failed to detect claudin 3 in RPE before E18. On E18, only trace amounts could be detected above background. Claudin 5 was not evident in the choroid (*not shown*). These data confirm the suitability of RT-PCR for examining primary cultures of RPE where the amount of mRNA is limiting.

To determine if the induction of claudin 5 was regulated by the neural retina, mRNA and protein were examined in a culture model of RPE development (Fig. 6). RPE was isolated from E7 and E14 embryos and cultured in the presence or absence of media conditioned by the E14 neural retinas. As documented in earlier studies, the transepithelial electrical resistance of the E14 cultures was twice that of the E7 cultures, and retinal conditioned medium increased the electrical resistance of each culture approximately two-fold (Ban & Rizzolo, 1997; Ban & Rizzolo, 2000a). Claudin 5 mRNA was 2-fold higher in E7 cultures when conditioned medium was present

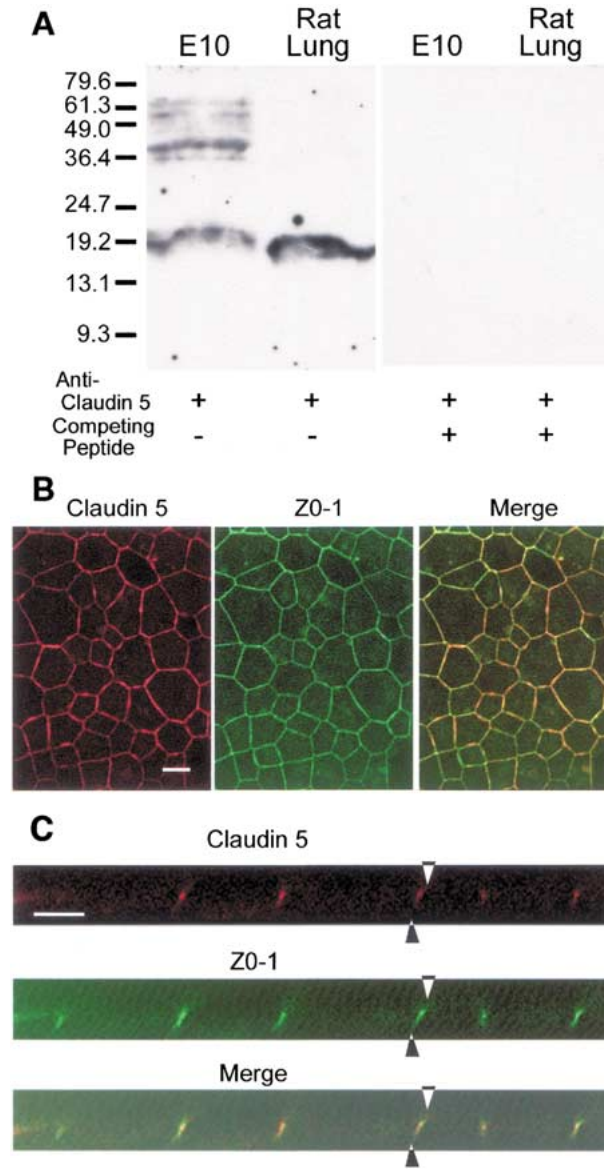

Fig. 3. Characterization of antibodies to claudin 5. (*A*) Immunoblots of RPE isolated from E10 embryos were probed with antibodies to claudin 5 or with antibodies that were preincubated with a more than 10-fold excess of the peptide used to raise the antibody. A lysate of rat lung was run as a positive control. Monomer and presumptive dimer and trimer bands were observed in chick. Each of these were absent in the competition control. Molecular weight standards in kiloDaltons are indicated at the left. (*B*) Primary cultures of E14 RPE were immunolabeled using antibodies to claudin 5 and ZO-1 followed by fluorescently labeled secondary antibodies. Confocal images are shown in the XY plane. In the merged images, orange/yellow indicates the overlap of the claudin 5 (red) and ZO-1 (green) labeling. (*C*) Confocal images in the XZ plane. Claudin 5 was more precisely localized to apical junctions, while some ZO-1 distributed along the lateral membranes. Downward arrowheads, apical membrane; Upward arrowheads, basal membrane; Bars, 10 μm.

($p < 0.05$) (Fig. 6a). By contrast, in E14 cultures conditioned medium decreased the amount of claudin 5 mRNA by 30% ($p < 0.02$). Similar results were

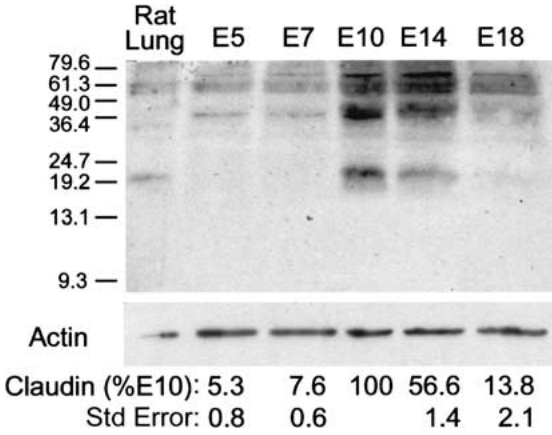

Fig. 4. Immunoblots show a transient expression of claudin 5 protein. RPE was isolated from embryos of the indicated age, and cell lysates were analyzed by immunoblotting with antibodies to claudin 5 and actin. The density of monomer (~19 kDa) and dimer (~36 kDa) bands of claudin 5 were combined. For each blotting experiment, actin labeling was used to normalize the intensity of the claudin 5 signal, which was expressed as a percent of the E10 signal. Molecular weight standards in kiloDaltons are indicated at the left. A representative blot and the statistics for three independent experiments are presented.

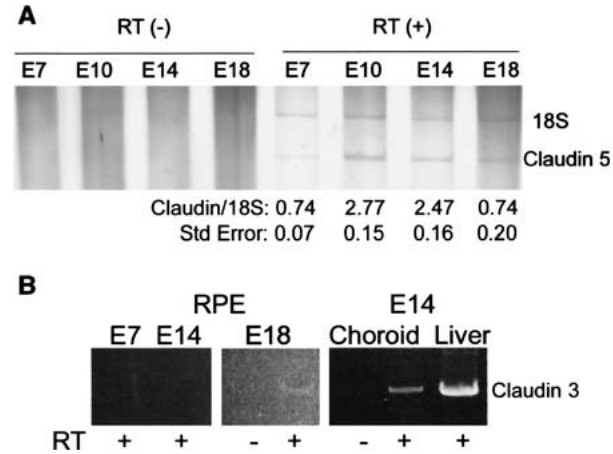

Fig. 5. Semiquantitative PCR shows that claudin 5- but not claudin 3- mRNA is transiently expressed during development. (*A*) Total RNA was isolated from RPE of the indicated age and amplified by PCR (15 cycles) using a mixture of primers for claudin 5 and 18S rRNA and competing (non-extendable) primers for 18S rRNA. The reaction products were resolved on 7% polyacrylamide gels and analyzed by silver staining. A representative gel shows that no reaction products were obtained in the absence of reverse transcriptase (RT−). In the presence of reverse transcriptase (RT+), products of the expected size were obtained for 18S rRNA and claudin 5. The claudin 5/18S ratio and standard error are based on three independent experiments.(*B*) Total RNA (0.5 μg) was isolated from RPE of the indicated age and from E14 choroid and liver and amplified by PCR for 32 cycles using primers for claudin 3. A PCR product obtained from choroid and liver was readily stained with ethidium bromide and confirmed by DNA sequencing. Trace amounts of product were observed with E18 RPE.

obtained by immunoblotting the protein (Fig. 6b). In E7 cultures, conditioned medium raised the steady-state level of claudin 5 by 2.8 fold, but in E14 cultures, it decreased the steady-state level by 18% ($p < 0.05$).

Discussion

Twenty claudin genes have been reported in the human genome (Genbank, Venter et al., 2001). As noted in the introduction, they show tissue specificity and even regional specificity within the same epithelium. This report indicates that claudins can be specific for individual stages during the differentiation of an epithelium. Because claudins lend specificity to the junctions, the changes in expression provide a molecular basis for the changes in selectivity that have been described for RPE development (Ban & Rizzolo, 2000a).

We identified two claudins along the outer blood-retina barrier. These claudins were highly conserved and displayed a high degree of sequence identity with their human orthologs. Further, many of the amino-acid substitutions were of a conservative nature. There were two notable deviations. The extracellular loops of chick claudin 5 were less basic than the mammalian orthologs. Second, the C-terminus of claudin 3 lacked a 6-amino-acid sequence that lay near the binding site for the PDZ1 domains of ZO-1, ZO-2 and ZO-3. This deletion would account for the lack of cross-reactivity with antibodies specific for claudin 3 (*data not shown*). Although claudin 5 was thought to be specific for certain endothelia (Morita et al., 1999b), it was undetected in choroidal vessels but was transiently expressed in RPE. In contrast, claudin 3 was absent or weakly expressed in RPE despite its presence in the choroid and many epithelia (Morita et al., 1999a; Rahner et al., 2001).

Embryonic days 7 and 14 span the intermediate stage of development, when decreases in permeability and changes in selectivity are known to occur (Ban & Rizzolo, 1997; Ban & Rizzolo, 2000a; Wilt & Rizzolo, 2001). The number of tight junctional strands is constant between E10 and E14, but the interval between E7 and E10 has not been studied (Kniesel & Wolburg, 1993). Rudimentary junctions are evident on E7. ZO-1 and occludin are present in an apical junctional complex that can retard the diffusion of horseradish peroxidase (Latker & Beebe, 1984; Williams & Rizzolo, 1997). Therefore, an increase in claudin 5 could represent an increase in strand number or branching, or it could enhance rudimentary strands formed initially by occludin (Furuse et al., 1998). Alternatively, claudin 5 could be replacing another claudin that was expressed earlier. Three independent measures indicate that claudin 5 increases. Northern blotting and semiquantitative PCR demonstrated an increase in the steady-state levels of claudin 5 mRNA. Immunoblotting demonstrated a corresponding increase in

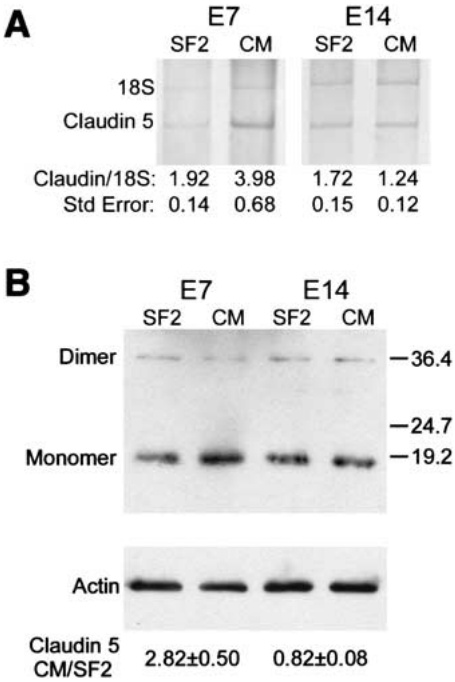

Fig. 6. Claudin 5 expression is regulated by diffusible factors produced by the neural retina. Primary cultures of E7 or E14 RPE were established on filters and maintained with growth medium (SF2) or E14 retinal conditioned medium (CM) in the apical medium chamber. (*A*) Semiquantitative PCR was performed as described in the legend to Figure 5. The claudin 5/18S ratio and standard error are based on four independent experiments. Conditioned medium increased the level of claudin 5 mRNA in cultures of E7 RPE, but decreased it in cultures of E14 RPE. (*B*) Immunoblots were prepared and analyzed as described in the legend to Figure 4. The ratio of claudin 5 expressed in the presence and absence of conditioned medium is indicated together with the standard error. Conditioned medium increased the steady-state levels of claudin 5 protein E7 cultures, but decreased them in E14, cultures. Molecular weight standards in kiloDaltons are indicated at the right.

steady-state levels of claudin 5. The increase in claudin 5 presages permeability changes that were observed in native tissue (Williams & Rizzolo, 1997). Horseradish peroxidase penetrated the tight junctions of RPE up to E10, but not thereafter.

Claudin 5 began to decrease between E10 and E14. The decrease in protein levels was statistically significant. Although there was a consistent trend, the decrease in mRNA levels did not reach significance for either Northern blotting or semiquantitative PCR. Therefore, we cannot rule out regulation of claudin 5 at the level of protein turnover. By all measures, there was a substantial decrease in claudin 5 between E10 and El8. In this case, claudin switching certainly occurs, because claudins form the junctional strands that are increasing in number and branching during this interval (Kniesel & Wolburg, 1993). Claudin 3 would be a likely candidate, as it is common in many epithelia (Rahner et al., 2001). Nonetheless, we could not detect it by northern blotting. Although by RT-PCR, E18 RPE exhibited trace amounts of claudin 3 mRNA, we cannot rule out contamination of the RPE by the adjacent choroid layer. It appears unlikely that claudin 3 alone could compensate for the loss of claudin 5 and the increase in junctional strands. It will be necessary to develop probes for the other 18 or more claudins that might participate in the E7-E10 or E10-E14 transitions (Venter et al., 2001).

A culture model that mimics the functional changes of the intermediate period can be regulated by medium conditioned by E14 neural retinas (Ban & Rizzolo, 1997; Ban & Rizzolo, 2000a). This model examines how the neural layers of the retina regulate the adjacent RPE layer (Rizzolo, 1997; Wilt & Rizzolo, 2001). Although the expression of occludin, ZO-1 isoforms and ZO-2 change during the intermediate stage, their expression is unaffected by conditioned medium (Ban & Rizzolo, 1997; Williams & Rizzolo, 1997; Wilt & Rizzolo, 2001). By contrast, conditioned medium regulated the expression of claudin 5 mRNA and protein in biphasic fashion. Claudin 5 was upregulated in E7 cells. Steady-state levels of both mRNA and protein increased substantially, which suggests an increase in gene transcription or stabilization of the claudin 5 mRNA. If anything, semiquantitative PCR and immunoblotting suggest that claudin 5 was slightly downregulated in E14 cultures. The effect on E14 cultures is consistent with the interpretation that the decrease observed in vivo between E10 and E14 results from a decrease in gene expression or mRNA stability. The contrasting effects of retinal conditioned medium on E7 and E14 cultures is consistent with previous data that demonstrate the presence of at least two active factors in retinal conditioned medium (Ban et al., 2000). E7 cells were shown to respond only to a low molecular weight factor (<10 kDa), while E14 cells responded only to a 49-kDa protein termed RCM49. Both factors required more than a day to exert their effect, which suggests they affected gene expression. Claudin 5 appears to be one such gene.

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