The apical and basal environments of the retinal pigment epithelium regulate the maturation of tight junctions during development

Christoph Rahner*, Masayuki Fukuhara*, Shaomin Peng‡, Shota Kojima and Lawrence J. Rizzolo§
Department of Surgery, Department of Ophthalmology and Visual Science, Yale University School of Medicine, New Haven, CT 06520-8062, USA

*These authors contributed equally to this work
‡Present address: Second Affiliated Hospital of Harbin Medical University, 246 Xuefu Road, Harbin, China
§Author for correspondence (e-mail: lawrence.rizzolo@yale.edu)

Accepted 24 February 2004
Journal of Cell Science 117, 3307-3318 Published by The Company of Biologists 2004
doi:10.1242/jcs.01181

Summary
A culture model has been established to study the gradual development of tight junctions during the embryogenesis of the chick retinal pigment epithelium. This study asks how closely the culture model reflects normal development and how the composition, structure and function of embryonic tight junctions are affected by the apical and basal environments. The study focused on the expression of claudins, the fine-structure of tight junctional strands and the transepithelial electrical resistance. Between embryonic days 7 and 14, patches of junctional strands gradually expanded and coalesced to form a continuous junction, in vivo. Although there was a corresponding increase in claudin expression, different claudins appeared at different times. In culture, the apical and basal environments acted synergistically to promote a continuous network of tight junctions with higher electrical resistance. Independently, pituitary extract or the secretory products of either embryonic fibroblasts or the retina promoted the formation of tight junctions. In combination, three effects were identified. With basally placed fibroblast conditioned medium, apical retinal medium increased transepithelial electrical resistance by affecting structure alone. With basally placed pituitary extract, apical retinal conditioned medium increased transepithelial electrical resistance by affecting structure and by modulating claudin expression in a manner that was consistent with development in vivo. Although embryonic day 7 and 14 cultures in retinal medium exhibited similar structure, the transepithelial electrical resistance of the embryonic day 14 cultures was higher. This higher transepithelial electrical resistance correlated with differences in claudin expression and localization. Therefore, this experimental model can isolate the effects of retinal secretions on structure and claudin expression, and can help us to determine how claudins affect function when structure is held constant.

Key words: Tight junctions, Retinal pigment epithelium, Retina, Epithelia, Development, Claudins

Introduction
The cells of an epithelial monolayer are bound to each of its neighbors by a circumferential band of junctions (Farquhar and Palade, 1963). Of these, the tight junction forms a partially occluding seal that retards the diffusion of solutes across the paracellular space (Cereijido and Anderson, 2001; Gonzalez-Mariscal et al., 2003; Tsukita et al., 2001). This barrier function of the tight junction enables the monolayer to establish and maintain concentration gradients between its apical and basal environments. The permeability and selectivity of tight junctions differ among the various epithelia and can be regulated. By transmission electron microscopy of conventional thin sections, tight junctions are observed as a series of ‘kisses’, where the lateral membranes of adjacent cells appear to fuse. When the lateral membrane is observed en face by freeze-fracture electron microscopy of chemically fixed tissue, these kissing points appear as a continuous network of branching and anastomosing strands that encircle each cell in the apical-most domain of the lateral plasma membrane.

Two approaches have been used to investigate the permeability and selectivity of tight junctions. One examines protein composition and post-translational modification (Balda et al., 2000; Balda and Matter, 2000; Gonzalez-Mariscal et al., 2003; Matter and Balda, 2003; Nitta et al., 2003; Tsukita and Furuse, 1999; Tsukita et al., 2001; Yu et al., 2003). The claudins are a family of 20-24 transmembrane proteins that form junctional strands and help determine permeability and selectivity. Epithelia of various tissues express subsets of these claudins (Rahner et al., 2001). Occludin is another transmembrane protein of the junctional strands that is ubiquitously expressed by epithelia. Changes in its phosphorylation correlate with changes in permeability (Antonetti et al., 1999; Sakakibara et al., 1997). The modulation of protein composition or post-translational modification could affect the properties of individual strands or the structural arrangement of the strands (Van Itallie et al., 2001).

Another approach examines the diversity of structure found in the tight junctions of different tissues (Claude, 1978). Three structural features affect permeability. First, there is an inverse correlation between the number of strands parallel to the plane of the monolayer and the permeability of the junction. Second,
diffusion can be retarded by narrowing the space between the lateral membranes of adjacent cells. Therefore, the distance between the apical-most and basal-most strand (depth) is inversely proportional to the permeability. Third, the junction is subdivided into compartments, which provides a means to amplify the barrier properties of the individual strands. Compartments are formed by the branching and anastomosing of junctional strands. An increase in the number of branch points (complexity) leads to more compartments and lower permeability. Nonetheless, structure alone fails to explain all the observed differences in permeability. Two strains of MDCK cells have similar structure, and yet there is a large difference in their permeability (Stevenson et al., 1988). The difference in the permeability of these strains results from differences in the expression of claudins (Colegio et al., 2002; Furuse et al., 2001).

We studied the development of tight junctions in vivo and in a culture model of embryonic development (Ban and Rizzolo, 2000; Peng et al., 2003). The retinal pigment epithelium (RPE) forms the outer blood-retinal barrier by separating the neural portion of the retina from the choriocapillaris (Marmor and Wolfensberger, 1998; Rizzolo, 1997; Wilt and Rizzolo, 2001). The embryonic development of vertebrate RPE can be divided into early, intermediate and late phases. The transition between the early and intermediate phases occurs when photoreceptors protrude through the outer limiting membrane of the retina (embryonic day 9, E9, in chick). The transition between the intermediate and late phases occurs when photoreceptors begin to elaborate outer segments (E15 in chick). Previous studies examined the maturation of gap and adherens junctions (Grunwald, 1996; Liu et al., 1997; Sandig and Kalnins, 1990), but studies of the tight junction are less complete. It has been reported that complexity of the tight junction does not change during the intermediate stage, but the number of parallel strands and junctional depth were not described (Kniesel and Wolburg, 1993). Although complexity did not change, the intermediate phase is when protein composition and permeability of tight junctions does change (Kojima et al., 2002; Williams and Rizzolo, 1997; Wilt and Rizzolo, 2001). Structural studies of the period before E10 have not been reported.

In a culture model of the early and intermediate stages, we showed that the permeability of cultures is sensitive to the apical and basal environments (Ban and Rizzolo, 2000; Peng et al., 2003). Like most epithelia, the RPE lies on a basement membrane and underlying capillary bed. Unlike most epithelia, the apical membrane directly apposes a tissue, the neural retina. Diffusible factors secreted by the neural retina act synergistically with basal factors to decrease the permeability of the cultured RPE. These studies suggested that permeability could be explained by a combination of slits (discontinuities) and pores in the junctional network in a model that resembles the dual model of Guo et al. (Guo et al., 2003).

The current study describes the normal differentiation of RPE tight junctional structure and the accompanying changes in the expression of the claudins. Against this standard, we assess development in culture, as it is influenced by changes in the apical and basal environments. The description of structure and claudin expression is combined with previous functional studies to understand the influence of the neural retina and choroid on structure-function relationships in the outer blood-retinal barrier.

Materials and Methods

Cell culture

RPE was isolated from E7, E10 and E14 White Leghorn chicken embryos and cultured on Transwell filters (Costar, Cambridge, MA), as described previously (Ban and Rizzolo, 1997; Peng et al., 2003). Unless noted, reagents were obtained from Sigma (St Louis, MO). Single cell suspensions were plated in SF2 medium. After one day, the medium in the basolateral chamber was replaced with SF2 or SF3 medium and the apical chamber was replaced with SF3 or rcsSF3 medium. SF3 is a completely defined medium (Peng et al., 2003). SF2 medium is SF3 supplemented with 50 μg/ml bovine pituitary extract (Upstate Biotechnologies). The rcsSF3 medium is SF3 that was conditioned by E14 neural retinas using previously described methods (Ban and Rizzolo, 1997). The transepithelial electrical resistance (TER) was used to monitor the function of the RPE tight junctions, as described previously (Peng et al., 2003).

In some experiments, the medium in the basolateral chamber was CEF medium, a one-to-one mixture of SF3 and medium conditioned by secondary cultures of chick embryonic fibroblasts (Morgan and Fekete, 1996). Briefly, fibroblasts were isolated from E10 embryos and cultured in Dulbecco’s modified Eagles medium supplemented with 10% fetal bovine serum and 2% chicken serum. To collect conditioned medium, the serum was reduced to 2% fetal bovine serum and 0.2% chicken serum. Previous experiments showed that this low concentration of serum in the basolateral chamber had no effect on the TER of cultured RPE (Peng et al., 2003).

Preparation and analysis of freeze-fracture replicas

Sheets of RPE and choroid were isolated from E7, E10 and E14 embryos. Briefly, eye cups were prepared by removing the anterior structures and the vitreous body and submerged in SF2 medium that was buffered with 10 mM HEPES, pH 7.2. The retina was removed and sheets of RPE/choroid were separated from the sclera using forceps. The sheets were fixed with 2.5% glutaraldehyde (Polyscience, Wamington, PA) in 0.1 M cacodylate buffer, pH 7.2 for 20 minutes at RT. Sheets were rinsed three times, 30 minutes each, in 0.1 M cacodylate buffer, and freeze-fracture replicas were prepared as described (Rahner et al., 1996). For each age, five replicas were prepared from each of 4-6 embryos. For in vitro experiments, cells were cultured as described above. After 9 days in culture, TER was measured and 3-5 filters for each culture conditions were fixed and processed as described above. For each experimental condition, five replicas were prepared from each filter.

Electron micrographs of the junctional complex were acquired on a Philips 410 electron microscope at a primary magnification of 38,000x. Electron micrographs were digitized at 1200 dpi with a Powerlook 1100 scanner (Umax Technologies, Fremont, CA) and processed with PhotoShop software (7.0). For constant orientation of the tight junctions, images were rotated to the appropriate angle. Parallel lines, spaced 0.2 μm apart, were superimposed perpendicular to the apical-basal axis of the cells. The number of strands intersecting these grid lines was counted and the junctional depth as the distance between the apical-most and basal-most junctional strands was measured. Strands were considered proper parts of the tight junction only if both ends were connected to the network of strands. The number of strands connected to the junctions at one end (open-ended) and unconnected (free-floating) were also recorded. The distance between the branch points of proper junctional strands was determined and the reciprocal was reported as the complexity of the junctions. Statistical analysis was performed using KaleidaGraph (v. 3.6) software. The Student’s t-test was used to determine the significance of pair-wise comparisons. A one-way analysis of variance with a Bonferroni post hoc test was used to compare sets of three or more specimens. For data that was not normally distributed, the Wilcoxon matched pairs test was used.
Qualitative and quantitative characterization of claudin mRNA expression

Total RNA was isolated from sheets of RPE that were isolated from E7, E10, E14 and E18 embryos or from RPE that was cultured as described above. To isolate total RNA, the RNeasy Protect kit from Qiagen (Valencia, CA) was used according to the manufacturer’s protocols. For nonquantitative studies, the polymerase chain reaction (31 cycles) was used to amplify claudin mRNA, which was visualized on ethidium bromide gels, as described previously (Kojima et al., 2002). We examined each of the claudins that has been reported for the chicken in the Institute for Genomic Research (http://www.tigr.org/) or GenBank Gallus gallus databases. These include claudins AL, 1, 2, 3, 4, 5, 10, 11, 12 and 15. Table 1 indicates the primers that were used. Primers for claudin AL were based on a chick GenBank sequence that was described as being similar to Xenopus claudin A. However, we observed that the inferred amino acid sequence of the reaction product was very similar to claudin 9. Closer examination of the inferred amino acid sequence for extracellular loop 1 suggests that this chick claudin has different functional properties than claudin A or claudin 9 (Cologli et al., 2003). Therefore, because of the original GenBank designation and the potential functional difference, we designated this claudin as claudin AL for A-like. The primers and Taqman probes were designed using Primer 3 software from the Whitehead Institute/MIT Center for Genome Research. For quantitative determinations, real-time reverse transcriptase-PCR (RT-PCR) was used to measure the expression of claudins mRNA in vivo and in vitro. For each claudin, a standard curve was determined as follows: The concentrations of linearized plasmids bearing the relevant sequence were quantified with BisBenzimide (Sigma) using a DyNA Quant 200 fluorimeter (Hoefer Pharmacia Biotech, San Francisco, CA). A dilution series of linearized plasmid was analyzed by RT-PCR using the Quantitect SYBR Green PCR kit (Qiagen) or Taqman Probes, as indicated in Table 1. Taqman probes for claudins 5’ ROX reporter and 3’ Blackhole Quencher-2 (BHQ-2), and 18S rRNA 5’ FAM reporter and 3’ BHQ-1) were purchased from Biosearch Tech. (Norato, CA). Data from multiple samples were normalized using 18S primers, and competitors from Ambion, according to the manufacturers protocols. A primer/competitor ratio of 1/15 was used to yield a signal for 18S RNA that was comparable to the claudin signal. For experiments using SYBR Green, parallel samples were assayed for rRNA or claudin mRNA. For experiments using Taqman probes, 18S RNA and claudin mRNA were assayed simultaneously in a multiplex reaction. For those claudins for which Taqman probes were prepared, similar results were obtained regardless of whether they were analyzed by uniplex reactions with SYBR Green or multiplex reactions using Taqman reporters.

Immunofluorescence

Specimens (RPE cultured on filters or flat mounts of RPE/choroid) were fixed in 100% ethanol at 4°C for 30 minutes. Where the cDNA sequence encoding the C-termius was known, rabbit anti-peptide antibodies were raised by Antibody Solutions (Palo Alto, CA). Briefly, a peptide was synthesized with an N-terminal cysteine to facilitate coupling to the carrier protein, keyhole limpet hemocyanin. The peptides were NH2-CRSETSYPPSRGYPKNAPST-OH (claudin 1), NH2-CMQKPKPSFSSYNLTGYV-OH (claudin 2) and NH2-CSARSLSAMEIDIP-OH (claudin 12). Polyclonal and monoclonal antibodies to claudin 5 (ZYMED, San Francisco, CA) bind the chick protein (Kojima et al., 2002). The rat monoclonal antibody R40.76 was used to label ZO-1 (Anderson et al., 1988). To test specificity, peptide competition was performed by preincubating the antiserum with 0.4 mg/ml of the peptide antigen. In multi-labeling experiments, competition for anti-claudin 5 and anti-ZO-1 was not observed. To exclude cross-reactivity during multi-labeling, ML-grade secondary antibodies conjugated with Cy2, Cy3 and Cy5 dyes were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Indirect immunofluorescence and image acquisition were performed, as described previously (Rahner et al., 2001).

Table 1. Primers for PCR

| Primer | cDNA 5’-3’/accession no. | Sequence |
|--------|--------------------------|----------|
| CLDN 1/TC84607* | L | GAGATGGTGATCTCATCGACC | 132-152 |
| R | AGGCCACCTCGTTGCAATCC | 495-514 |
| TM | CTGGTGTGTGTTGTTGCTGTGAC | 353-378 |
| CLDN 2/TC66199* | L | GAGGTCTCTGCTGTCTCCTC | 593-612 |
| R | ACTCATTCTGGCGTCTTGC | 971-990 |
| CLDN3/AF334677* | L | CCAAGATCACCATGGTCTCC | 972-991 |
| R | CACCCAGGGTGTGGAAAT | 1065-1084 |
| CLDN 4/AY35420* | L | ATGCCCTGCTCCTACATCA | 552-569 |
| R | CTGTTGATACACTTCCGAG | 669-688 |
| CLDN 5/AF34678* | L | AGCCATTACCCAGGTTCATCC | 1378-1398 |
| R | AAGCCAGTGCACTTGACCG | 1701-1720 |
| TM | TTGTCGCTGCTCCAGACCCG | 1503-1524 |
| CLDN 10/TC50298* | L | TGGAGGCTCCTACTCTGACATCA | 371-390 |
| R | GATGCGGCTCCTATTTATGCG | 434-453 |
| CLDN 11/TC69344* | L | GGAGCTCCTCATACTCCCCT | 476-495 |
| R | GGGGAAGAAACAACCTGGT | 840-858 |
| CLDN 12/TC58599* | L | GATGTTGATGACAGGATCCTTTC | 287-306 |
| R | GTGGTACCAACAGGATGTCG | 403-422 |
| TM | TGGACACGCTATGCCCTCCTAGCCATA | 345-369 |
| CLDN 15/TC72240* | L | TATGCTGTCAGCTGCTGTG | 1095-1114 |
| R | CAAGATGTCATCAGGACAA | 1220-1239 |
| CLDN A/BN49612* | L | TTGGATGACTCTGCCGTCCTCA | 252-271 |
| R | CATGATGAGGGTGTTGACCA | 352-371 |
| 18S/M10098* | TM | TTGCTAAGCAGCCACTTGCTCCTAA | 1545-1570 |

Primers are designated as L, left; R, right; TM, Taqman. The accession numbers are for the *TIGR or ‡GenBank databases.

Results

Development of tight junctions in vivo
A description of how RPE tight junctions develop in vivo is required to relate the culture model to the normal development of the tissue. Tight junctions were examined in RPE isolated from E7, E10 and E14 embryos. On E7, single strands and patches of strands were occasionally observed. By contrast, gap junctions were commonly observed at the apical end of the lateral membranes. For example, freeze-fracture replicas showed gap junctions and microvilli, but no tight junctional strands (Fig. 1A). When observed, single strands were commonly associated with a gap junction at their tips (Fig. 1B). Occasionally, strands were assembled into patches (Fig. 1C).
To quantify the structural parameters of these patches, we placed grid lines perpendicular to the plane of the monolayer and counted the number of strands aligned parallel to the plane of the monolayer (Fig. 2A, Table 2). The depth of the junctions was also measured as the perpendicular distance between the apical-most and basal-most strand (Fig. 2B, Table 2). On E7, the patches averaged 2.5±0.3 strands. When two or more strands were present, the depth averaged 0.15±0.02 μm. The complexity of the interconnected strands was 6.8±0.5 branch points/μm. The standard error for the complexity was low, because ~1000 strand segments were measured. However, the distance between branch points was very variable to yield a broad, flat distribution with a standard deviation of 6.0.

Similarly, broad distributions were observed for each sample reported in this study. Many of the strands observed could not contribute to the function of the tight junction because they were unattached at one end (20%) or were free-floating (9%). On E10, strand-less regions subjacent to the apical microvilli were rare, but two types of tight junctional structures were evident (Fig. 1D,E). Regions with discontinuities gave rise to a high percentage of open-ended and free-floating strands (Table 2). Other regions exhibited a network of continuous strands. Like the patches observed on E7, the continuous and discontinuous regions averaged 2.3±0.2 strands with an average depth of 0.12±0.02 μm (Fig. 2, Table 2). The complexity (5.1±0.4 branch points/μm) was somewhat lower than observed in E7 RPE, because the regions with discontinuities were less complex. Like E7, 23% of strands were either free-floating or attached to the junction at only one end. An analysis of variance indicated that there were significant differences in complexity among E7, E10 and E14 RPE (P<0.001).
Regulation of tight junction maturation

The transition from the intermediate to the late phase of development occurs when the outer segments of the photoreceptors begin to form. This occurs on E15 for chick RPE. Therefore, E14 is near the end of the intermediate stage. A continuous network of tight junctional strands was always observed (Fig. 1F). The percentage of open-ended and free-floating strands decreased considerably, and the complexity of the junctions increased to 9.1±0.2 branch points/μm (Table 2). The average number of strands increased to 5.6±0.2 and the average depth of the junctions increased to 0.31±0.01 μm (Fig. 2). Unlike the younger tissues, >90% of the strands were fully incorporated into the tight junction. In summary, when junctional strands were observed they were always at the apical end of the lateral membrane, as marked by microvilli in the field. As development proceeded through the intermediate phase, isolated patches of tight junctional strands were assembled into a continuous network that significantly increased in strand number, junctional depth and complexity (P<0.001).

Expression of claudins in vivo

We examined the claudins because they readily form tight junctional strands in vitro (Furuse et al., 1998). Of the claudins known to be expressed by chickens, RPE appeared to express mRNA for claudins AL, 1, 2, 5, 11 and 12 (Fig. 3A,C). The mRNAs of claudins 3, 4, 10 and 15 were only evident in other tissues. To determine the relative amounts of each claudin, the expression of claudin mRNA was quantified using the RT-PCR reaction at different stages of development. Data in Fig. 3C confirms earlier studies that showed claudin 5 mRNA increased until E10, but decreased slightly thereafter (Kojima et al., 2002). By contrast, claudins 1, 2 and AL continued to increase through E18 (when the retina appears fully differentiated, histologically). Consequently, the ratio of the different claudin mRNAs changed during development. On E7, claudins 5 and 12 were the most prominent, as the other claudins were barely detectable. On E10 both claudin 1 and 5 mRNAs were each 10x greater than claudin 12 mRNA, and by E18 the number of copies of claudin AL mRNA exceeded that of claudin 5 mRNA. While claudin 1 increased 1000-fold between E7 and E18, claudins 2 and AL increased only 10- and 60-fold, respectively. On E18, claudin 1 represented approximately 83% of the total claudin mRNA. Claudin AL represented 9% and claudin 5 mRNA represented 6% of the total, whereas claudins 2 and 12 represented only 1%

Because PCR is very sensitive, it might detect potential contamination by the neural retina or the choroid. Claudins 5, 11 and 12 were expressed weakly in the neural retina, but strongly in the choroid (Fig. 3A). Because the choroid was
much more difficult to dissect from the RPE, it had the greater potential to contaminate the preparation. From culture data (not shown), we estimate that there is approximately 0.01 ng of total RNA per cell, which suggests that claudin 11 is present in less than one copy per cell and is probably a contaminant. By contrast, claudin 5 has been detected immunologically in RPE (Kojima et al., 2002), and the same was observed for claudin 12 in this study.

Where possible, indirect immunofluorescence was used to examine claudin expression at the protein level (Fig. 3D). We were able to raise specific rabbit polyclonal antibodies to claudins 1, 2 and 12 and use commercial antibodies to claudin 5. The sensitivity of the antibodies generated in this study was too low to detect claudin in chick RPE by immunoblotting, but a strong signal localized to the tight junction was obtained by immunofluorescence. Claudin 1 was undetected in E7 RPE, but in E14 RPE it was found in junctions and the cytoplasm. As for each of these antibodies, the peptide used as the antigen blocked all claudin 1 immunoreactivity without affecting the immunoreactivity of the other anti-claudin antibodies (not shown). Claudins 2 and 5 increased between these ages, but claudin 12 was unchanged. Notably, the immunofluorescence was punctate along the tight junction only in the E7 RPE in vivo, which is consistent with the lack of a continuous junction, as observed in freeze-fracture replicas (Fig. 1A-C).

**Table 3. Transepithelial electrical resistance (TER) of cultures used in this study**

|                | SF3/SF3 | rcSF3/SF3 | SF3/SF2 | rcSF3/SF2 | SF3/SF3CEF | rcSF3/SF3CEF |
|----------------|---------|-----------|---------|-----------|------------|-------------|
| E7             | 23.9±0.6| 52.9±3.3  | 23.4±0.4| 68.1±6.4  | –          | –           |
| E10            | –       | –         | 23.1±0.6| 60.8±1.6  | –          | –           |
| E14            | 16.2±2.6| 74.7±5.3  | 33.5±0.7| 121.9±2.5 | 69.7±2.7   | 229.7±5.5   |

Data were pooled from three experiments using 3-6 filters per experiment. The contents of the apical/basolateral media chambers are indicated. The TER in Ω cm² and s.e.m. are indicated.

Effect of retinal conditioned medium in culture on structure

Previous data show that in minimal media (SF3) RPE attaches weakly to the substrate, and only E7 cells display an epithelioid appearance. The major effect of E14 retinal conditioned medium (rcSF3) was to promote an epithelial phenotype in E14 cultures and a continuous network of tight junctional strands in E7 and E14 cultures (Peng et al., 2003). We quantified the effect of rcSF3 on the morphology of the tight junctions by using freeze-fracture electron microscopy. The TERs of the cultures used for these studies are shown in Table 3. Compared with E7 RPE in vivo, the E7 cultures exhibited more patches of tight junctional strands (Figs 4, 5). There were 3.6±0.3 strands with a depth of only 0.19±0.03 μm. This represents a slight increase in strand number, but not depth, compared with the patches of strands of E7 RPE in vivo (P<0.05). The complexity and percentage of open-ended and free-floating strands was also similar to E7 in vivo (Table 4). The rcSF3 appeared to eliminate discontinuities in the network thereby yielding a more homogeneous junction. The depth and strand number increased to the levels observed for E14 RPE in vivo. The complexity also increased, but was slightly less than the complexity of E14 RPE in vivo (P<0.05).

Tight junctional strands were rare in cultures of E14 RPE that were maintained in SF3 alone. These cells had a nonepithelioid appearance with an abundance of stress fibers (Peng et al., 2003). These cultures had the highest percentage of free-floating strands. When patches of interconnected strands were observed (Fig. 4), they were lower in strand number and complexity than in E14 RPE in vivo (P<0.001), but the depth was similar (Fig. 5; Table 4). When rcSF3 was in the apical medium chamber, the cultures displayed an epithelial phenotype. A continuous network of tight junctional strands was evident, although there were more open-ended and free-floating strands than in E14 RPE in vivo. The depth was...
and complexity increased (Figs 6, 7; Table 4). Notably, medium had little effect on strand number, but the depth increased (P<0.001) or rcSF3 (○). In E7 cultures, the junctions were heterogeneous with respect to strand number and depth. Although the junctional networks remained heterogeneous, retinal conditioned medium increased the number of strands and the depth. In E14 cultures, the rare junctions that were observed in the absence of rcSF3 had a structure that was similar to those found in the presence of rcSF3. In E14 cultures, the principal effect was to convert the rare patches into the continuous, circumferential network that typifies tight junction morphology.

unaffected, compared with the patches formed in SF3. By contrast, the number of strands and complexity increased significantly, but were less than that of RPE in vivo (P<0.001). Complexity in the E14 cultures was significantly less than that in the E7 cultures (P<0.001) in rcSF3, but the number of strands and the depth of the junctions were similar. Despite the lower complexity, the E14 cultures had the higher TER (Table 3). In summary, E14 retinal conditioned medium converted the tight junctions of E7 and E14 cultures into structures that approximated the junctions of E14 in vivo, but the higher TER of the E14 cultures was unexplained by these structural parameters.

Effect of bovine pituitary extract in the basal environment on structure

Despite the ability of rcSF3 to induce an epithelial phenotype, to stimulate the production of strands and to assemble them into a continuous network, the low TER indicated that the cultures were relatively leaky. Previous studies indicated that retinal factors acted synergistically with pituitary factors to decrease the permeability of RPE in culture (Peng et al., 2003). In E7 cultures, SF2 in the basal medium chamber and SF3 in the apical chamber supported a nearly continuous, homogeneous network of junctional strands (Figs 6, 7). The number of parallel strands was higher in SF2, and the average depth increased (P<0.001). Nonetheless, the junctions were still very leaky (Table 3) and discontinuities were often present. When SF2 was in the basal medium chamber, apical rcSF3 medium had little effect on strand number, but the depth and complexity increased (Figs 6, 7; Table 4). Notably, discontinuities were no longer observed and the junctions appeared similar to those found in E14 RPE in vivo.

In E10 cultures, substitution of basal SF3 with SF2 supported an epithelial monolayer with a very heterogeneous junctional complex. There was a broad distribution of parallel strands with as many as 12 strands observed. Similarly, there was a broad distribution of junctional depths (Figs 6, 7; Table 4). When, in addition, rcSF3 replaced SF3 in the apical medium chamber, a more homogeneous junction was observed. The junctions averaged 6.1±0.2 parallel strands with an average depth of 0.44±0.2 μm. Discontinuities were not evident. These structural characteristics and the TER resembled those of E7 cultures in basal SF2 and apical SF3.

The E14 cultures also exhibited a heterogeneous junctional complex when SF2 was added to the basal medium chamber and SF3 was added to the apical chamber (Figs 6, 7; Table 4). There was a broad distribution of junctional strands and a bimodal distribution of junctional depth. Discontinuities in the network were evident. When rcSF3 replaced SF3 in the apical medium chamber, discontinuities disappeared, and the tight junctions became more homogeneous. Recall that rcSF3 was made by using E14 retinas to condition SF3 medium. Notably, the average number of parallel strands and junctional depth were similar among all the cultures in rcSF3 and were close to the structure of E14 RPE in vivo. Despite these similarities and the lower complexity in the E14 cultures, the E14 cultures were substantially less permeable (Table 3) (Peng et al., 2003). In
in the choroid. Secondary cultures of chick embryonic fibroblasts (CEF) were used to make a conditioned medium that was mixed 1:1 with SF3. The growth medium for CEF cells contains a low concentration of serum. Consistent with Peng et al. (Peng et al., 2003), control experiments showed that unconditioned medium had no effect on the TER (data not shown). The presence of CEF medium in the basal chamber promoted the formation of an epithelial monolayer in E14 cultures with plentiful microvilli and a TER comparable to E14 cultures in rcSF3/SF3 (Table 3). The combination of apical rcSF3 and basal CEF medium was synergistic and yielded a TER that exceeds the 138 W·cm² expected for in vivo RPE (Gallemore and Steinberg, 1993).

Absent rcSF3, CEF conditioned medium formed a continuous network of junctions that averaged 5.7±0.3 parallel strands and 0.45±0.2 µm deep, but note that the distribution was bimodal (Figs 8, 9; Table 4). The inclusion of apical rcSF3 led to junctions that averaged 8.3±0.6 parallel strands and were 0.72±0.06 µm deep. Although the distributions remained bimodal, the mode that was most in vivo-like was much more prominent. These increases were accompanied by an increase in complexity. Like the interaction with pituitary extract, the effects of CEF medium and rcSF3 on function were synergistic. However, with CEF rcSF3 also increased the number of strands and junctional depth.

### Effect of culture conditions on the expression of claudins

The same claudin mRNAs expressed in vivo were found in each culture condition. In addition, trace amounts of claudins 3, 4 and 11 were occasionally detected, but always at less than rs.
Regulation of tight junction maturation

one copy per cell. The striking finding was that the mRNAs for claudins 5 and 12 were expressed in far greater amounts than in vivo (Fig. 10A-C). For example, when E7 RPE was cultured in the presence of apical rcSF3 and basal SF3, there was a 600-fold excess of claudin 5 mRNA, and the other claudins were also evident in greater amounts (Fig. 10A). By contrast, the E14 cultures expressed claudins 1, 2 and AL mRNAs in amounts similar to those found on E14 in vivo. Even though claudin 5 and 12 expression decreased in the E14 cultures, they were still in great excess compared with RPE in vivo. Despite the overexpression of claudin 5 and 12 mRNA, strands were only observed in freeze-fracture replicas at the apical end of the lateral membranes.

Retinal conditioned medium effected claudin expression most when pituitary extract was included in the basolateral medium chamber (Fig. 10B). The effect varied with the claudin and the embryonic age of the RPE. Regardless of embryonic age, rcSF3 increased the expression of claudin 1 (3×) and claudin AL (10×) to levels comparable to expression in vivo. By contrast, the effects on claudins 2, 5 and 12 depended on age. Consistent with the patterns observed in vivo, rcSF3 stimulated the expression of claudin 2 mRNA (7×) in E14 cultures, but had little effect on claudin 2 in E7 cultures. Retinal conditioned medium modestly stimulated the expression of claudin 5 mRNA in the E7 cultures, but had little – if any – effect on the E14 cultures. Further, rcSF3 lowered the expression of claudin 12 mRNA only in E14 cultures.

Even though both cultures have continuous junctions of similar structure, the TER of E14 RPE cultured with rcSF3/SF2 was higher than that of E7 RPE (Figs 6, 7; Table 3). Although the E14 cultures expressed only slightly more claudin 1 mRNA, immunocytochemical localization of claudin 1 shifted dramatically from cytoplasmic to junctional pools (Fig. 10D). A similar shift was observed for claudin 12 even though the steady-state level of claudin 12 mRNA decreased. The distribution of claudin 2 and 5 was unchanged. Therefore, E7 cells stimulated by E14 retinal conditioned medium in culture were able to express claudins not observed in vivo at this embryonic stage, but were unable to predominantly target them to the tight junctions.

The CEF conditioned medium minimized the effects of rcSF3 (Fig. 10C). Although the expression of claudin AL was modestly induced, the regulation of other claudins was minimal. Inconsistent with the in vivo observations, claudin 5 mRNA was slightly induced rather than suppressed.

Discussion

These studies show that the neural portion of the retina secretes diffusible factors that regulate the structure and composition of RPE tight junctions. Furthermore, the signaling pathways that are activated by this apical stimulation interact with signaling pathways that are activated by factors presented to the basolateral side of the monolayer. The culture model described here can be adapted for studying three aspects of this regulation: regulation of claudin gene expression, regulation of permeability in the absence of structural changes and regulation of permeability with minimal changes in claudin expression.
In vivo, the tight junctions of chick RPE develop gradually. On E7, there were occasional free-floating strands, or patches of strands. The strands were always observed in proximity to microvilli, which indicates that they were confined to the apical end of the lateral membranes. The strands were often associated with gap junctions that became incorporated into mature tight junctions, as described for many species (Hudspeth and Yee, 1973). By E10, patches of tight junctional strands began to coalesce into a continuous network, which just precedes the time when RPE blocked the transmonolayer diffusion of horseradish peroxidase (Williams and Rizzolo, 1997). Between E10 and E14, free-floating and open-ended strands were incorporated into the junctional complex; this was accompanied by an increase in the number of strands, junctional depth and complexity. These results differ from those of Kniesel and Wolburg (Kniesel and Wolburg, 1993), who reported only on complexity. They observed that complexity did not increase until after E15. This discrepancy may relate to our observation that E10 is very close to the transition when the RPE forms a barrier to horseradish peroxidase. Because these authors did not report studies of earlier ages, it is possible that under their incubation conditions the E10 embryos were slightly more mature than ours.

During the gradual assembly of strands into junctions, the expression of the tight junctional proteins changes. Previous studies showed that ZO-1 and occludin were present from E3, although the expression of ZO-1 isoforms and the association of occludin with the apical junctional complex did change (Williams and Rizzolo, 1997; Wilt and Rizzolo, 2001). Occludin increased its affinity for the junctions in parallel with the expression of claudins described here, and may reflect the incorporation of occludin into tight junctional strands formed by claudins (Furuse et al., 1998). Because claudins lend specificity to the permeability of tight junctions (Colegio et al., 2002; Colegio et al., 2003; Furuse et al., 2001; Simon et al., 1999), changes in claudin expression during RPE development suggest changes in function. On E7, the nascent strands were composed primarily of claudins 5 and 12, similar to endothelial junctions (Nitta et al., 2003). As development proceeded, the steady-state levels of the various claudin mRNAs changed continuously. Immunocytochemistry indicated a second level of regulation. Although claudin immunoreactivity increased in
parallel with mRNA, claudins 2 and 5 were detected only in the junctional region, whereas claudins 1 and 12 (but no junctional strands) were also found outside the junctional region on E7.

We devised a primary cell culture system to model development and found that RPE tight junctions were sensitive to retinal secretions (Ban and Rizzolo, 1997; Ban et al., 2000; Peng et al., 2003; Rizzolo, 1991). Although E7 RPE could form an epithelial monolayer when cultured in minimal medium (SF3), E14 RPE required more environmental cues, which could be provided by pituitary extract, diffusible factors secreted by chick embryonic fibroblasts or diffusible factors secreted by the neural retina. The current study shows that the cultures expressed only the claudins that are normally found in RPE, but the mRNAs for claudins 5 and 12 were grossly overexpressed. Nonetheless, these claudins did not appear by immunocytochemistry to be overexpressed, which suggests that translational or post-translational mechanisms prevented their overexpression. This contrasts with exogenous gene expression experiments, where overexpression of claudin led to ectopic patches of strands along the basolateral membranes (McCarthy et al., 2000).

The addition of pituitary extract or CEF conditioned medium minimally affected the expression of claudin mRNA, but the structure of the junctions in each supplement was significantly different. Each supplement increased the number and the depth of the junctions. Discontinuities persisted in the pituitary extract cultures, and the tight junctions were less deep and had fewer strands than E14 RPE in vivo. By contrast, the cultures in CEF conditioned medium exhibited junctions that were highly heterogeneous, with some regions exhibiting many more strands and greater depth than found in vivo. Both pituitary extract and CEF conditioned medium acted synergistically with rcSF3 to raise the TER of the cultures, but by different mechanisms. With pituitary extract, the rcSF3 medium sealed discontinuities and modulated the expression of claudins in a manner that was consistent with the patterns observed during development in vivo. By contrast with basal CEF medium, apical retinal conditioned medium reorganized the tight junctional strands to a less heterogeneous structure without substantial changes in the expression of the claudins. However, these junctions were deeper, with more strands, than found in native E14 RPE, and the TER was twice as high as expected for newborn chick RPE (Gallemore and Steinberg, 1993). Therefore, CEF conditioned medium suppressed the ability of retinal factors to regulate the expression of claudins, but not the ability of retinal factors to regulate structure.

It is surprising that apically applied retinal factors promoted the epithelial phenotype, because this function is generally ascribed to basolateral interactions (Drubin and Nelson, 1996; Eaton and Simons, 1995; Rodriguez-Boulan and Powell, 1992). We focused on the interactions of retinal conditioned medium with pituitary extract, because in these conditions the structure of the junctions and the pattern of claudin expression were most in vivo-like. Regardless of whether RPE was isolated from E7, E10 and E14 embryos, the structure of the tight junctions that formed in the presence of E14 retinal conditioned medium was closest to that of E14 RPE in vivo. Nonetheless, the TER was higher for E14 cultures, even though the lower complexity of the junctions in the E14 cultures would predict a lower TER. Previous studies that used basal pituitary extract suggested that retinal conditioned medium changed the selectivity for small organic compounds that vary in charge (Ban and Rizzolo, 2000; Peng et al., 2003). The current study suggests that the change reflects the closing of large slits (discontinuities) in the junctional network, which would be nonselective, thereby forcing tracers like mannitol and N-acetylneuraminic acid through smaller slits and pores that would be more sensitive to charge (Guo et al., 2003; Tang and Goodenough, 2003).

Although rcSF3 had no effect on the expression of ZO-1 or occludin (Ban and Rizzolo, 1997; Wilt and Rizzolo, 2001), rcSF3 regulated claudin expression in an age-dependent fashion. For example, retinal factors induced E7 and E14 cultures to increase the synthesis of claudins 1 and AL mRNAs to the in vivo levels of E14 RPE, but the effect on the other claudins depended on the culture. Consistent with the pattern of normal development, claudin 5 mRNA was induced only in E7 cultures, but claudin 2 was induced only in E14 cultures. These data suggest that this culture model is suitable for investigating how retinal factors regulate gene expression at different developmental stages. The immunocytochemical localization of the claudins illustrates a second regulatory mechanism. Besides the different ratios of claudin mRNA, the intracellular distribution of claudins 1 and 12 differed between the E7 and E14 cultures maintained in rcSF3. For these claudins only, a substantial nonjunctional pool was observed in the E7 cultures. Although it would be difficult to quantify, these observations suggest that the relative proportions of the various claudins in the tight junctions differ between the E7 and E14 cultures. Although these differences failed to affect structure, they could account for the observed differences in TER and the diffusion of tracers (Colegio et al., 2003; Van Itallie et al., 2003). The increased expression of claudin 2 is important. Claudin 2 increases the permeability of tight junctions to sodium (Van Itallie et al., 2003). Essentially, our TER measurement is inversely proportional to the Na+ conductance, because that is the most plentiful ion in our medium. The presence of claudin 2 would explain the low TER of RPE tight junctions, despite their well-developed structure and low permeability to mannitol (Ban and Rizzolo, 2000; Peng et al., 2003). The physiologic significance is that RPE is an absorptive epithelium. The mechanism that drives the apical-to-basal movement of water is an apical-to-basal transcellular transport of Cl− that is balanced by a diffusion of Na+ across the paracellular space (Edelman and Miller, 1991). Therefore, the induction of claudin 2 by retinal factors is consistent with the in vivo function of the tissue.

In summary, the neural portion of the retina secretes factors that regulate the permeability of tight junctions by modulating claudin expression and the structure of tight junctions. The signaling pathways that mediate these apical signals interact with several pathways that mediate different basolateral signals. Variations of the culture model presented here provide a means to identify and dissect this presumptive signaling network. These studies have important implications for RPE transplantation, where normal RPE is introduced into an environment that has been altered by disease (Grierson et al., 1994). Retinal degeneration and the accompanying alteration of the choroid would affect both the apical and basal signaling pathways that regulate RPE tight junctions and presumably cell polarity.
supported in part by a National Institutes of Health grant, DK34135, to James Anderson (Yale University). The authors thank James Anderson and members of his laboratory for helpful discussion and Lukas Landmann (University of Basel, Switzerland) for the use of his freeze-fracture facility.

References

Anderson, J. M., Stevenson, B. R., Jesaitis, L. A., Goodenough, D. A. and Mooseker, M. S. (1988). Characterization of ZO-1, a protein component of the tight junction from mouse liver and Madin-Darby canine kidney cells. J. Cell Biol. 106, 1141-1149.

Antonetti, D. A., Barber, A. J., Hollinger, L. A., Wolpert, E. B. and Gardner, T. W. (1999). Vascular endothelial growth factor induces rapid phosphorylation of tight junction proteins occludin and zona occluden 1. A potential mechanism for vascular permeability in diabetic retinopathy and tumors. J. Biol. Chem. 274, 23463-23467.

Balda, M. S. and Matter, K. (2000). Transmembrane proteins of tight junctions. Semin. Cell Dev. Biol. 11, 281-289.

Balda, M. S., Flores-Maldonado, C., Cereijido, M. and Matter, K. (2000). Multiple domains of occludin are involved in the regulation of paracellular permeability. J. Cell Biochem. 78, 85-96.

Ban, Y. and Rizzolo, L. J. (1997). A culture model of development reveals multiple properties of RPE tight junctions. Mol. Vis. 31, 3-18.

Ban, Y. and Rizzolo, L. J. (2000). Differential regulation of tight junction permeability during development of the retinal pigment epithelium. Am. J. Physiol. 279, C744-C759.

Ban, Y., Wilt, S. D. and Rizzolo, L. J. (2000). Two secreted retinal factors regulate different stages of development of the outer blood-retinal barrier. Brain Res. Dev. Brain Res. 119, 259-267.

Cereijido, M. and Anderson, J. M. (2001). Tight Junctions. p. 792. Boca Raton, FL: CRC Press.

Claupe, P. (1973). The intercellular junctional complexes during the development of the blood-retinal barrier. Anat. Rec. 179, 81-91.

Furuse, M., Sasaki, H., Fujimoto, K. and Tsukita, S. (1998). Multiple domains of occludin are involved in the regulation of paracellular permeability. J. Cell Biochem. 78, 85-96.

Furuse, M., Sasaki, H., Fujimoto, K. and Tsukita, S. (2003). Large-scale remodeling of the blood-brain barrier in claudin-5-deficient mice. J. Cell Biol. 161, 653-660.

Kojima, S., Rahner, C., Peng, S. and Rizzolo, L. J. (2002). Claudin 5 is transiently expressed during development of the retinal pigment epithelium. J. Membr. Biol. 186, 81-88.

Liu, X., Mizoguchi, A., Takeichi, M., Honda, Y. and Ide, C. (1997). Developmental changes in the subcellular localization of R-cadherin in chick retinal pigment epithelium. Histochem. Cell Biol. 108, 35-43.

Marmor, M. F. and Wolfensberger, T. J. (1998). The Retinal Pigment Epithelium. Function and Disease. New York, NY: Oxford University Press.

Matter, K. and Balda, M. S. (2003). Holey barrier, claudins and the regulation of brain endothelial permeability. J. Cell Biol. 161, 459-460.

McCarthy, K. M., Francis, S. A., McCormack, J. M., Lai, J., Rogers, A. J., Skare, I. B., Lynch, R. D. and Schneeberger, E. E. (2000). Inducible expression of claudin-1-myc but not occludin-VSV-G results in aberrant tight junction strand formation in MDCK cells. J. Cell Sci. 113, 3387-3398.

Morgan, B. A. and Fekete, D. M. (1996). Manipulating gene expression with replication-competent retroviruses. In Methods in Avian Embryology, Vol. 51 (ed. M. Bronner-Fraser), pp. 186-219. New York, NY: Academic Press.

Nitta, T., Hata, M., Gotoh, S., Seo, Y., Sasaki, H., Hashimoto, N., Furuse, M. and Tsukita, S. (2003). Size-selective loosening of the blood-brain barrier in claudin-5-deficient mice. J. Cell Biol. 161, 653-660.

Peng, S., Rahner, C. and Rizzolo, L. J. (2003). Apical and basal regulation of the permeability of the retinal pigment epithelium. Invest. Ophthal. Mol. Vis. Sci. 44, 808-817.

Rahner, C., Stieger, B. and Landmann, L. (1996). Structure-function correlation of tight junctional impairment after intrahepatic and extrahepatic cholestasis in rat liver. Gastroenterology 110, 1564-1578.

Rahner, C., Mitic, I. L. and Anderson, J. M. (2001). Heterogeneity in expression and subcellular localization of claudins 2, 3, 4, and 5 in the rat liver, pancreas, and gut. Gastroenterology 120, 411-422.

Rizzolo, L. J. (1991). Basement membrane stimulates the polarized distribution but not the Na,K-ATPase in the retinal pigment epithelium. Cell Regul. 2, 939-949.

Rizzolo, L. J. (1997). Polarity and the development of the outer blood-retinal barrier. Histol. Histopathol. 12, 1057-1067.

Rodriguez-Boulan, E. and Powell, S. K. (1992). Polarity of epithelial and neuronal cells. Annu. Rev. Cell Biol. 8, 395-427.

Sandig, M. and Kalnins, V. I. (1990). Morphological changes in the zona adherens during embryonic development of chick retinal pigment epithelial cells. Cell Tissue Res. 259, 455-461.

Simon, D. B., Lu, Y., Choate, K. A., Velazquez, H., Al-Sabban, E., Praga, M., Tang, V. W. and Goodenough, D. A., Skare, I. B., Lynch, R. D. and Schneeberger, E. E. (1999). Paracellin-1, a renal tight junction protein required for paracellular Mg2+ resorption. Science 285, 103-106.

Stevenson, B. R., Anderson, J. M., Goodenough, D. A. and Mooseker, M. S. (1988). Tight junction structure and ZO-1 content are identical in two strains of Madin-Darby canine kidney cells which differ in transepithelial resistance. J. Cell Biol. 107, 2401-2408.

Tang, V. W. and Goodenough, D. A. (2003). Paracellular ion channel at the tight junction. Biophys. J. 84, 1660-1673.

Tsukita, S. and Furuse, M. (1999). Occludin and claudins in tight-junction strands, leading or supporting players? Trends Cell Biol. 9, 268-273.

Tsukita, S., Furuse, M. and Itoh, M. (2001). Multifunctional strands in tight junctions. Nat. Rev. Mol. Cell. Biol. 2, 285-293.

Van Itallie, C., Rahner, C. and Anderson, J. M. (2001). Regulated expression of claudin-4 decreases paracellular conductance through a selective decrease in sodium permeability. J. Clin. Invest. 107, 1319-1327.

Van Itallie, C. M., Fanning, A. S. and Anderson, J. M. (2003). Reversal of charge selectivity in cation or anion-selective epithelial lines by expression of different claudins. Am. J. Physiol. Renal Physiol. 285, F1078-F1084.

Williams, C. D. and Rizzolo, L. J. (1997). Removal of junctional complexes during the development of the blood-retinal barrier. Anat. Rec. 249, 380-388.

Wilt, S. D. and Rizzolo, L. J. (2001). Unique aspects of the blood-brain barrier. In Tight Junctions (ed. J. M. Anderson and M. Cereijido), pp. 415-443. Boca Raton, FL: CRC Press.

Yu, A. S., Enck, A. H., Lencer, W. I. and Schneeberger, E. E. (2003). Claudin-8 expression in Madin-Darby canine kidney cells augments the paracellular barrier to cation permeation. J. Biol. Chem. 278, 17350-17359.

Knesel, U. and Wolburg, H. (1993). Tight junction complexity in the retinal pigment epithelium of the chicken during development. Neurosci. Lett. 149, 71-74.

Rahner, C., Peng, S. and Rizzolo, L. J. (2002). Claudin 5 is transiently expressed during development of the retinal pigment epithelium. J. Membr. Biol. 186, 81-88.