The tight junction protein claudin-b regulates epithelial permeability and sodium handling in larval zebrafish, *Danio rerio*

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Submitted 23 August 2012; accepted in final form 29 January 2013

Kwong RWM, Perry SF. The tight junction protein claudin-b regulates epithelial permeability and sodium handling in larval zebrafish, *Danio rerio*. Am J Physiol Regul Integr Comp Physiol 304: R504–R513, 2013. First published January 13, 2013; doi:10.1152/ajpregu.00385.2012.—The functional role of the tight junction protein claudin-b in larval zebrafish (*Danio rerio*) was investigated. We showed that claudin-b protein is expressed at epithelial cell–cell contacts on the skin. Translational gene knockdown of claudin-b protein expression caused developmental defects, including edema in the pericardial cavity and yolk sac. Claudin-b morphants exhibited an increase in epithelial permeability to the paracellular marker polyethylene glycol (PEG-4000) and fluorescein isothiocyanate-dextran (FD-4). Accumulation of FD-4 was confined mainly to the yolk sac and pericardial cavity in the claudin-b morphants, suggesting these regions became particularly leaky in the absence of claudin-b expression. Additionally, Na⁺ efflux was substantially increased in the claudin-b morphants, which contributed to a significant reduction in whole-body Na⁺ levels. These results indicate that claudin-b normally acts as a paracellular barrier to Na⁺. Nevertheless, the elevated loss of Na⁺ in the morphants was compensated by an increase in Na⁺ uptake. Notably, we observed that the increased Na⁺ uptake in the morphants was attenuated in the presence of the selective Na⁺/Cl⁻ cotransporter (NCC) inhibitor metolazone, or during exposure to Cl⁻-free water. These results suggested that the increased Na⁺ uptake in the morphants was, at least in part, mediated by NCC. Furthermore, treatment with an H⁺-ATPase inhibitor bafilomycin A1 was found to reduce Na⁺ uptake in the morphants, suggesting that H⁺-ATPase activity was essential to provide a driving force for Na⁺ uptake. Overall, the results suggest that claudin-b plays an important role in regulating epithelial permeability and Na⁺ handling in zebrafish.

claudin-b; epithelial permeability; sodium; tight junction proteins; zebrafish

TIGHT JUNCTIONS (TJs) are important components regulating the paracellular movement of solutes and water in vertebrates. TJs are composed of four major transmembrane proteins: occludin, claudins, tricellulins, and junctional adhesion molecule. However, claudins are thought to be the major determinant of the epithelial barrier functions (35). To date, more than 20 claudin isoforms have been identified in mammals (16), as well as in teleost fish (9, 24, 27, 32), and the expression of these claudin isoforms appears to be cell- and tissue-specific. Studies using mammalian cell models have shown that the various claudin isoforms exhibit marked differences in their permeability characteristics, presumably establishing unique properties for different epithelia and endothelia (34, 37). It has also been demonstrated that some claudin isoforms can form ion-specific seals or pores to regulate the paracellular movement of ions (1, 20). The ion selectivity of claudins is suggested to be determined, in part, by the presence of charged amino acid residues on the first extracellular domain (10).

Recent evidence indicates that certain claudin isoforms provide important and distinct functions in developing zebrafish (3, 31, 42). For example, knockdown of claudin-5 or -15, which are expressed predominantly in endothelial and intestinal cells, causes developmental defects in the blood-brain barrier and intestinal lumen, respectively (3, 42). Another tight junction protein claudin-b also is expressed in a tissue-specific manner; predominant sites of expression include the forebrain, optic vesicle, and skin (17, 23). The skin is the major route for ionic and osmotic fluxes in developing zebrafish; however, the role of claudin-b in epithelial barrier function is not clear. Zebrafish claudin-b is orthologous to mammalian claudin-4, and overexpression of claudin-4 in a Madin-Darby canine kidney (MDCK) cell line was shown to induce epithelial resistance and reduce paracellular Na⁺ movement (36). Additionally, knockdown of claudin-4 in a MDCK cell line using siRNA increased paracellular permeability and Na⁺ movement (20).

Several recent studies have provided evidence that claudin-b promotes a tight epithelium and regulates paracellular Na⁺ movement in teleost fish. For example, expression of claudin-b increased in the gill of goldfish (*Carassius auratus*) and zebrafish during acclimation to ion-poor conditions (7, 26). The increased abundance of branchial claudin-b has been suggested to be associated with a reduction in passive Na⁺ loss (26). In a goldfish gill cell culture, treatment with ion-poor water also decreased the permeability of the paracellular marker PEG-4000 (7). An increase in the branchial expression of claudin-b was also reported in zebrafish exposed to acidic water, a condition known to stimulate passive Na⁺ loss (24). Recently, Engelund et al. (12) demonstrated that transfection of Atlantic salmon (*Salmo salar L.*) claudin-30 (orthologous to claudin-b) in a MDCK cell line increased transepithelial resistance and decreased paracellular permeability to Na⁺. Together, the results of these studies suggest that claudin-b plays an important role in the regulation of epithelial permeability and paracellular Na⁺ movement. However, there is no direct in vivo evidence supporting a physiological role of claudin-b on epithelial barrier functions.

With the above background, we hypothesized that claudin-b is involved in the regulation of paracellular permeability and Na⁺ movement in zebrafish. We used a reverse genetics approach to prevent the translation of claudin-b protein in developing zebrafish and evaluated the epithelial permeability using paracellular markers [e.g., PEG-4000 and fluorescein isothiocyanate-dextran 4000 (FD-4)]. In addition, we examined the effects of claudin-b knockdown on unidirectional Na⁺ fluxes, as well as the mRNA expression of Na⁺-transport-
related genes. Because our data suggested that knockdown of claudin-b increased Na\(^+\) loss, which was compensated by an increase in Na\(^+\) uptake, we also explored the potential mechanisms underlying this increase using Na\(^+\) transport inhibitors.

**MATERIALS AND METHODS**

**Fish.** Adult zebrafish (*Danio rerio* Hamilton-Buchanan 1822) were purchased from Big Al’s Aquarium Services (Ottawa, ON, Canada) and kept in the University of Ottawa Aquatic Care Facility, where they were maintained in plastic tanks supplied with aerated, dechloraminated city of Ottawa tap water at 28°C. Fish were subjected to a constant 14:10-h light-dark photoperiod and fed daily until satiation with no. 1 crumble-Zeigler (Aquatic Habitats, Apopka, FL). Morpholino and sham-injected embryos (detailed below) were reared in 50-ml petri dishes supplemented with dechloraminated city of Ottawa tap water. The ionic composition of the water was Na\(^+\) = 0.78 mM; Cl\(^-\) = 0.4 mM; Ca\(^2+\) = 0.25 mM; K\(^+\) = 0.025 mM; pH 7.6. All of the flux measurements were conducted with this water, except where mentioned otherwise. The petri dishes were kept in incubators set at 28.5°C. All experiments were performed on fish at 3 days postfertilization (dpf) except for the Na\(^+\) and Cl\(^-\) measurements where 2 dpf fish were also analyzed after manual removal of chorion. The experiments were conducted in compliance with guidelines of the Canadian Council of Animal Care and after the approval of the University of Ottawa Animal Care Committee (protocol BL-226).

**Microinjection of antisense morpholino nucleotides.** A morpholino oligonucleotide (5'-CCG GTT GAT GCC ATG CTT TTT CGT T-3'; Genetools, Philomath, OR) was designed to bind to the translation start site of zebrafish claudin-b (GenBank ID: NM131763.2). A BLAST (Basic Local Alignment Search Tool) analysis revealed no sequence similarity between the morpholino and other claudin homologs. The morpholino was diluted in a Danieu buffer [58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO\(_4\), 0.6 mM Ca(NO\(_3\))\(_2\), 5.0 mM HEPES (pH 7.6)] plus 0.05% phenol red before injection. A “sham” group was injected with a standard control morpholino (5'-CCCT GTT ACC TCA GGT ACA ATT TAT A-3'; GeneTools) prepared as the claudin-b morpholino. Two to four nanograms of morpholino (1 nl) was injected into one-cell stage zebrafish embryos using a microinjector system (model IM 300; Narishige, Long Island, NY). Because injection of 4 ng morpholino caused severe developmental defects (see RESULTS), all flux measurements were performed on fish injected with 2 ng morpholino (described below). Only fish that showed no gross morphological abnormalities were used in flux experiments.

**Western blot analysis and immunohistochemistry.** Methods for Western blot analysis were similar to those reported previously (26). Briefly, 20 larvae were pooled as one sample (n = 1) and homogenized in a RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl, and 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride) plus protease inhibitor cocktail (Roche, Minneapolis, MN). Extracted protein was then loaded onto a 12% SDS-PAGE and transferred to a PVDF membrane (Bio-Rad, Hercules, CA). The membrane was blocked for 2 h in 5% BSA and probed with homologous anti-claudin-b (1:250 dilution) at 4°C overnight. The custom polyclonal claudin-b antibody was raised in rabbit (21st Century Biochemicals, Marlboro, MA) against a C-terminal region (CATPRSEASAPSGKNF) of zebrafish claudin-b protein (accession no. NM 131763). The specificity of this claudin-b antibody was validated previously (26). Subsequently, the membrane was probed with 1:15,000 goat anti-rabbit secondary antibody (Pierce, Rockford, IL) for 2 h at room temperature, and the immunoreactive bands were detected using enhanced chemiluminescence (SuperSignal West femto chemiluminescent substrate; Pierce). To check for equal protein loading, the membrane was reprobed with 1:4,000 β-actin antibodies (A2066; Sigma, St. Louis, MO) for 2 h at room temperature after stripping with Re-Blot Plus solution (Millipore, Billerica, MA). The intensity of the bands was estimated using ImageJ (30), and the expression of claudin-b was normalized to that of β-actin.

For immunostaining of claudin-b, zebrafish larvae were fixed in 100% methanol and then blocked with 10% BSA plus 1.0% Tween in PBS for 2 h at room temperature. The fish were incubated with the claudin-b antibody (1:250 dilution) in PBS with 0.2% Tween at 4°C overnight, and then incubated in a Alexa Fluor 488-coupled goat anti-rabbit IgG (1:500 dilution; Invitrogen, Carlsbad, CA) for 2 h at room temperature in the dark. The images were acquired using a Zeiss LSM 5 Pascal/AxioVert 200 confocal microscope (with an Alexa Fluor-488 filter), which was fitted on an inverted microscope equipped with a 63 × Plan-Apochromat 1.4 oil immersion objectives (Carl Zeiss, Jena, Germany).

**Phenotypic evaluation of claudin-b morphant.** At 3 dpf, larvae injected with 0, 2, or 4 ng morpholino were anesthetized using 5% MS-222, and then randomly distributed into single wells of a 6-well plate (n = 5 per well). A total of 90 fish was analyzed from three independent experiments. Embryos were examined with a light microscope (model SZX12, Olympus, Center Valley, PA), and images were captured using a CCD camera (Diagnostic Instruments, Sterling Heights, MI).

**Whole body permeability of PEG-4000 and FD-4.** Control or morpholino-injected larvae were transferred to a water-filled 2.0-ml microfuge tube and exposed to 2 μCi/ml [\(^{14} \)H]PEG-4000 (American Radiolabeled Chemicals, St. Louis, MO) for 6 h. The final concentration of PEG-4000 in the water was 0.5 mM. Water samples were taken before and after the fluxes. At the end of the flux period, fish were washed in isolate-free water, and three larvae were pooled as one sample (n = 1). The fish were then digested with 1 N HNO\(_3\) at 60°C for 48 h, and the radioactivity in the digest, as well as the water samples, were measured using a liquid scintillation counter (LS-6500; Beckman Coulter, Mississauga, ON, Canada), following the addition of scintillation cocktail (BioSafe-II; Research Products International, Mt. Prospect, IL).

To evaluate the absorption of FD-4 (Sigma-Aldrich), 3 dpf larvae (either injected with control or claudin-b morpholino; n = 20 for each group) were incubated in the dark overnight with 50 μg/ml of FD-4 and then washed briefly. Images were obtained on a Zeiss LSM 5 Pascal/AxioVert 200 confocal microscope, as described above, using a 10× EC Plan-Neofluar 0.3 Ph1 objective (Zeiss).

**Measurements of sodium fluxes.** To measure Na\(^+\) efflux, 20 fish (n = 1) at 2 or 3 dpf were incubated with 2 μCi/ml \(^{22} \)Na\(^+\) (Perkin Elmer, Woodbridge, ON, Canada) for 6 h, and then washed briefly in isotope-free water. The fish were transferred to a microfuge tube containing 1.5 ml water, and 250 μl water was sampled every 30 min for 2 h. Subsequently, 10 fish were collected for radioactivity measurement, as described above, and another 10 fish were used to determine the whole-body Na\(^+\) concentration (detailed below) for calculation of internal specific activity.

For measurement of Na\(^+\) influx, fish were exposed to 0.5 μCi/ml \(^{22} \)Na\(^+\) for 2 h. The final concentration of Na\(^+\) in the water was 0.78 mM. At the end of the flux period, fish were washed in isolate-free water, and three fish were pooled as one sample (n = 1). The fish were then digested, and the radioactivity in the digest, as well as the water samples, were measured, as described previously. To examine the potential involvement of NCC (see DISCUSSION), the uptake of Na\(^+\) in the shams and morphants was measured in Cl\(^-\)-free water. The water was prepared with double-deionized water supplemented with either NaCl (control water; Ci = 0.78 mM) or Na\(_2\)SO\(_4\) (Cl\(^-\)-free water), plus CaSO\(_4\)\(_2\)H\(_2\)O, MgSO\(_4\)\(_7\)H\(_2\)O, K\(_2\)HPO\(_4\) and KH\(_2\)PO\(_4\). The final concentrations of Na\(^+\), Ca\(^2+\), Mg\(^2+\) and K\(^+\) in the water were (in mM) 0.78, 0.25, 0.15, and 0.3, respectively.

**Measurement of whole body sodium and chloride levels.** To measure the whole body Na\(^+\) and Cl\(^-\) content, 10 fish were pooled (n = 1), killed with an overdose of MS-222, and briefly rinsed in double deionized water. The fish were then digested with 1 N HNO\(_3\) at 70°C for 48 h, and diluted appropriately with deionized water. The total
Na⁺ concentration was measured by flame emission spectrophotometry (Spectra AA 220FS; Varian, Palo Alto, CA), and verified using certified Na⁺ standards (Fisher Scientific, Waltham, MA). The Cl⁻ concentration was measured colorimetrically following the protocol of Zall et al. (41) adapted for microplate reader.

**Pharmacological analysis of the increased sodium uptake in the morphants.** To examine the potential pathways contributing to the increased Na⁺ uptake in the claudin-b morphants at 3 dpf (see RESULTS), control, or morpholino-injected larvae were exposed to metolazone (Sigma), 5-(N-ethyl-N-isopropyl) amiloride (EIPA; Sigma), or bafilomycin A1 (LC Laboratories, Woburn, MA). Metolazone, EIPA, and bafilomycin A1 are selective inhibitors for Na⁺/Cl⁻cotransporter, Na⁺/H⁺ exchanger, and H⁺-ATPase, respectively. The inhibitors were dissolved in DMSO (final concentration = 0.2%), and vehicle controls were performed in all experiments. The experimental conditions were similar to those described previously for larval zebrafish (13, 25). In brief, fish were incubated with the inhibitors (100 µM metolazone, 100 µM EIPA, or 1 µM bafilomycin A1) for 30 min, and the fish were then exposed to 0.5 µCi/ml ²²Na⁺. The uptake of Na⁺ was measured as described above (2-h flux period), except for bafilomycin A1 treatment where the flux period was reduced to 1 h.

**Real-time PCR.** To evaluate the potential modulation of Na⁺ transport-related genes in the claudin-b morphants, the mRNA expression of NCC (slc12a10.2), NHE3b (slc9a3b), and H⁺-ATPase were evaluated. Total RNA was extracted using TRIzol (Invitrogen), and genomic DNA was removed with DNase I (Invitrogen), following the manufacturer’s guidelines. First-strand cDNA was synthesized using RevertAid H Minus reverse transcriptase (Fermentas, Burlington, ON, Canada) and random hexamer primers. Primer sets used in the present study are summarized in Table 1. RT-quantitative PCR (qPCR) assays were performed using a Bio-Rad CFX96 qPCR system with Brilliant III SYBR Green Master Mix (Agilent Technologies, Santa Clara, CA). All RT-qPCR was performed using the following conditions: 95°C for 3 min, 40 cycles of 95°C for 20 s and 58°C for 20 s, with final extension for 5 min at 72°C. Data were normalized to the expression of 18S and were presented relative to the sham group.

**Calculations and statistical analysis.** The rate of PEG-4000 or Na⁺ influx was calculated by

\[
J_{in} = \frac{cpm}{SA \times n \times t}
\]

where cpm is counts per minute measured in the fish, SA is the specific activity in the water (PEG-4000; cpm/pmol; Na⁺: cpm/nmol), n is the number of fish, and t is the duration of the experiment in hours.

The Na⁺ efflux rate was calculated by

\[
J_{out} = \frac{1}{SA \times n} \frac{dW}{dr}
\]

where SA is the internal specific activity of Na⁺ (cpm/nmol), n is the number of fish, W is the total radioactivity in the water (cpm), and t is the duration of efflux (h).

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**RESULTS**

**Protein expression of claudin-b is reduced after morpholino knockdown.** To examine the effects of morpholino injection on claudin-b protein expression, Western blot analysis and immunohistochemistry were performed. As shown in Fig. 1A, the protein expression of claudin-b in lysates of 3 dpf zebrafish larvae was reduced with increasing concentrations of morpholino injected. The expression of a housekeeping gene β-actin remained relatively steady regardless of the amount of morpholino injected. Subsequent quantification revealed that the expression of claudin-b protein was significantly reduced following 2 ng or 4 ng morpholino injections (Fig. 1B). Confocal immunohistochemistry revealed that the claudin-b protein was expressed between cell-cell contacts on the skin (i.e., yolk sac; Fig. 1C). A representative z-stack confocal video showing the claudin-b expression on the skin of yolk sac is displayed in the Supplemental Movie SM1 on the journal Web site. Claudin-b expression was virtually absent on the skin after injection with 2 (Fig. 1D) or 4 ng morpholino (not shown).

**Knockdown of claudin-b causes developmental defects.** The developmental effects of claudin-b knockdown on 3 dpf zebrafish were evaluated. Sham-injected larvae showed normal morphological development (Fig. 2A). Some claudin-b morphants following the 2-ng morpholino injection exhibited a relatively mild swelling of the yolk sac and a slight curvature of the tail (Fig. 2B). However, injection with 4 ng morpholino caused a severe curvature of the tail and pronounced edema in the pericardial cavity and yolk sac. Compared with the shams (Fig. 2D), a number of claudin-b morphants following 4 ng morpholino injection exhibited an increased accumulation of blood around the yolk sac (Fig. 2E). The developmental effects of claudin-b knockdown appeared to be dose-dependent (Fig. 2F). In general, more fish exhibited edema in the yolk sac and curved tail following 2 or 4 ng morpholino injection. About 70% of fish showed no gross morphological abnormalities after injection with 2 ng morpholino; however, only 30% of fish showed normal morphological development following 4 ng morpholino injection (Table 2). Compared with fish injected with 0 or 2 ng morpholino, a significantly higher number of fish exhibited accumulation of blood around the yolk sac,

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**Table 1. Primer sets used in the present study**

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<th>Primers Reference</th>
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<td>Na⁺/H⁺ exchanger</td>
<td>FWD: 5'-TGG AGA CAG CCG CGC CTC TAG C-3'</td>
<td>(40)</td>
</tr>
<tr>
<td>(NHE3b: slc9a3b)</td>
<td>REV: 5'-TGT GCC CTC TCT CTT TTT GGC-3'</td>
<td></td>
</tr>
<tr>
<td>H⁺-ATPase</td>
<td>FWD: 5'-GAG GAA CTA CCG CTT CCA-3'</td>
<td>(5)</td>
</tr>
<tr>
<td>(atp6v1a)</td>
<td>REV: 5'-CGA CCA CCA ATG AGA ACA TGG-3'</td>
<td></td>
</tr>
<tr>
<td>Na⁺/Cl⁻ cotransporter</td>
<td>FWD: 5'-GCC CCC AAA GTT TTC CAG TT-3'</td>
<td>(38)</td>
</tr>
<tr>
<td>(NCC; slc12a10.2)</td>
<td>REV: 5'-TAA GCA CCA AGA GGC TCC TTG-3'</td>
<td></td>
</tr>
<tr>
<td>18S</td>
<td>FWD: 5'-GGA GCC GGT ATT CCC ATG ACC-3'</td>
<td></td>
</tr>
<tr>
<td></td>
<td>REV: 5'-GGA GGA GGT CCT TGG ACC-3'</td>
<td>(24)</td>
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whether claudin-b played a role in regulating Na\(^+\) absorption into the yolk sac and, thus, presumably increased absorption of FD-4. The increased intensity of fluorescence after FD-4 exposure in 2 dpf morphants, respectively. About 90% of the morphants exhibited increased intensity of fluorescence after FD-4 exposure and, thus, presumably increased absorption of FD-4. The majority of FD-4 appeared to be absorbed into the yolk sac and pericardial cavity.

**Claudin-b knockdown increases paracellular permeability.** To examine whether claudin-b contributed to a tight epithelium, the absorption of the paracellular markers PEG-4000 and FD-4 were evaluated (both have a molecular mass of 4,000 Da). Knockdown of claudin-b significantly increased the permeability of PEG-4000 (Fig. 3). However, no statistical significance in the permeability to a smaller solute PEG-400 (molecular mass: 400 Da) was observed between sham and morphants (absorption of 30 nM PEG-400 in the sham and morphants was 0.10 ± 0.01 and 0.11 ± 0.01 fmol-fish\(^{-1}\)-h\(^{-1}\), respectively; data not shown). Figure 4. A and B show representative images of FD-4 fluorescence in shams and claudin-b morphants, respectively. About 90% of the morphants exhibited increased intensity of fluorescence after FD-4 exposure and, thus, presumably increased absorption of FD-4. The majority of FD-4 appeared to be absorbed into the yolk sac and pericardial cavity.

**Claudin-b knockdown increases sodium loss.** To examine whether claudin-b played a role in regulating Na\(^+\) handling, the efflux and influx of Na\(^+\) following claudin-b gene knockdown were investigated. The influx rate of Na\(^+\) was markedly increased in the claudin-b morphants at 2 and 3 dpf (Fig. 5A). The influx rate of Na\(^+\) in the 3 dpf morphants (but not in 2 dpf morphants) was significantly increased (Fig. 5B).

**Claudin-b knockdown reduces whole-body sodium but not chloride levels.** Because we observed a change in Na\(^+\) fluxes in the claudin-b morphants, further experiments were performed to examine the whole-body Na\(^+\) levels in the morphants. A significant reduction in the whole-body Na\(^+\) content was observed in 2 dpf morphants, whereas no statistical difference was observed at 3 dpf (Fig. 6A). The whole body Cl\(^-\) level was also measured to evaluate whether the increased Na\(^+\) loss in the morphants was accompanied by an increase in Cl\(^-\) loss. At 2 dpf, there was no statistical difference in the whole body Cl\(^-\) level between sham and morphants (Fig. 6B). However, a significant increase in the whole-body Cl\(^-\) level was observed in 3 dpf morphants.

**Pharmacological examination of the mechanisms for the increased sodium uptake in the claudin-b morphants.** To examine the potential mechanism contributed to the increased Na\(^+\) uptake in the 3 dpf claudin-b morphants, the effects of specific Na\(^+\)-transport inhibitors metolazone (inhibitor for Na\(^+\)/Cl\(^-\)-cotransporter, NCC), EIPA (inhibitor for Na\(^+\)/H\(^+\) exchanger, NHE) and bafilomycin A1 (inhibitor for H\(^+\)-ATPase) on Na\(^+\) uptake were evaluated. Exposure to metolazone significantly reduced Na\(^+\) uptake in the morphants (Fig. 7A), whereas EIPA did not have any apparent effect (Fig. 7B). The uptake of Na\(^+\) in sham and morphants was significantly reduced by bafilomycin A1 exposure (Fig. 7C).

**Chloride-dependent sodium uptake in the claudin-b morphants.** Further experiments were performed to examine the involvement of NCC (dependence on ambient Cl\(^-\)) in increasing Na\(^+\) uptake in the 3 dpf claudin-b morphants. During exposure to Cl\(^-\)-free water, the uptake rate of Na\(^+\) was decreased in both sham and claudin-b morphants (Fig. 8). The Na\(^+\) uptake rate in the claudin-b morphants exposed to Cl\(^-\)-free water was comparable to the shams in control water but was significantly higher than the shams in Cl\(^-\)-free water. Two-way ANOVA revealed that there was no significant interaction between morpholino knockdown and exposure to Cl\(^-\)-free water.

**Claudin-b morphants exhibit an increase in the mRNA expression of sodium/chloride cotransporter.** Real-time PCR was performed to examine the mRNA expressions of the Na\(^+\) uptake-related genes, NCC (slc12a10.2), NHE3b (slc9a3b), and H\(^+\)-ATPase (atp6v1a), in the 3 dpf claudin-b morphants. The mRNA expression of NCC (slc12a10.2) was significantly increased in the claudin-b morphants, whereas the NHE3b (slc9a3b) and H\(^+\)-ATPase (atp6v1a) mRNA levels remained unchanged (Fig. 9).

**DISCUSSION**

**Overview.** Claudins can either form paracellular barriers or pores (37), and it is well documented that the expression of barrier-forming claudin orthologs in fish is increased during acclimation to dilute environments (7, 11, 26, 32, 33). Al-

![Figure 1](http://ajpregu.physiology.org/)
though the permeability properties for most claudins in teleost fish remain largely unknown, previous studies have shown that claudin-b or claudin-30 (salmon claudin-30 is orthologous to zebrafish claudin-b) may regulate passive ion losses in fish (6, 12, 24, 26, 32). Importantly, Engelund et al. (12) demonstrated that transfection of claudin-30 in a MDCK cell line increased transepithelial resistance and reduced paracellular Na\textsuperscript{+}/H\textsuperscript{+} movement. In the present study, we examined the physiological importance and functional characteristics of claudin-b in zebrafish larvae. By employing a specific morpholino to block the translation of claudin-b in developing zebrafish, we demonstrated that claudin-b is essential in maintaining a tight epithelium. We also showed that diffusive Na\textsuperscript{+} loss was substantially increased in the claudin-b morphants, providing direct in vivo evidence that claudin-b acts as a paracellular barrier to Na\textsuperscript{+}.

Knockdown of claudin-b causes developmental defects and increases epithelial permeability. In agreement with previous findings (17), we observed that claudin-b protein was expressed at similar levels in normal and claudin-b morphant zebrafish larvae. 

Table 2. Phenotypic analysis of 3 dpf zebrafish following claudin-b morpholino knockdown

<table>
<thead>
<tr>
<th>Morpholino, ng</th>
<th>Normal</th>
<th>Pericardial Edema</th>
<th>Yolk Sac Edema</th>
<th>Blood Accumulation Around the Yolk Sac</th>
<th>Curved Tail</th>
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<tbody>
<tr>
<td>0</td>
<td>100 ± 0(^a)</td>
<td>0 ± 0(^a)</td>
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<tr>
<td>2</td>
<td>71 ± 8(^b)</td>
<td>0 ± 0(^a)</td>
<td>13 ± 7(^b)</td>
<td>0 ± 0(^a)</td>
<td>16 ± 3(^b)</td>
</tr>
<tr>
<td>4</td>
<td>28 ± 9(^c)</td>
<td>16 ± 4(^b)</td>
<td>25 ± 3(^b)</td>
<td>9 ± 4(^b)</td>
<td>22 ± 7(^b)</td>
</tr>
</tbody>
</table>

Data are expressed as mean % ± SE and are representative of three independent experiments with \( n = 90 \) for each group. \(^ {a,b,c}\)Values labeled with different letters represent a statistical difference within the same category of defect (one-way ANOVA, followed by a post hoc Holm-Sidak test; \( P < 0.05 \)).
pressed predominantly in several tissues/organs in zebrafish larvae (e.g., forebrain, olfactory vesicle, neuromasts; not shown), including the skin. We observed that the claudin-b protein was expressed between cell-cell contacts on the skin, suggesting its potential function as an epithelial barrier. The permeability of PEG-4000 (a paracellular permeability marker) was increased in the claudin-b morphants, indicating that claudin-b contributes to a tight epithelium. Interestingly, we observed that the permeability of a smaller solute (PEG-400) was unaffected by claudin-b knockdown (data not shown). A recent study using adult zebrafish also demonstrated that acclimation to ion-poor conditions increased claudin-b expression without reducing the permeability of PEG-400 (26). Therefore, it would appear that for nonionic solutes, claudin-b preferentially restricts the paracellular movement of larger molecules. These findings are similar to those reported for the tight junction protein claudin-5, which was found to effectively block the paracellular movement of FD-4 (4,000 Da) but not fluorescein (376 Da) across the blood-brain barrier endothelium in developing zebrafish (39).

Embryos injected with differing amounts of morpholino appeared to exhibit similar phenotypes, although the incidence of defects and the degree of severity were dose-dependent. In general, a phenotype characterized by a curved tail and edema in the pericardial cavity and yolk sac was observed. In vertebrates, Na⁺/H⁺ is known to be essential for stimulating cell proliferation and protein synthesis during development, and it has been shown that Na⁺ depletion could result in growth retardation in developing rats (18). Therefore, it is possible that the tail malformation in the claudin-b morphants was associated with Na⁺ imbalance (discussed below) during early stages of development. Similar developmental defects were also observed in zebrafish larvae experiencing ZO-3 knockdown (22). ZO-3 is a scaffolding protein that interacts with transmembrane tight junction proteins and cytoskeleton actin, and is suggested to play a role in maintaining epithelial integrity (22). Using a fluorescent paracellular marker FD-4, we observed that the accumulation of FD-4 was confined mainly to the yolk sac and pericardial cavity in the claudin-b morphants. This finding suggests that these regions became particularly leaky in the absence of claudin-b expression. Therefore, the increased fluid accumulation in the pericardial cavity and yolk sac possibly resulted from a loss of epithelial integrity after the claudin-b knockdown (e.g., increased water fluxes through paracellular routes). Excessive water influx may have subsequently disrupted the formation or integrity of blood vessels, leading to blood accumulation around the yolk sac. Together, the phenotypes of claudin-b morphants appeared to be a consequence of the loss of the tight epithelial barrier and the compromised osmoregulatory function.

Claudin-b is involved in regulation of diffusive sodium loss. Freshwater fish are hyperionic to their external environment and, thus, are challenged by a continual diffusive loss of Na⁺. Although the precise mechanisms regulating diffusive Na⁺ loss remain poorly understood, several previous studies have

![Graph showing PEG-4000 uptake rates](image1)

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Fig. 3. Knockdown of claudin-b increases whole-body permeability to PEG-4000 in zebrafish larvae. The influx rate of polyethylene glycol-4000 (PEG-4000) in sham and claudin-b morphants at 3 dpf. *Significant difference in influx rates between shams and claudin-b morphants (Student’s t-test, P < 0.05). Values are expressed as means ± SE; n = 12.

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![Image showing FD-4 absorption](image2)

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Fig. 4. Knockdown of claudin-b increases permeability of FD-4 in zebrafish larvae. Representative images from confocal laser scanning microscopy showing the absorption of fluorescein isothiocyanate dextran 4000 (FD-4; green) in sham (A) and claudin-b morphant (B) zebrafish at 3 dpf. Compared with the shams, the absorption of FD-4 was higher in the claudin-b morphants. The absorption of FD-4 appeared to be confined to the pericardial cavity and yolk sac.
suggested that Na\(^+\) loss is, at least in part, governed by tight junction proteins (8, 12, 14, 24, 28). We previously observed that in ion-poor conditions, the branchial expression of claudin-b was increased in adult zebrafish, which appeared to be associated with a reduction in passive Na\(^+\)/H\(^+\) loss (26). In the present study, we demonstrated that knockdown of claudin-b markedly increased Na\(^+\)/H\(^+\) loss. A significant increase in the whole-body Na\(^+\)/H\(^+\) content was also observed in 2 dpf morphants. These findings clearly indicate that claudin-b acts as a paracellular Na\(^+\)/H\(^+\) barrier. A decrease in the paracellular Na\(^+\)/H\(^+\) permeability has been reported in a MDCK cell line expressing the Atlantic salmon claudin-30 (12) or the mammalian claudin-4 (36). Notably, the expression of claudin-30 or claudin-4 in the MDCK cell line was found to have no effect on Cl\(^-\)/H\(^+\) permeability (12, 36). Here, we also observed that the increased Na\(^+\) loss in the morphants was not accompanied by a reduction in the whole body Cl\(^-\) level, further supporting the notion that claudin-b forms a selective paracellular barrier to Na\(^+\).

Interestingly, we also observed that the claudin-b morphants compensated for the elevated Na\(^+\) loss by increasing Na\(^+\)/H\(^+\) uptake at 3 dpf. To date, at least three mechanisms for Na\(^+\) uptake have been proposed for zebrafish; electroneutral Na\(^+\)/H\(^+\)/H\(^+\)/H\(^+\) exchange (via NHE3), Na\(^+\)/Cl\(^-\) cotransport (via NCC; i.e., slc12a10.2), and coupling of Na\(^+\) absorption with H\(^+\) secretion driven by a V-type H\(^+\)-ATPase (e.g., atp6v1a) (for review, see Ref. 21). Although the physiological importance of NCC in Na\(^+\) uptake is still under debate (21), there is increasing evidence that NCC may facilitate Na\(^+\) uptake in FW fish (19, 29, 38). In larval zebrafish, Wang et al. (38) reported that NCC mRNA is expressed starting at 1 dpf, and its expression appears to increase during development. Additionally, treatment with metolazone, a specific NCC inhibitor, reduced Na\(^+\) uptake in zebrafish larvae (38). A 40% reduction in Na\(^+\) uptake was also reported in adult goldfish during exposure to Cl\(^-\)-free water (29). In the present study, we observed that the whole body Cl\(^-\) level was significantly increased in the morphants at 3 dpf, which appeared to be associated with the increase in the mRNA expression of NCC. Similarly, Wang et al. (38) reported that morpholino gene knockdown of NCC reduced the whole body Cl\(^-\) level in larval zebrafish. We also observed that treatment with metolazone significantly reduced Na\(^+\) uptake in the morphants.

![Graph A](image1.png)  
**A** Efflux  
Sham  
MO  
2 dpf 3 dpf  
Na\(^+\) efflux rate (pmol/fish/h)  
-100 0 100 200 300 400  
*  

![Graph B](image2.png)  
**B** Influx  
Sham  
MO  
2 dpf 3 dpf  
Na\(^+\) influx rate (pmol/fish/h)  
0 100 200 300 400  
*  

Fig. 5. Knockdown of claudin-b increases Na\(^+\) loss in zebrafish larvae. The efflux (**A**) and influx (**B**) rate of Na\(^+\) in shams and claudin-b morphants at 2 or 3 dpf. Values are expressed as means ± SE; n = 6. Asterisks indicate a significant difference in flux rates between sham and morphants on the same developmental stage (Student’s t-test; P < 0.05).

![Graph C](image3.png)  
**A** Whole-body Na\(^+\) content  
Sham  
MO  
2 dpf 3 dpf  
Whole-body Na\(^+\) content (nmol/fish)  
0 2 4 6 8 10 12 14  
*  

![Graph D](image4.png)  
**B** Whole-body Cl\(^-\) content  
Sham  
MO  
2 dpf 3 dpf  
Whole-body Cl\(^-\) content (nmol/fish)  
0 2 4 6 8 10 12 14  
*  

Fig. 6. Knockdown of claudin-b reduces whole-body Na\(^+\) but not Cl\(^-\) content in zebrafish larvae. The whole-body level of Na\(^+\) (**A**) and Cl\(^-\) (**B**) in shams and claudin-b morphants at 2 or 3 dpf. Values are expressed as means ± SE; n = 6. *Significant difference in flux rates between sham and morphants on the same developmental stage (Student’s t-test; P < 0.05).
ally, the elevated Na\(^{+}\) uptake in the morphants was attenuated during exposure to Cl\(^{-}\)-free water. These findings suggest that the elevated Na\(^{+}\) loss in the morphants was probably compensated by activation of NCC. However, it is noteworthy that in Cl\(^{-}\)-free water, the rate of Na\(^{+}\) uptake in the morphants remained significantly higher than in the shams. Therefore, in addition to NCC, other Na\(^{+}\)-transporting pathways may be contributing to the increased Na\(^{+}\) uptake. For example, it is thought that H\(^{+}\)-ATPase activity is important in Na\(^{+}\) uptake in these particular fish under these specific experimental conditions (Na\(^{+}\) = 0.78 mM, Cl\(^{-}\) = 0.4 mM, Ca\(^{2+}\) = 0.25 mM; pH 7.6). In contrast, Esaki et al. (13) showed that both EIPA and amiloride (another NHE inhibitor) reduced Na\(^{+}\) uptake in 55 hpf larval zebrafish main-

ATPase activity was essential to provide a driving force for increasing Na\(^{+}\) uptake in the morphants. Taken together, the results suggested that both NCC and H\(^{+}\)-ATPase played prominent roles in increasing Na\(^{+}\) uptake in the claudin-b morphants.

Recently, Kumai and Perry (25) reported that the NHE inhibitor EIPA inhibited Na\(^{+}\) uptake in 4 dpf zebrafish reared under acidic conditions (pH 4.0), whereas it had no effect in fish reared in normal (circumneutral pH) water. Similarly, we also observed that EIPA did not affect Na\(^{+}\) uptake in shams or claudin-b morphants, suggesting that NHE did not play a prominent role in Na\(^{+}\) uptake in these particular fish under these specific experimental conditions (Na\(^{+}\) = 0.78 mM, Cl\(^{-}\) = 0.4 mM, Ca\(^{2+}\) = 0.25 mM; pH 7.6). In contrast, Esaki et al. (13) showed that both EIPA and amiloride (another NHE inhibitor) reduced Na\(^{+}\) uptake in 55 hpf larval zebrafish main-

Fig. 7. Evidence for the contribution of Na\(^{+}\)/Cl\(^{-}\)-cotransporter and H\(^{+}\)-ATPase in increasing Na\(^{+}\) uptake in the zebrafish larvae following claudin-b knockdown. The effects of the Na\(^{+}\)/Cl\(^{-}\)-cotransporter inhibitor metolazone (A), Na\(^{+}\)/H\(^{+}\) exchanger inhibitor 5-(N-ethyl-N-isopropyl) amiloride (EIPA) (B), and H\(^{+}\)-ATPase inhibitor bafilomycin A1 (C) on the Na\(^{+}\) uptake rate in shams and claudin-b morphants at 3 dpf. Values are expressed as means ± SE; n = 6. Bars labeled with different letters represent a statistical difference (two-way ANOVA, followed by a post hoc Holm-Sidak test; P < 0.05).

Fig. 8. The increased Na\(^{+}\) uptake in the zebrafish larvae following claudin-b knockdown is attenuated during exposure to Cl\(^{-}\)-free water. The Na\(^{+}\) uptake rate in shams and claudin-b morphants (3 dpf) during exposure to either control (Cl\(^{-}\) level = 0.78 mM) or Cl\(^{-}\)-free water. Values are expressed as means ± SE; n = 6. Bars labeled with different letters represent a statistical difference (two-way ANOVA, followed by a post hoc Holm-Sidak test; P < 0.05).

Fig. 9. The mRNA expression of Na\(^{+}\)/Cl\(^{-}\)-cotransporter is increased in the zebrafish larvae following claudin-b knockdown. The mRNA expression levels of Na\(^{+}\)/Cl\(^{-}\}-cotransporter (NCC; slc12a10.2), Na\(^{+}\)/H\(^{+}\) exchanger (NHE3b; slc9a3b) and H\(^{+}\)-ATPase (atp6v1a) in shams and claudin-b morphants at 3 dpf. Values are expressed as means ± SE; n = 6. *Significant difference between sham and morphants (Student’s t-test; *P < 0.05).
tained in water with similar Na⁺ levels and pH (Na⁺ = 0.68 mM, Cl⁻ = 0.8 mM, Ca²⁺ = 0.16 mM; pH 6.7–7.4) to our study. Notably, Boisen et al. (4) demonstrated that EIPA either was without effect on Na⁺ uptake in adult zebrafish acclimated to hard water (Na⁺ = 1.48 mM, Cl⁻ = 1.62 mM, Ca²⁺ = 3.2 mM; pH 8.2) or actually increased Na⁺ uptake in soft water (Na⁺ = 0.035 mM, Cl⁻ = 0.04 mM, Ca²⁺ = 0.004 mM; pH 6.0). In that same study, the authors also reported that amiloride reduced Na⁺ uptake only in fish acclimated to hard water (4). These conflicting results regarding the role of NHE in Na⁺ uptake are unclear but may suggest that zebrafish use different mechanisms for Na⁺ uptake depending on the development stage, environmental pH, and ionic compositions. With respect to bafilomycin and EIPA eliciting different Na⁺ uptake responses, it is possible that the inhibitory effect of bafilomycin on Na⁺ uptake is linked to a Na⁺ uptake mechanism, which is not associated with NHE function (e.g., Na⁺ entry through channels electrically coupled to H⁺-ATPase).

Perspectives and Significance

The present study provides the first in vivo evidence that the tight junction protein claudin-b plays a critical role in maintaining a tight cutaneous epithelium in developing zebrafish. We demonstrated a significant increase in Na⁺ efflux in claudin-b morphants, indicating that claudin-b acts as a paracellular barrier against Na⁺ loss. Nevertheless, the elevated Na⁺ loss in the morphants was compensated by an increase in Na⁺ uptake, which appeared to be associated with activation of NCC and H⁺-ATPase activity. The present study on the epithelial barrier functions of claudin-b, as well as previous research on the role of claudin-5 (42) and claudin-15 (3) in neuroepithelial and gut permeability, highlights the diverse functional properties of the various claudin isoforms in developing zebrafish. In humans, loss of claudin expression and function has been linked to various disorders, such as salt wasting and nephrocalcinosis (2); the present study suggests that zebrafish may serve as an alternate model system to understand claudin-related pathophysiology of ion handling in vivo.

ACKNOWLEDGMENTS

We thank J. Bradshaw and V. Saxena for their technical assistance.

GRANTS

This study was supported by Natural Sciences and Engineering Research Council (NSERC) Discovery and NSERC Research Tools and Innovation Grants to S. F. Perry.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

R.W.M.K and S.F.P. are responsible for conception and design of the research; R.W.M.K. performed the experiments and analyzed the data; R.W.M. K. and S.F.P. interpreted the results of the experiments; R.W.M.K. drafted the manuscript; R.W.M.K and S.F.P. edited and revised the manuscript; R.W.M.K and S.F.P. approved the final version of the manuscript.

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