Claudin-8d is a cortisol-responsive barrier protein in the gill epithelium of trout

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Abstract

The influence of claudin (Cldn) 8 tight junction (TJ) proteins on cortisol-mediated alterations in gill epithelium permeability was examined using a primary cultured trout gill epithelium model. Genes encoding three Cldn-8 proteins (*cldn-8b*, *-8c* and *-8d*) have been identified in trout and all are expressed in the model gill epithelium. Cortisol treatment 'tightened' the gill epithelium, as indicated by increased transepithelial resistance (TER) and reduced paracellular [³H]polyethylene glycol (MW 400 Da; PEG-400) flux. This occurred in association with elevated *cldn-8d* mRNA abundance, but no alterations in *cldn-8b* and *-8c* mRNA abundance were observed. Transcriptional knockdown (KD) of *cldn-8d* inhibited a cortisol-induced increase in Cldn-8d abundance and reduced the 'epithelium tightening' effect of cortisol in association with increased paracellular PEG-400 flux. Under simulated *in vivo* conditions (i.e. apical freshwater), *cldn-8d* KD hindered a cortisol-mediated reduction in basolateral to apical Na⁺ and Cl⁻ flux (i.e. reduced the ability of cortisol to mitigate ion loss). However, *cldn-8d* KD did not abolish the tightening effect of cortisol on the gill epithelium. This is likely due, in part, to the effect of cortisol on genes encoding other TJ proteins, which in some cases appeared to exhibit a compensatory response. Data support the idea that Cldn-8d is a barrier protein of the gill epithelium TJ that contributes significantly to corticosteroid-mediated alterations in gill epithelium permeability.

Key Words

- ▶ osmoregulation
- ▶ fish gill
- ▶ tight junction
- ▶ paracellular permeability
- ▶ corticosteroid

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Introduction

The tight junction (TJ) complex acts as a selectively permeable intercellular occlusion barrier in vertebrate epithelia and contributes to the regulated paracellular movement of solutes between fluid compartments (Farquhar & Palade 1963, Van Itallie & Anderson 2006). The TJ complex is composed of numerous transmembrane and cytoplasmic proteins, but it is now generally accepted that transmembrane claudin (CLDN) TJ proteins that were first identified by Furuse and coworkers (1998) play a central role in establishing the permselectivity conditions

of TJ occlusion (Günzel & Yu 2013). In tetrapods, the CLDN family is large (i.e. ~27 proteins in mammals), but in teleost fishes, duplication events have resulted in a considerable expansion (Loh *et al.* 2004), and more than half of the +50 *cldn* genes found in a teleost are expressed in the gill (Chasiotis *et al.* 2012b, Kolosov *et al.* 2013). In aquatic vertebrates, the gill presents a large surface area that directly interfaces with surrounding water. The gill plays a multifaceted role in regulating solute movement between environment and blood (Evans *et al.* 2005),

and this includes limiting as well as finely controlling passive solute movement (Chasiotis *et al.* 2012b).

In freshwater (FW), a particularly important task for the gill epithelium is to limit diffusional ion loss (ion efflux) down electrochemical gradients into hypotonic surroundings (Evans *et al.* 2005). It has been proposed that cortisol, the principal circulating corticosteroid in teleost fishes, plays a crucial role in curbing passive ion loss across the gill epithelium in FW as cortisol has been shown to elicit a dose-dependent increase in transepithelial resistance (TER) across gill epithelium models derived from FW fish gills, and this occurs in association with reduced flux of the paracellular permeability marker [³H]polyethylene glycol (PEG) (a proxy for paracellular permeability), as well as reduced Na⁺, Cl⁻ and Ca²⁺ efflux rates (Kelly & Wood 2001, 2002, 2008). This tightening effect of cortisol on the teleost fish gill epithelium is consistent with many observations of corticosteroid-induced barrier tightening in diverse vertebrate organs (e.g. Zettl *et al.* 1992, Stelwagen *et al.* 1999, Antonetti *et al.* 2002, Förster *et al.* 2005, Kwong & Perry 2013) and is closely associated with alterations in the abundance of specific TJ proteins of the gill, including those of the Cldn superfamily (Chasiotis *et al.* 2010, Chasiotis & Kelly 2011b, Kelly & Chasiotis 2011, Kolosov *et al.* 2017).

The TJ protein CLDN-8 exists as a single isoform in mammals and has a putative Na⁺ barrier function in the nephron, urinary bladder and colon (Kiuchi-Saishin *et al.* 2002, Yu *et al.* 2003, Acharya *et al.* 2004, Li *et al.* 2004, Angelow *et al.* 2007, Amasheh *et al.* 2009). In teleosts, four genes encoding Cldn-8 proteins have been described: *cldn-8a*, *-8b*, *-8c* and *-8d* (Loh *et al.* 2004). These genes exhibit species-specific expression patterns in various fish organs (Kolosov *et al.* 2013), but in species studied so far, *cldn-8d* is seen to be abundant in the gill (Loh *et al.* 2004, Bagherie-Lachidan *et al.* 2009, Chasiotis & Kelly 2012, Kolosov *et al.* 2014). In this regard, it is of particular interest that following cortisol treatment of a primary cultured gill epithelium model, the abundance of mRNA encoding Cldn-8d, as well as Cldn-8d protein has been reported to increase (Chasiotis & Kelly 2011b, Kelly & Chasiotis 2011, Kolosov *et al.* 2017). Because this occurs in association with reduced epithelium permeability, it would suggest that Cldn-8d is a barrier-forming TJ protein in the teleost fish gill epithelium that plays an important role in corticosteroid-associated changes in paracellular solute movement. However, there is no functional evidence to support this idea. Nevertheless, other reports

suggest a barrier-forming role for Cldn-8d as well as other Cldn-8 isoforms in fishes. For example, *cldn-8b* and *-8d* (but not *cldn-8c*) transcript abundance increase dramatically during cultured trout gill epithelium formation as well as during the development of model gill epithelium resistive properties, and this occurs in association with a significant decrease in paracellular [³H]PEG-400 flux (Kolosov *et al.* 2014). Furthermore, transcript abundance of *cldn-8b*, *-8c* and *-8d* has been shown to significantly increase in the fish gill following acclimation or exposure to an ion-poor environment (Chasiotis *et al.* 2012a, Kolosov & Kelly 2016), where ion efflux rates (i.e. passive ion loss across the gill TJ complex) are documented to significantly decrease (e.g. Perry & Laurent 1989). Most recently, it has been suggested that Cldn-8d may contribute to a *cldn-31* KD-associated increase in gill epithelium permeability because *cldn-31* KD appears to prevent a cortisol-stimulated increase in Cldn-8d abundance (Kolosov *et al.* 2017).

Taken together, previous observations suggest that Cldn-8d may play an important endocrine-mediated role in adjusting epithelial permeability of ionoregulatory epithelia in fishes and mounting evidence also suggests that Cldn-8d as well as other Cldn-8 isoforms may contribute to the barrier properties of the gill epithelium. Therefore, the goal of this study was to use a primary cultured model gill epithelium to further consider a role for Cldn-8 isoforms in corticosteroid-induced FW fish gill tightening, and more specifically, provide functional insight that goes beyond the currently documented causal association between cortisol, gill epithelium tightening and *cldn-8d*/Cldn-8d.

Materials and methods

Experimental animals

Rainbow trout (*Oncorhynchus mykiss*, Walbaum 1792; obtained from Humber Springs Trout Hatchery, Orangeville, ON, Canada), were held in 600L opaque polyethylene tanks supplied with flow-through dechlorinated freshwater (FW, composition in µM: [Na⁺] 590, [Cl⁻] 920, [Ca²⁺] 760, [K⁺] 43, pH 7.35). Photoperiod was 12L:12D and fish were fed *ad libitum* once daily (Martin Profishent, Elmira, ON, Canada). Animal care and experimental procedures were in accordance with an approved York University Animal Care Committee protocol, which conformed to the guidelines of the Canadian Council on Animal Care.

Procedures for the preparation and culture of a model gill epithelium

The primary cultured model gill epithelium used in these studies was prepared using methods originally developed by Wood and Pärt (1997) and described in detail by Kelly *et al.* (2000). The model is composed of gill pavement cells (PVCs) only and begins with a period of flask culture, following which cells are harvested and seeded on to semi-permeable polyethylene terephthalate filter inserts (0.9 cm² area, 0.4 µm pore, 1.6 × 10⁶ pore/cm² density; BD Falcon TM, BD Biosciences, Mississauga, ON, Canada). Cell culture inserts were kept in companion cell culture plates (Falcon BD) and the apical and basolateral compartments of the culture system contained L15 media supplemented with 6% fetal bovine serum.

Cultured preparations were exposed to cortisol in accordance with methods previously outlined by Kelly and Wood (2001). In brief, a concentrated stock solution of cortisol was prepared by dissolving cell culture-grade hydrocortisone 21-hemisuccinate sodium salt (Sigma-Aldrich) in phosphate-buffered saline (PBS, pH 7.7). Aliquots of stock solution were stored at -30°C. Upon each media change, stock cortisol solution was added to cell culture media in order to yield a final media cortisol concentration of 500 ng/mL. Media containing cortisol was added to the basolateral side of cultured preparations only. Similar levels of circulating cortisol have been reported in stressed rainbow trout (Barton & Iwama 1991).

Measurement of transepithelial resistance (TER) and [³H] PEG-400 flux

TER of insert-cultured preparations was monitored with chopstick electrodes (STX-2) attached to a custom-modified EVOM epithelial voltohmmeter (World Precision Instruments, Sarasota, FL, USA). Paracellular flux of [³H]PEG-400 (American Radio Chemicals, St. Lois, MO, USA) was measured and calculated according to Wood and coworkers (1998).

Quantitative real-time PCR (qPCR) analysis of *cldn-8* mRNA abundance

Transcript abundance of *cldn-8b*, *-8c* and *-8d* in control and cortisol-treated model gill epithelia was determined using primer sets and techniques described in detail by Kolosov and coworkers (2014). In brief, total RNA was isolated from gill tissue using TRIzol Reagent (Invitrogen Canada Inc., Burlington, ON, Canada)

according to manufacturer's instructions and quantified using a Multiskan Spectrum UV/Vis microplate spectrophotometer (Thermo Fisher Scientific). Total RNA (2 µg) was treated with DNase I (Amplifications Grade, Invitrogen Canada, Inc.) and used for cDNA synthesis with SuperScriptTM III reverse transcriptase and Oligo(dT)12–18 primers (Invitrogen Canada, Inc.). Transcript abundance of *cldn-8b* (GenBank accession # BK008770.1; forward: ACGACTCCCTCCTGGCTCT and reverse: GAGACCCATCCGATGTAGA; predicted amplicon size 185 bp), *cldn-8c* (GenBank accession # BK008771.1; forward: GCTTGATGTGCTGCTCTC and reverse: CCCAGAGGTCAGGAGGA; predicted amplicon size 201 bp) and *cldn-8d* (GenBank accession # BK007966.1; forward: GCAGTGTAAGTGTACGACTCTCTG and reverse: CACGAGGAACAGGCATCC; predicted amplicon size 200 bp) was examined with SYBR Green I Supermix (Bio-Rad Laboratories Canada Ltd) in a Chromo4TM Detection System (CFB-3240; Bio-Rad Laboratories Canada Ltd). Reaction conditions were 1 denaturation cycle (95°C, 4 min), followed by 40 cycles of: denaturation (95°C, 30 s), annealing (*cldn-8b* 56°C; *cldn-8c* 60°C; *cldn-8d* 60°C, 30 s) and extension (72°C, 30 s). A dissociation curve analysis was carried out after each qPCR run to confirm the synthesis of a single PCR product. Rainbow trout elongation factor 1- α (*ef-1a*; GenBank accession # AF498320.1; forward: GGCAAGTCAACCACCACAG and reverse: GATACCACGCTCCCTCTCAG; predicted amplicon size 159 bp) was used as a reference gene. The use of *ef-1a* as a reference for gene of interest normalization was validated by statistically comparing *ef-1a* threshold cycles between treatments to confirm that experimental conditions had not significantly altered the values. Transcript encoding rainbow trout *cldn-8a* was not examined in this study because we have been unable to identify a *cldn-8a* homolog in this species.

Western blot, immunocytochemistry and Cldn-8d protein quantification

Western blot analysis of Cldn-8d was conducted using methods previously described by Chasiotis and coworkers (2010). Briefly, insert-cultured cells were rinsed with ice-cold PBS (4°C), and then incubated in a lysis buffer (10 mM Tris-HCl, pH=7.5, 1 mM EDTA, 0.1 mM NaCl, 1 mM PMSF) with 1:200 protease inhibitor cocktail (Sigma-Aldrich Canada Ltd.). Epithelial tissue was then collected after disruption by repeatedly passing cells in solution through a 26G syringe needle. A supernatant was collected following centrifugation (10,000 g for

10 min, 4°C) and protein concentration of this solution was determined using a Bradford Assay (Sigma-Aldrich Canada Ltd.). A total of 5 µg protein was then used for Western blotting using a 12% SDS-PAGE gel. Wet transfer was performed to transfer protein samples from the gel to a polyvinylidene difluoride (PVDF) membrane at 100 V for 1 h. Following transfer, the membrane was incubated with 5% skimmed milk solution for 1 h at room temperature (RT), after which the membrane was incubated overnight at RT with a rabbit anti-Cldn-8d custom synthesized antibody. The custom synthesized Cldn-8d polyclonal antibody was raised in rabbit against a synthetic peptide (Ac-SYFDPAYDEKAAKSC-amide) corresponding to a 16–29 amino acid region of rainbow trout Cldn-8d (New England Peptide, LLC, Gardner, MA, USA) (1:1000 dilution in Tris-buffered saline with Tween; TBS-T, 10 mM Tris, 150 mM NaCl, 0.05% Tween-20, pH=7.4). Signal detection was performed by incubating the membrane with a horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (Bio-Rad Laboratories Canada Ltd.; 1 h, RT). Antigen reactivity was examined with Clarity Western ECL Blotting Substrate (Bio-Rad Laboratories Canada Ltd.; 5 min at RT). Imaging of Cldn-8d immunoreactivity (Cldn-8d-ir) was conducted using a Chemi-Doc MP System (Bio-Rad Laboratories Canada Ltd.). Specificity of the custom-made primary antibody was confirmed using a peptide pre-absorption technique described by Bui and Kelly (2014).

Procedures for the immunolocalization of Cldn-8d in cultured gill epithelia are outlined in detail in Chasiotis and Kelly (2011a). Briefly, insert-cultured gill epithelium preparations were fixed with 3% paraformaldehyde, permeabilized with ice-cold methanol for 5 min at –20°C, rinsed with Triton X-100 in phosphate-buffered saline (PBS) and incubated with antibody-dilution buffer (ADB; 10% goat serum, 3% BSA and 0.05% Triton X-100 in PBS; 1 h at RT). Preparations were then incubated with the custom anti-Cldn-8d antibody (as detailed previously; overnight at RT, 1:100 in ADB). After washing in PBS, epithelia were incubated with a TRITC-labeled goat anti-rabbit antibody (1:500 in ADB; Jackson ImmunoResearch Laboratories; 1 h at RT). Preparations were then mounted on slides with mounting medium containing 4',6-diamidino-2-phenylindole (DAPI) for nuclear visualization. Images of Cldn-8d-ir and DAPI fluorescence were captured using a differential interference contrast (DIC) and laser-scanning confocal microscopy with an Olympus BX-51 in conjunction with a Fluoview unit and MellesGriot green and red argon lasers (Olympus Canada). As a negative

control, epithelia were prepared as previously described in the absence of primary antibody.

For quantification of Cldn-8d protein abundance, Western blot analysis was used to image Cldn-8d-ir as described previously. Membranes were then incubated in stripping buffer, blocked with 5% skim milk solution as outlined above and incubated with mouse monoclonal anti-actin JLA20 antibody (Developmental Studies Hybridoma Bank, Iowa City, USA), as a reference protein. Actin immunoreactivity was imaged and used to normalize Cldn-8d abundance after validating, by statistical comparison, that actin abundance was not influenced by experimental treatment. Cldn-8d and actin protein abundance were quantified using ImageJ software (National Institutes of Health, Java 1.6.0_45 (64-bit)).

Transcriptional knockdown (KD) of *cldn-8d*

Methods followed for transcriptional KD of *cldn-8d* have been reported by Kolosov and coworkers (2017). Custom-made double-stranded RNA (dsRNA) spanning nucleotides 64–548 of the *cldn-8d* transcript (GenBank accession # BK007966.1) was prepared using a T7 RiboMAX Express RNAi System (Promega) according to the manufacturer instructions. dsRNA concentration was determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific) and 15–60 pmol was used for *cldn-8d* KD in cultured preparations combined with Lipofectamine 3000 transfection reagent (Invitrogen Canada Inc.). A range of 15–60 pmol of dsRNA was found to be effective; therefore, subsequent experiments were conducted with 15 pmol dsRNA, where epithelia were first transfected 24 h after seeding on to cell culture inserts.

For analysis of *cldn-8d* transcript in transfected tissue, total RNA was isolated and cDNA synthesized as previously described (see Quantitative real-time PCR (qPCR) analysis of *cldn-8* mRNA abundance section). The presence of *cldn-8d* mRNA was then determined by reverse transcriptase PCR (RT-PCR). In order to detect transcriptional KD of *cldn-8d*, a separate set of primers for rainbow trout *cldn-8d* were designed using the coding sequence (forward: 5'-TACGACGAGAAAGCAGCAAA-3' and reverse: 5'-ATTGGGATGATGGTGGTGAC-3', predicted amplicon size ~490 bp). PCR conditions used were as follows: denaturation at 95°C, 4 min, followed by 40 cycles of: (1) denaturation (95°C, 30 s), (2) annealing (60°C, 30 s) and (3) extension (72°C, 30 s). RT-PCR product was examined using agarose gel electrophoresis.

Ion flux measurements under asymmetrical culture conditions

Using ion-selective microelectrodes (ISMe) constructed according to Donini and O'Donnell (2005), net flux rates of Na⁺ were determined under asymmetrical cultures conditions (i.e. apical FW/L15 basolateral) using apical FW collected at the start and at the end of a 2-h flux period. When introduced to the experimental setup, apical FW was sterile, temperature equilibrated and the same composition as fish holding water (see Experimental animals section). Flux rates (efflux; basolateral to apical movement) were calculated as the difference between ion concentration at the beginning and the end of FW exposure over time and surface area. To measure Cl⁻ flux rates, the same samples were used to examine (Cl⁻) colorimetrically as described by Zall and coworkers (1956).

Transcriptional analysis of genes encoding cortisol-responsive *cldns*

To examine how *cldn-8d* KD influenced the transcriptional response of other cortisol-responsive *cldns*, *cldn-3a* (BK007964), -6 (KF445436), -7 (BK007965), -12 (BK007967), -20a (BK009404), -23a (BK008775), -28b (EU921670), -30 (BK007968) and -31 (BK007969) were chosen for examination in accordance with the observations of Kelly and Chasiotis (2011), Kolosov and coworkers (2014) and Gauberg and coworkers (2017). Transcript abundance of *cldns* were examined by qPCR using techniques as outlined above, previously published primer sets (Kelly & Chasiotis 2011, Kolosov *et al.* 2014, Gauberg *et al.* 2017) and cDNA were prepared using symmetrically cultured cells.

Statistical analysis

Statistical analysis was conducted using a Student's *t*-test or a two-way ANOVA protocol coupled with a multiple comparison test where appropriate, in SigmaPlot (version 11) statistical software. Statistical significance was based on the observation of a fiduciary limit of $P < 0.05$.

Results

Effect of cortisol on model gill epithelium permeability, *cldn-8* isoform mRNA abundance and Cldn-8d abundance

Cortisol treatment of the cultured gill epithelium increased TER (Fig. 1A) and decreased paracellular

Figure 1

Cortisol reduces gill epithelium permeability and selectively alters claudin (*cldn*) 8 isoform transcript abundance. Effect of cortisol on (A) transepithelial resistance, (B) [³H]PEG-400 flux and (C) *cldn-8b*, -8c and -8d transcript abundance. All data are expressed as mean values $\pm$ s.e.m. ($n=5$). Transcript abundance of *cldn-8b*, -8c and -8d is normalized using rainbow trout elongation factor 1a as a reference gene and transcript abundance of *cldn-8* genes in cortisol-treated preparations (open bars) are expressed relative to control preparations assigned a value of 100 (closed bars). An asterisk indicates significant difference ($P < 0.05$) between cortisol-treated and control epithelia as determined by an unpaired Student's *t*-test.

[³H]PEG-400 flux (Fig. 1B). Cortisol treatment did not alter the transcript abundance of *cldn-8b* or -8c, but significantly increased *cldn-8d* transcript abundance (Fig. 1C). A custom-synthesized antibody raised against rainbow trout Cldn-8d resolved as a single band of ~38 kDa by Western blot analysis of gill epithelium tissue (Fig. 2A). Cldn-8d localized to regions of cell-to-cell contact around the periphery of cultured gill epithelium cells (Fig. 2B). Cortisol treatment significantly increases Cldn-8d abundance in the cultured gill epithelium preparation (Fig. 2C).

Figure 2

Claudin (Cldn)-8d protein localizes to regions of cell-to-cell contact in the gill epithelium and increases in abundance following cortisol treatment. (A) Western blot analysis of a custom-synthesized rainbow trout Cldn-8d antibody in primary cultured gill epithelium tissue revealed a single immunoreactive band of ~38 kDa in total (T) and membrane (M) fractions but not in the cytosolic (Cyt) fraction. This band was not present following a peptide block procedure (+peptide). In panel (B), Cldn-8d (red) can be seen immunolocalized to regions of cell-to-cell contact around the periphery of cultured gill cells and nuclear DAPI staining can be seen in blue (scale bar = 100 µm). Panel (C) shows the effect of cortisol on Cldn-8d abundance as determined by Western blot analysis. Data in panel (C) are expressed as mean values $\pm$ s.e.m. ($n=5$) and a significant difference ($P<0.05$) between cortisol-treated and control epithelia as determined by an unpaired Student's *t*-test, is indicated by an asterisk. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JME-17-0108>.

Effect of cortisol and *cldn-8d* KD on model gill epithelium permeability

Using dsRNA to KD *cldn-8d* resulted in complete elimination of transcript 24 h post transfection (Fig. 3A). This occurred at the three doses tested (i.e. 15, 30 and 60 pmol dsRNA). An amplicon of expected size was seen in untreated controls and in cultured preparations

transfected with negative control dsRNA designed for a bacterial transcript (Fig. 3A). Cldn-8d protein abundance was examined 24, 48 and 72 h after dsRNA transfection and was found to be significantly reduced (~50%) and stable vs epithelia transfected with negative control dsRNA (Fig. 3B). In control and cortisol-treated preparations, *cldn-8d* KD significantly reduced cortisol-stimulated TER (Fig. 3C). A significant increase in paracellular PEG-400

Figure 3

Transcriptional knockdown (KD) of *cldn-8d* in the gill epithelium reduces Cldn-8d abundance and increases permeability of control and cortisol-treated preparations. The effect of *cldn-8d* KD on (A) RT-PCR amplification of *cldn-8d* 24 h following transcriptional KD and (B) Cldn-8d protein abundance as well as (C) transepithelial resistance and (D) paracellular $[^3\text{H}]\text{PEG-400}$ flux across a primary cultured model gill epithelium. Panel (A) shows RT-PCR-generated *cldn-8d* amplicons run on an agarose gel. Tissue for PCR analysis was harvested 24 h following transcriptional KD with dsRNA (Ct=untreated control, B=bacterial nonsense dsRNA, and 15, 30, and 60= dose, in pmol, of dsRNA used for *cldn-8d* KD). In panels (B, C and D), all data are expressed as mean values $\pm$ s.e.m. In panel (B), Cldn-8d abundance is shown for tissue collected 24, 48 and 72 h following *cldn-8d* KD ($n=4/\text{group}$). An asterisk indicates significant difference ($P<0.05$) between control and *cldn-8d* KD groups as determined by an unpaired Student's *t*-test. In panels (C) and (D), permeability endpoints were determined using epithelium preparations bathed on both sides with L15 medium that had plateaued (i.e. mature preparations, $n=8/\text{group}$); an asterisk indicates the significant difference due to cortisol treatment, while daggers indicate significance due to *cldn-8d* KD, as determined by a two-way ANOVA coupled with a Holm-Sidak *post hoc* test.

flux was also observed in cortisol-treated *cldn-8d* KD preparations (Fig. 3D).

Effect of cortisol and *cldn-8d* KD on model gill epithelium permeability, Cldn-8d abundance and ion flux rates under simulated *in vivo* conditions

In cortisol-treated cultured gill epithelium preparations exposed to simulated *in vivo* conditions (i.e. apical FW/basolateral L15), TER was elevated (Fig. 4A) and Cldn-8d abundance was increased vs control epithelia (Fig. 4B). In addition, net Na⁺ and Cl⁻ flux rates (i.e. basolateral to apical ion movement; equivalent to ion loss) were decreased (Fig. 4C and D). However, under the same asymmetrical culture conditions, *cldn-8d* KD reduced a cortisol-stimulated increase in TER (Fig. 4A) and Cldn-8d abundance (Fig. 4B). Flux (i.e. basolateral to apical movement) rates of Na⁺ and Cl⁻ were elevated by *cldn-8d* KD in control preparations (Fig. 4C and D), and the cortisol-induced reduction in ion flux rates as described previously was also significantly impaired by *cldn-8d* KD (Fig. 4C and D). Nevertheless, when comparing *cldn-8d* KD control and cortisol-treated preparations, an effect of cortisol could still be seen (Fig. 4A, B, C and D).

Effect of cortisol and *cldn-8d* KD on cortisol-responsive model gill epithelium *cldn* isoform mRNA abundance

Cortisol treatment significantly elevated the transcript abundance of *cldn-3a*, -6, -7, -12, -20a, -23a, -28b,

-30 and -31 in the model gill epithelium (Fig. 5). In *cldn-8d* KD preparations, a significant increase in the transcript abundance of these *cldns* (vs control *cldn-8d* KD epithelia) was also observed for all isoforms except for *cldn-3a*. In the absence of cortisol, *cldn-8d* KD resulted in an increase in *cldn-7*, -12 and -20a mRNA abundance. An increase in mRNA abundance of *cldn-7* and -12 was also observed as a result of *cldn-8d* KD in the presence cortisol (i.e. when comparing cortisol vs cortisol *cldn-8d* KD groups). However, the same trend was absent for *cldn-20a*, which exhibited no significant difference between cortisol and cortisol *cldn-8d* KD preparations.

Discussion

Overview

Data from this study provide functional evidence that Cldn-8d plays an important role in cortisol-induced gill epithelium tightening and that Cldn-8d may be the only cortisol-responsive Cldn-8 isoform in the gill epithelium of rainbow trout. More specifically, *cldn-8d* KD limited, but did not eliminate, the tightening effect of cortisol on a model gill epithelium composed of PVCs. These observations are not unexpected because there are numerous gill TJ proteins that are transcriptionally responsive to cortisol and many of these encode either putative or known barrier-forming TJ proteins. Furthermore, the ability of *cldn-8d* KD to limit but not abolish cortisol-induced tightening of the primary cultured model PVC epithelium used in this study is

Figure 4

Transcriptional knockdown (KD) of *cldn-8d* in the gill epithelium inhibits cortisol-induced alterations in permeability, Cldn-8d abundance and ion flux rates ($\equiv$ ion loss) under simulated *in vivo* conditions. The effect of *cldn-8d* KD and cortisol treatment on (A) transepithelial resistance (TER), (B) Cldn-8d abundance, (C) net Na⁺ flux ($\equiv$ Na⁺ efflux) and (D) net Cl⁻ flux ($\equiv$ to Cl⁻ efflux) in cultured gill epithelium preparations exposed to asymmetrical culture conditions (i.e. simulated *in vivo* conditions; apical freshwater/basolateral L15) for 2 h. All data are expressed as mean values $\pm$ S.E.M. (A) $n=5$ /group, (B) $n=10$ /group, (C) $n=5$ /group, (D) $n=5$ /group. An asterisk indicates a statistically significant difference ($P<0.05$) between control and cortisol-treated groups while a dagger denotes a significant difference due to *cldn-8d* KD, as determined by a two-way ANOVA coupled with a Holm-Sidak post hoc test.

Figure 5

Transcriptional knockdown (KD) of *cldn-8d* in the gill epithelium alters the transcript abundance of select cortisol-responsive *cldn* isoforms. The effect of *cldn-8d* KD and cortisol treatment on transcript abundance of *cldn-3a*, -6, -7, -12, -20a, -23a, -28b, -30 and -31 in a primary cultured gill epithelium model. Transcript abundance of each *cldn* isoform was normalized using trout elongation factor 1a as a reference gene and expressed relative to the untreated control group (control) assigned a value of 100. Data are expressed as mean values $\pm$ S.E.M. ($n=5$ or 6/group). An asterisk indicates a statistically significant difference ($P < 0.05$) between control and cortisol-treated groups while a dagger denotes a significant difference due to *cldn-8d* KD, as determined by a two-way ANOVA coupled with a Holm-Sidak post hoc test.

consistent with the effect of *cldn-31* KD, which was also shown to have a similar effect on model gill epithelium permeability (Kolosov *et al.* 2017). However, although Cldn-31 contributed to cortisol-induced epithelium tightening, some ambiguity remains as to how much of a role it plays because *cldn-31* KD prevented a cortisol-induced increase in Cldn-8d abundance, which may have enhanced increased permeability (Kolosov *et al.* 2017). In contrast, this study suggests that *cldn-8d* KD compromises gill PVC epithelium integrity without negatively affecting other cortisol-responsive *cldns* in PVCs, including *cldn-31*. The importance of Cldn-8d as a cortisol-responsive barrier protein in the gill epithelium TJ complex can ultimately be seen in asymmetrically exposed (apical FW) KD preparations that manifest a weakened ability to mitigate Na^+ and Cl^- flux ($\equiv$ ion loss). These observations support the idea that in a natural setting where the gill epithelium is exposed to FW on the apical surface, Cldn-8d contributes significantly to gill epithelium tightness and that this contribution can be modulated by alterations in circulating cortisol levels.

The effect of cortisol on permeability and *cldn-8* isoform abundance in the gill epithelium and characterization of Cldn-8d

The tightening effect of corticosteroids on vertebrate epithelia/endothelia is well documented across a broad

array of tissue barrier types, but was first reported for a fish epithelium using a cultured trout gill model (Kelly & Wood 2001). It has since been shown to occur in cultured gill epithelia derived from other species (Kelly & Wood 2002, Chasiotis & Kelly 2011b, 2012) as well as intact fish (Kwong & Perry 2013), and this takes place in association with changes in the molecular physiology of the TJ complex (Chasiotis *et al.* 2010, Kelly & Chasiotis 2011, Kolosov & Kelly 2013). However, from a mechanistic standpoint, almost nothing is known about how different TJ components contribute to this endocrine-mediated physiological change. In this study, we confirm that gill epithelium *cldn-8d* mRNA abundance increases in response to cortisol, and that this occurs in association with reduced paracellular PEG flux (a proxy for paracellular permeability). In addition, we are able to suggest that *cldn-8d* may be the only cortisol-responsive *cldn-8* isoform in the trout gill epithelium because neither *cldn-8b* nor -8c mRNA alter following cortisol treatment. Whether this will be a species and/or organ specific phenomenon remains to be determined as we are unaware of any other study that has examined the effect of corticosteroids on *cldn-8b* or -8c.

Cldn-8d was found to localize around the periphery of cultured gill epithelium cells at regions of cell-to-cell contact. This is consistent with the localization of a bicellular TJ protein in a vertebrate epithelium. Using

Western blot analysis, Cldn-8d resolved as a single band of ~38 kDa. This is larger than the ~25 kDa MW of mammalian CLDN-8, which was first described by Morita *et al.* (1999), but it is not unusual for a CLDN TJ protein. For example, CLDN-16 and CLDN-23 are ~34 and ~32 kDa, respectively (Simon *et al.* 1999, Katoh & Katoh 2003). It was also confirmed that Cldn-8d abundance increases following cortisol treatment, which is consistent with previous observations (Kolosov *et al.* 2017).

The effect of *cldn-8d* KD and cortisol on gill epithelium permeability

Transcriptional KD of *cldn-8d* significantly reduced Cldn-8d protein abundance at the first time point examined (i.e. 24 h following transfection). At this time, no RT-PCR-amplified *cldn-8d* amplicon was observed in cultured preparations and protein abundance was ~50% reduced. This reduction in Cldn-8d abundance was observed for at least 72 h following *cldn-8d* KD. There did not appear to be any time-dependent change in Cldn-8d abundance following *cldn-8d* KD. Because the estimated half-life of Cldn-8d in a vertebrate *in vitro* model (as calculated using ProtParam ExPaSy online tool; <http://web.expasy.org/cgi-bin/protparam/protparam>) is ~30 h, this apparent absence of time-dependent change could be due to an orchestrated balance of residual, pre-KD Cldn-8d protein during the early stages of KD and the appearance of new protein as the system recovers during the later stages of KD. Nevertheless, *cldn-8d* KD (and a reduction in Cldn-8d abundance) was coupled with a reduction in TER of gill epithelium preparations both in the presence and absence of cortisol. However, a significant effect of *cldn-8d* KD on paracellular PEG flux was only seen in cortisol-treated preparations. In this case, these results obscure a definitive conclusion regarding the nature of Cldn-8d as a putative barrier protein, although they do support the idea that Cldn-8d plays an important barrier-forming role in cortisol-induced gill epithelium tightening. But the aforementioned results are generated using model preparations held under symmetrical culture conditions (i.e. L15 apical/L15 basolateral), which does not present the demands experienced by a tissue exposed to solute asymmetry such as that faced by the FW fish gill epithelium (i.e. apical FW/basolateral blood). Therefore, more compelling evidence that Cldn-8d is a barrier protein in the gill epithelium can be seen when considering how *cldn-8d* KD preparations cope with exposure to apical FW. In control *cldn-8d* KD preparations, decreased TER was

observed as before, and this was coupled with an increase in net Na⁺ and Cl⁻ flux, which in these preparations is equivalent to paracellular efflux rates or ion loss (Wood *et al.* 1998). The ability of cortisol to mitigate ion loss under asymmetrical conditions was compromised by *cldn-8d* KD, and taken together, these observations reinforce the idea that Cldn-8d is a barrier-forming TJ protein in fishes or at least in the gill epithelium of trout.

Because efflux rates of Na⁺ and Cl⁻ across *cldn-8d* KD preparations increase equally (resulting in unchanged P_{Na}/P_{Cl}), it would suggest that Cldn-8d in the cultured trout gill epithelium acts as an indiscriminate barrier to Na⁺ and Cl⁻ movement. This contrasts with the ion- and charge-selectivity that is often reported for CLDN TJ proteins, including CLDN-8 (Günzel & Yu 2013). However, it is likely to be more complicated than this as mammalian CLDN-8 is reported to act as both a Na⁺ barrier and Cl⁻ pore depending on cell type (Yu *et al.* 2003, Hou *et al.* 2010) as well as contribute to the formation of an impermeable TJ barrier in the urinary bladder (Acharya *et al.* 2004). The role of Cldn-8d in the electrically tight cultured gill epithelium would certainly be consistent with the latter. However, it should also be noted that the model gill epithelium used in this study is composed of PVCs only, and the role of Cldn-8d in gill ionocytes might not be indiscriminate. It has been reported that *cldn-8d* transcript abundance is greater in teleost gill ionocytes vs PVCs (Chasiotis *et al.* 2012a, Kolosov *et al.* 2014), which could be suggestive of different function. It has also been observed that environmental change can differently alter *cldn-8d* mRNA abundance in gill PVCs and ionocytes (Chasiotis *et al.* 2012a). Therefore, further work investigating the idea of a cell-specific role for Cldn-8d in the fish gill is warranted.

The contribution of Cldn-8d to cortisol-mediated tightening of the gill epithelium

In the rainbow trout gill epithelium, *cldn-8d* has been reported to be particularly responsive to corticosteroid treatment (Kelly & Chasiotis 2011), and the response to cortisol appears to be mediated through both glucocorticoid and mineralocorticoid receptors (Kelly & Chasiotis 2011). Transcript encoding Cldn-8d is a comparatively abundant *cldn* isoform in the gill of a number of teleost species, including rainbow trout (Bagherie-Lachidan *et al.* 2009, Chasiotis & Kelly 2012, Kolosov *et al.* 2014). Therefore, it seems logical to consider that cortisol-induced changes in *cldn-8d* abundance may be indicative of a prominent role

for Cldn-8d in cortisol-induced gill epithelium tightening. This idea is supported by data in the current study, which show that when cortisol treatment is coupled with a *cldn-8d* KD-induced reduction in Cldn-8d abundance, the gill epithelium does not exhibit the same increase in TER and reduction in PEG-400 permeability that can be observed in cortisol-treated control preparations. However, relative to control *cldn-8* KD preparations, cortisol-treated *cldn-8d* KD preparations still exhibit higher TER and lower PEG-400 permeability. There are likely to be several reasons for this. The first is that other cortisol-responsive *cldn* isoforms encoding proteins that are currently thought to possess barrier function, continue to exhibit an unperturbed increase in abundance following cortisol treatment of *cldn-8d* KD preparations. These include *cldn-6*, *-20a*, *-23a*, *-28b*, *-30* and *-31*, the latter of which encodes Cldn-31, which has recently been confirmed as barrier forming in the gill TJ complex (Kolosov *et al.* 2017). In addition to Cldns, other TJ proteins that have been reported as cortisol-responsive and barrier forming, such as occludin (Chasiotis *et al.* 2010) and tricellulin (Kolosov & Kelly 2013) may also have contributed to a persistent, but suppressed cortisol-induced decrease in permeability of *cldn-8d* KD preparations. A second reason for observed changes in permeability is that despite effective transcriptional KD, some Cldn-8d is still present in the gill epithelium model. This continues to be responsive to cortisol treatment and most likely contributes to a reduction in permeability. Finally, there is some evidence to suggest that select Cldns may compensate for *cldn-8d* KD, because the abundance of *cldn-7*, *-12* and *-20a* mRNAs in cortisol-treated *cldn-8d* KD epithelia are greater than cortisol-treated control preparations. Transcript encoding Cldn-7 was also reported to mount a compensatory response to *cldn-31* KD in the gill epithelium (Kolosov *et al.* 2017), but alterations in *cldn-7* abundance have been associated with epithelial restructuring and the development of confluence rather than the development of resistive properties (Chasiotis *et al.* 2012a, Kolosov *et al.* 2014). The roles of *cldn-12* and *-20a* in gill barrier function are also unclear. For example, previous studies have reported both increased and decreased gill *cldn-12* mRNA abundance in association with environmental change that requires enhanced barrier function (Chasiotis & Kelly 2011b, Chasiotis *et al.* 2012a, Kolosov *et al.* 2014). Furthermore, *cldn-20a* has only recently been described in trout as cortisol-responsive in the skin (Gauberg *et al.* 2017), and in this regard, remains to be investigated in

the gill. Studies on other vertebrates provide little insight as to the potential compensatory roles of *cldn-7*, *-12* and *-20a* in the manipulated gill epithelium. For instance, CLDN-7 has been reported to act as both an anion barrier and pore (Günzel & Yu 2013), while *in vitro* studies suggest that CLDN-12 may modulate paracellular Ca⁺⁺ movement (Fujita *et al.* 2008). In contrast, CLDN-20 in human breast cancer cells is involved in aggressive phenotype development and in chick embryos, development of the retinal pigment epithelium (Rizzolo *et al.* 2007, Martin *et al.* 2014). Therefore, the only clear picture is that if any compensation did occur, it was not enough to counteract increased ion flux in cortisol-treated *cldn-8d* KD epithelia when the model epithelium was exposed to apical FW. Nevertheless, it seems clear that Cldn-8d plays an important role in cortisol-induced tightening of the cultured gill epithelium and under natural conditions this could be important in order to moderate passive ion loss under conditions where a stressor-induced elevation in circulating cortisol occurs.

Conclusion and perspectives

The idea that cortisol can regulate paracellular solute movement across the FW fish gill epithelium by modulating the physiological properties of the gill TJ complex is well established. However, mechanistic understanding of this phenomenon is almost non-existent. Using a functional approach, this study highlights a barrier-forming role for Cldn-8d between rainbow trout gill epithelium PVCs that is enhanced by cortisol. Moreover, data suggest that other Cldn-8 isoforms in the PVCs of the rainbow trout gill may not be involved in corticosteroid-induced gill epithelium tightening. Given the importance of the gill epithelium as a barrier to solute movement in ectothermic aquatic vertebrates, and the central role of cortisol in teleost fish physiology, these observations bring into focus an emerging sophistication that will require thorough examination. This is important when we take into consideration the critical link between endocrine-mediated alterations in the ionoregulatory physiology of aquatic vertebrates and the ability of these organisms to maintain homeostasis in a challenging environment.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

D K and S P K conceived the study. D K conducted experiments and collected data. D K analyzed the data. S P K and D K drafted and edited the manuscript.

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