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RESEARCH ARTICLE

Tight junction protein gene expression patterns and changes in transcript abundance during development of model fish gill epithelia

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ABSTRACT

In vertebrates, tight junction (TJ) proteins play an important role in epithelium formation and development, the maintenance of tissue integrity and regulation of TJ permeability. In this study, primary cultured model gill epithelia composed of pavement cells (PVCs) were used to examine TJ protein transcript abundance during the development of epithelium confluence and epithelium resistive properties. Differences in TJ protein expression patterns and transcript abundance between gill models composed of PVCs and models composed of PVCs and mitochondrion-rich cells (MRCs) were also examined. Marked alterations in TJ protein transcript abundance were observed as cells developed to confluence in flask-cultured model gill epithelia. In contrast, during the formation of tissue resistance in insert-cultured epithelia (i.e. epithelia cultured on a permeable substrate), changes in TJ protein mRNA abundance were conservative, despite paracellular marker flux decreasing by orders of magnitude. In both cases significant changes in *claudin-8b*, *-8d*, *-27b*, *-28b* and *-32a* transcript abundance were observed, suggesting that temporal alterations in the abundance of these genes are important end points of model gill epithelium integrity. When MRCs were present in cultured gill models, the mRNA abundance of several TJ proteins significantly altered and *claudin-10c*, *-10d* and *-33b* were only detected in preparations that included MRCs. These data provide insight into the role of select TJ proteins in the formation and development of gill epithelia and the maintenance of gill barrier properties. In addition, observations reveal a heterogeneous distribution of claudin TJ proteins in the gill epithelial cells of rainbow trout.

KEY WORDS: Tricellulin, Claudin, Occludin, Mitochondrion-rich cell, Pavement cell, Paracellular permeability, Transepithelial resistance, Flask cell culture, Insert cell culture

INTRODUCTION

In the vertebrate epithelium, the tight junction (TJ) complex is an apicolateral belt-like reticular network of strands that: (1) separates the apical and basolateral membrane domain of an epithelial cell and (2) connects adjacent epithelial cells to act as a selectively permeable paracellular diffusion barrier (Farquhar and Palade, 1963; Staehelin, 1973; Gonzalez-Mariscal et al., 2001; Anderson and Van Itallie, 2006). TJs can be bicellular (bTJs) and connect two neighboring epithelial cells or tricellular (tTJs) and form a link between three epithelial cells at regions of tricellular contact (Günzel and Fromm, 2012). bTJs run alongside each other around the cell periphery and form the majority of the paracellular interface between epithelial cells and the apical environment (Anderson and Van Itallie, 2006). In contrast, tTJs run vertically in an apical-to-basal direction (Higashi et al., 2013). bTJs and tTJs consist of transmembrane TJ proteins and intracellular scaffolding TJ proteins (Günzel and Fromm, 2012). Transmembrane TJ proteins possess extracellular loops that link within the intercellular cleft, and intracellular domains that connect with scaffolding TJ proteins (Günzel and Fromm, 2012). Extracellular loop characteristics regulate the passage of material across the paracellular pathway, and cortical proteins can act as a link between the intracellular domain of transmembrane TJ proteins and the cell cytoskeleton (Citi et al., 2012; Van Itallie et al., 2009; Günzel and Fromm, 2012). Transmembrane TJ proteins of the claudin (Cldn) superfamily localize exclusively to bTJs and considerable evidence supports the view that bTJ Cldn composition is chiefly responsible for tissue- and cell-specific paracellular permeability properties of vertebrate epithelia (for a review see Günzel and Yu, 2013). In contrast, members of the recently proposed angulin family of proteins, consisting of tricellulin (Tric), lipolysis-stimulated lipoprotein receptor, and immunoglobulin-like domain-containing receptors 1 and 2, appear to make up the tTJ of vertebrates (Higashi et al., 2013). tTJ proteins have also been reported to contribute to the barrier properties of vertebrate epithelia (Furuse et al., 2012; Ikenouchi et al., 2005; Korompay et al., 2012; Krug et al., 2009; Masuda et al., 2010; Ohkuni et al., 2009; Takasawa et al., 2013; Kolosov and Kelly, 2013).

The gill occupies a central role in a fish's ability to maintain homeostasis (Evans et al., 2005). This is partly because of TJs and the paracellular permeability properties of the gill epithelium, which regulate the paracellular movement of solutes between extracellular fluid and the surrounding aquatic environment (for a review see Chasiotis et al., 2012a). Recent studies have shown select TJ proteins of the gill epithelium to be sensitive to environmental change as well as endocrine factors that mediate the systemic response of fishes to varying environmental conditions (for a review see Chasiotis et al., 2012a). These TJ proteins include a number of claudin as well as transmembrane TJ proteins such as occludin (Ocln) and Tric (Chasiotis et al., 2012b; Kolosov and Kelly, 2013). In addition, cortical proteins such as zonula occludens 1 (ZO-1) have been reported to alter in response to environmental or systemic change (Chasiotis et al., 2012b). However, despite an increasing number of studies examining gill epithelium TJ proteins in relation to changes in the external environment, the contribution of gill epithelium TJ proteins to the establishment and maintenance of paracellular permeability properties in this tissue remains poorly understood.

Primary cultured model gill epithelia provide a platform for studying the paracellular permeability characteristics of the gill epithelium (for a review see Wood et al., 2002). These models are

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List of abbreviations

bTJ	bicellular TJ
Cgn	cingulin
Cldn	claudin
DSI	double-seeded insert
FW	freshwater
MRC	mitochondrion-rich cell
NKA	Na^+-K^+-ATPase
Ocln	occludin
PEG	polyethylene glycol
PVC	pavement cell
SSI	single-seeded insert
SW	seawater
TER	transepithelial resistance
TJ	tight junction
Tric	tricellulin
tTJ	tricellular TJ
ZO-1	zonula occludens 1

simplified flat epithelia 'reconstructed' on permeable cell culture inserts using gill epithelium pavement cells (PVCs) (Avella and Ehrenfeld, 1997; Chasiotis and Kelly, 2011a; Chasiotis and Kelly, 2011b; Kelly and Wood, 2002; Wood and Pärt, 1997) or both PVCs and gill epithelium mitochondrion-rich cells (MRCs) (Fletcher et al., 2000). The characteristics of model gill epithelia allow the architectural complexity and cellular heterogeneity of the gill to be managed while the paracellular permeability properties of the gill epithelium remain very similar to those found in the intact gill (for a review see Wood et al., 2002). Permeability characteristics of model gill epithelia can be measured by electrophysiological techniques and/or by radiotracer (paracellular permeability marker) studies. More specifically, electrophysiological end points such as transepithelial electrical resistance (TER) can be used to monitor the development of epithelia, so that once a plateau in TER is observed, epithelia can be used for experimental manipulation. Recent work has shown that primary cultured gill epithelium models can be used to provide insight into the molecular physiology of the gill TJ complex (Bui and Kelly, 2011; Bui et al., 2010; Chasiotis and Kelly, 2011a; Chasiotis and Kelly, 2011b; Chasiotis et al., 2010; Chasiotis et al., 2012b; Kelly and Chasiotis, 2011; Kolosov and Kelly, 2013). But studies to date have examined experimental end points following the establishment of confluence and/or the development of stable resistive properties in these epithelial models (for a review see Chasiotis et al., 2012a). Therefore, TJ protein gene expression patterns during the formation of a confluent gill epithelium or during the development of gill epithelium resistive properties have yet to be examined. Given the significant changes in structure and function that accompany epithelium formation and the establishment of stable electrophysiological characteristics of cultured gill epithelia (for a review see Wood et al., 2002), it can be hypothesized that temporal differences in the abundance of transcript encoding TJ proteins during the development of gill epithelium confluence and/or the development of gill epithelium resistive properties will be observed. In addition, it also seems reasonable to contend that if differences in transcript abundance occur, these are likely to reflect the relative importance of TJ proteins in each process. Furthermore, because gill epithelium models can either be composed exclusively of PVCs or composed of PVCs and MRCs, these models present an opportunity to examine whether genes encoding TJ proteins are present exclusively in MRCs by virtue of their absence in PVC models and presence in models composed of both PVCs and MRCs. Therefore, the goal of this study was to characterize TJ protein gene expression patterns in model gill epithelia during the development of confluence, to characterize the resistive properties and to examine gene expression patterns of TJ proteins in models composed of PVCs versus those composed of both PVCs and MRCs.

RESULTS

TJ protein gene expression profiles in different tissues of rainbow trout

The mRNA abundance of all *cldn* genes studied varied in the different tissues of rainbow trout (*Oncorhynchus mykiss*) in which they were found (Fig. 1). These differences were generally orders of magnitude, but in a few cases reasonably consistent levels of mRNA were found between most tissues examined (e.g. *cldn-8c*, *-12* and *-29a*). Cytosolic scaffolding proteins cingulin (Cgn) and ZO-1 were also reasonably consistent (Fig. 1). Of the 21 *cldn* genes examined, mRNA of 10 were found to be expressed in all discrete tissues examined (*cldn-3a*, *-5a*, *-8b*, *-8c*, *-10e*, *-12*, *-13*, *-23a*, *-29a* and *-31*). In contrast, mRNA of 11 *cldn* genes were found to be absent from one or more tissues examined. In some instances (i.e. *cldn-10c*, *-10d*, *-32a* and *-33b*), mRNA was found in relatively few tissues (e.g. in gill, kidney and skin). mRNAs encoding Cgn and ZO-1 were also found to be expressed in all tissues examined in this study (Fig. 1).

Flask-cultured gill epithelial cells: epithelium development and TJ protein mRNA abundance

A sub-confluent layer of primary cultured gill epithelial cells were in cell culture flasks 24 h following cell seeding (Fig. 2A). By 48 h, a relatively confluent layer of primary cultured gill epithelial cells was present, but no obvious junctions between epithelial were observed (Fig. 2B). A confluent epithelial cell layer that exhibited a typical, but faint, 'cobblestone' or 'mosaic' pattern indicating the formation of junctions between primary cultured gill epithelial cells was seen by 72 h (Fig. 2C). This pattern appeared to intensify by 120 h (Fig. 2D) and was visually prominent by 192 h (Fig. 2E) and 240 h (Fig. 2F) following the introduction of gill epithelial cells into cell culture flasks.

With the exception of *cldn-1* (data not shown), significant alterations in the transcript abundance of all TJ proteins examined could be observed at some point over the 240 h flask-culture period in this study. During the first 72 h of flask culture when epithelia were forming a confluent layer (see Fig. 2), mRNA encoding Cldn-3a, -7, -8b, -8d, -13, -23a, -27b, -28b and -30 significantly increased (Fig. 3). Transcripts encoding Ocln, Cgn and ZO-1 were also observed to significantly increase in flask-cultured gill epithelial cells during the first 72 h of the experiment (Fig. 4). In many cases (e.g. *cldn-3a*, *-7*, *-23a*, *-30*, *ocln* and *cgn*) the observed increase in mRNA abundance during the first 72 h of flask culture was modest, i.e. in the range of a 1.5- to 2.5-fold elevation over cells that had been in culture for 24 h. However, *cldn-27b* mRNA exhibited an approximate 6-fold increase in abundance, whereas *cldn-8b*, *-8d* and *-28b* mRNA were found to increase 12- to 15-fold during the first 72 h of flask culture (Fig. 3). In contrast to these changes, transcript encoding Cldn-5a, -10d, -12, -31, -32a and -33b significantly decreased in gill epithelial cells during the first 72 h of flask culture whereas mRNA encoding Cldn-6, -8c, -10c, -10e, -29a and Tric did not significantly alter (Figs 3, 4). Of particular note was that transcripts encoding Cldn-10d, -31 and -33b fell to barely detectable levels after 72 h flask culture (Fig. 3).

Following an elevation in mRNA abundance during the first 72 h of flask culture, *cldn-8b*, *-8d*, *-27b* and *-28b* transcript levels remained relatively constant until 240 h (Fig. 3), whereas the abundance of *cldn-3a*, *-7*, *-23a*, *-30*, *ocln* and *cgn* mRNA significantly dropped by 240 h (Figs 3, 4). Nevertheless, mRNA

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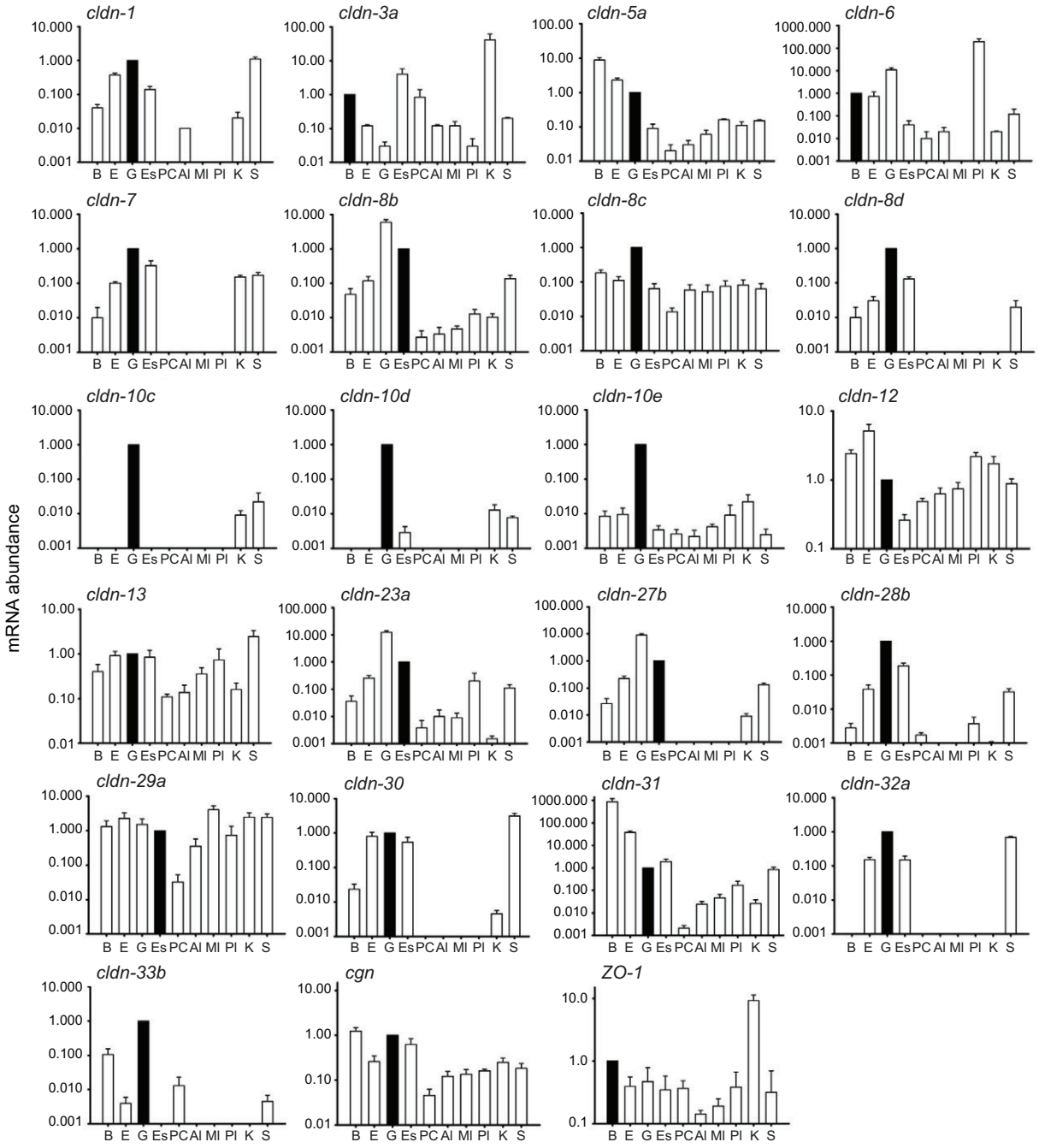

Fig. 1. mRNA expression profiles of tight junction proteins in discrete tissues of rainbow trout (*Oncorhynchus mykiss*). Claudin (*cldn*), cingulin (*cgn*) and zonula occludens-1 (*zo-1*) transcripts were examined in brain (B), eye (E), gill (G), esophagus (Es), pyloric caeca (PC), anterior intestine (AI), middle intestine (MI), posterior intestine (PI), kidney (K) and skin (S) tissue by quantitative real-time PCR (qRT-PCR) analysis. Transcript abundance was normalized to that of β-actin and expressed as fold change relative to a reference tissue (indicated by black bar) assigned a value of 1.0. All reference tissues exhibited a qRT-PCR threshold cycle value of ~25. Data are expressed as means ± s.e.m. (*N*=4).

levels of these latter genes were generally not lower than levels measured at 24 h (Figs 3, 4). One exception to this was *cldn-30* mRNA, for which the levels were lower at 240 h than they were at 24 h (Fig. 3). Over the remaining 240 h culture period in flasks, *cldn* transcripts that significantly declined during the first 72 h of culture either continued to decline (*cldn-32a*), remained relatively stable at lowered levels (*cldn-12* and *-31*), significantly elevated again (*cldn-5a*) or disappeared entirely (*cldn-10d* and *-33b*). Transcript encoding Cldn-10c was also found to be undetectable by the end of the 240 h flask-culture period, and *cldn-10e* mRNA abundance was extremely low (Fig. 3). A progressive decline in transcript levels of *cldn-8c*, *-29a* and *-32a* was observed in gill cells over the entire 240 h flask-culture period, resulting in significantly lower mRNA levels following 240 h of culture versus 24 h (Fig. 3).

Single-seeded insert (SSI) preparations composed of gill PVCs only: TER, [^{3}H] polyethylene glycol (PEG)-400 flux and TJ protein mRNA abundance during development

After seeding flask-cultured cells on to permeable cell culture inserts, cells formed an epithelium that, over time, had a typical sigmoidal

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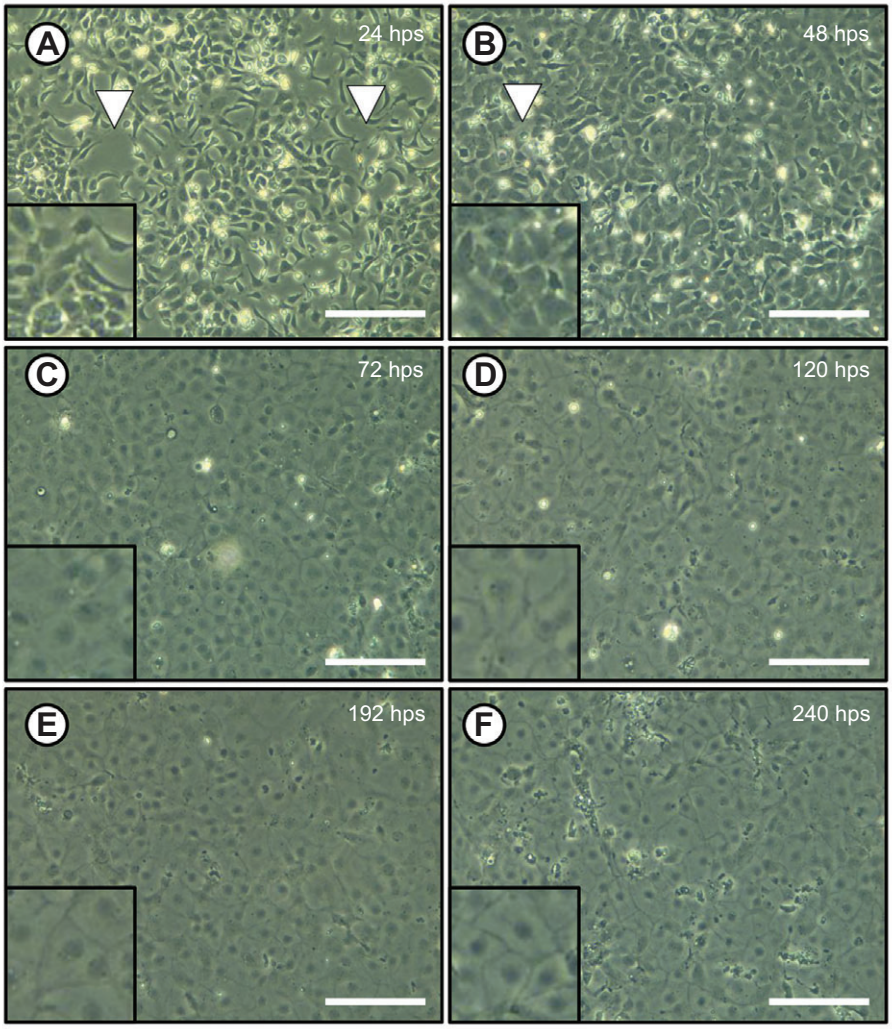

Fig. 2. Primary cultured rainbow trout gill epithelial cell development. (A) 24, (B) 48, (C) 72, (D) 120, (E) 192 and (F) 240 h post-seeding (hps) of trypsinated gill cells into cell culture flasks. In each panel, an inset shows a magnified region of the cultured cells. In A and B, regions where cells were absent are indicated by white arrowheads. Cells were confluent by 72 hps and as can be seen in C–F, cell borders became more distinct as the culture period progressed. Scale bars: 150 µm.

TER development curve (Fig. 5A). These epithelia were composed of PVCs only. A plateau in TER of 4345±517 Ω cm^2 was observed ~108 h after seeding cells on to inserts. Over the same time period, [^{3}H] polyethylene glycol (PEG)-400 flux across gill epithelia progressively reduced from $59.11\times10^{-7}\pm8.21\times10^{-7}$ cm s^{-1} at 24 h to $1.54\times10^{-7}\pm0.18\times10^{-7}$ cm s^{-1} at 108 h (Fig. 5B).

Transcript encoding a number of TJ proteins did not significantly alter in insert-cultured gill PVCs over the 108 h experimental period of this study. More specifically, mRNAs encoding Cldn-1, -3a, -7, -13 and -30 (Fig. 6), as well as Tric, Cng and ZO-1 (Fig. 7), did not exhibit any significant alterations over the period observed. In contrast, *cldn-6*, *-8b*, *-8d*, *-23a*, *-27b*, *-28b*, *-29a*, *-31* and *ocln* mRNAs all increased either at some point during the insert-culture period (*cldn-6*, *-8b*, *-29a*, *-31* and *ocln*) or progressively over the duration of the 108 h culture period (*cldn-8d*, *-23a*, *-27b* and *-28b*; Figs 6, 7). Only *cldn-5a*, *-10e* and *-32a* mRNA abundance was found to be significantly lower in PVCs at the end of the insert-culture period (Fig. 6), whereas *cldn-8c* and *-12* mRNAs were observed to decrease during the early part of the insert culture period, but were not significantly different at the end of the culture period (108 h) versus the beginning of the culture period (24 h; Fig. 6). Transcript encoding Cldn TJ proteins that were no longer detectable at the end of the flask-culture period (i.e. *cldn-10c*, *-10d* and *33b*), were not detected in insert-cultured gill PVCs at any point during the experimental period.

SSI (PVC) and double-seeded insert (DSI; PVC + MRC) preparations: a comparison of TJ protein mRNA abundance

The TER of insert-cultured SSI epithelia was lower than that of insert-cultured epithelia composed of both PVCs and MRCs (i.e. DSI epithelia; Fig. 8A). The presence of MRCs (ionocytes) in DSI epithelia was confirmed by the presence of Na^+/K^+-ATPase immunoreactive cells in these preparations (Fig. 8C). In contrast, cells that exhibited prominent Na^+/K^+-ATPase immunoreactivity were absent from SSI preparations (Fig. 8B).

A comparison of the mRNA abundance of TJ proteins between SSI and DSI epithelia revealed several marked differences between the preparations (Fig. 9). Of particular interest were *cldn-10c* and *-10d* mRNAs, which were not detectable by the end of the flask culture period (Fig. 3) or in SSI epithelia, were present in DSI epithelia (Fig. 9). Transcript encoding Cldn-33b was intermittently present in DSI preparations, and when it was present it was at very low levels (Fig. 9). Transcript encoding Cldn-31, which significantly declined during the flask culture period and was present at low levels in SSI epithelia, exhibited an ~60-fold elevation in DSI versus SSI preparations (Fig. 9). Transcript encoding other TJ proteins such as Cldn-3a, -5a, -6, -8c, -8d, -10e, -28b, -29a and Cgn were moderately but significantly elevated in DSI versus SSI epithelia (2-fold to 4-fold increase; Fig. 9). In contrast, *cldn-1*, *-8b*, *-12*, *-23a*, *-27b*, *-32a*, *ocln*, *tric* and *zo-1* mRNA abundance did not significantly alter between SSI and DSI preparations, whereas

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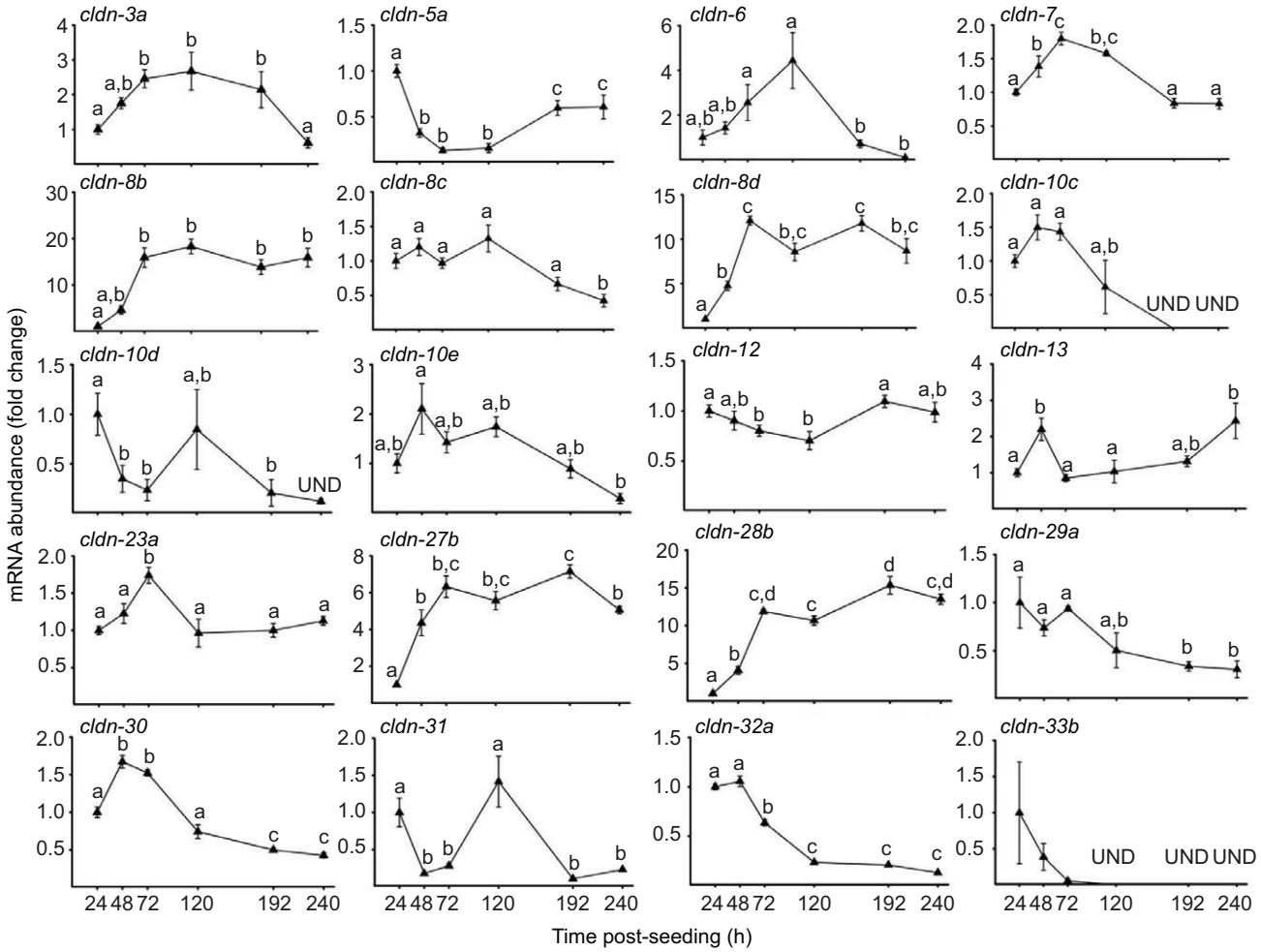

Fig. 3. Temporal changes in transcript abundance of claudin (Cldn) TJ proteins in primary cultured rainbow trout gill cells grown in cell culture flasks. Cells were sampled at time points that corresponded to those of the images in Fig. 2 (i.e. 24, 48, 72, 120, 192 and 240 h post-seeding isolated rainbow trout gills cells into flasks). TJ protein mRNA abundance was normalized to that of EF-1α, and expressed relative to values measured at 24 h, which were assigned a value of 1.0. Data are expressed as means ± s.e.m. (*N*=5 or 6). Different lowercase letters denote significant differences between samples collected at different time points. UND, undetected.

cldn-7, *-13* and *-30* mRNA abundance significantly decreased in DSI versus SSI epithelia (Fig. 9).

Relative abundance of *cldn* mRNAs in flask-, SSI- and DSI-cultured gill cells

The relative abundance of *cldn* mRNAs in cultured gill epithelial cells at the end of the flask (240 h post-seeding; hps), SSI (108 hps) and DSI (~108 hps) culture periods are shown (Fig. 10). Differences in the relative *cldn* mRNA abundance within each cultured gill epithelium preparation were approximately seven orders of magnitude (Fig. 10). In addition, differences in the order of *cldn* mRNA abundance (i.e. most to least abundant) could be observed between preparations. The main differences in relative *cldn* mRNA abundance between preparations were primarily between flask- and insert-cultured epithelia. For example, in flask-cultured gill cells (and in order), mRNA encoding Cldn-1, -30, -28b, -32a, -8b and -7 were found to be most abundant (Fig. 10A). However, in inserts (i.e. both SSI and DSI), *cldn-30* mRNA was the most abundant and from a relative standpoint, *cldn-1* mRNA was one order of magnitude lower in inserts (Fig. 10). In addition, the relative abundance of *cldn-8b* mRNA also decreased by over one order of magnitude in insert-cultured cells (SSI and DSI) versus cells in flasks (Fig. 10). However, another major difference between preparations was the absence of *cldn-10c*, *-10d* and *-33b* mRNA in flask- and SSI-cultured gill epithelial cells (Fig. 10). Nevertheless, there was no relative change in abundance of mRNAs encoding several Cldn proteins (i.e. Cldn -8d, -12, -13, -23a and -27b) between preparations (Fig. 10).

DISCUSSION

Overview

This study presents gene expression patterns of TJ proteins in a broad array of rainbow trout tissues as well as primary cultured rainbow trout model gill epithelia. In addition, the study also provides a first look at temporal changes in TJ protein transcript abundance of model gill epithelia during the formation of tissue confluence and during the development of epithelium resistive properties. The hypothesis that temporal differences in TJ protein transcript abundance will occur during the development of gill epithelium confluence and/or the development of gill epithelium resistive properties can be accepted. For example, transcripts encoding several Cldn proteins (i.e. Cldn-8b, -8d, -27b and -28b) are substantially elevated during the formation of tissue confluence on a solid substrate (i.e. in cell culture flasks), whereas others are

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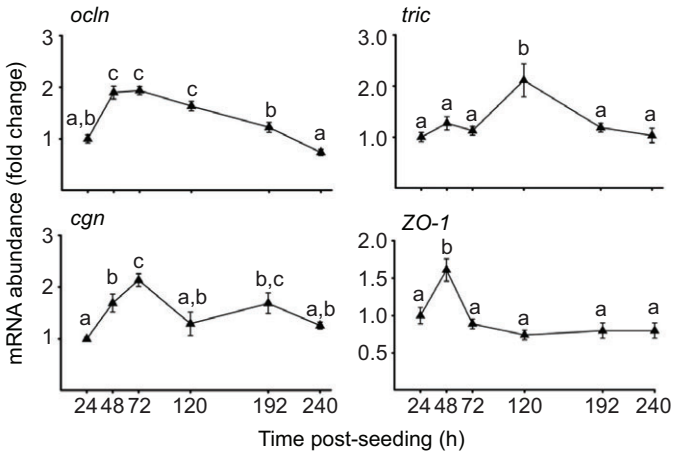

Fig. 4. Temporal changes in transcript abundance of occludin (*ocln*) tricellulin (*tric*), cingulin (*cgn*) and zonula occludens 1 (*zo-1*) in primary cultured rainbow trout gill cells grown in cell culture flasks. Cells were sampled at time points that corresponded to those of the images in Fig. 2 (i.e. 24, 48, 72, 120, 192 and 240 h post-seeding isolated gills cells into flasks). Transcript abundance was normalized to that of EF-1α, and expressed relative to values measured at 24 h which were assigned a value of 1.0. Data are expressed as means ± s.e.m. (*N*=5 or 6). Different lowercase letters denote significant differences between groups of flasks collected at different time points.

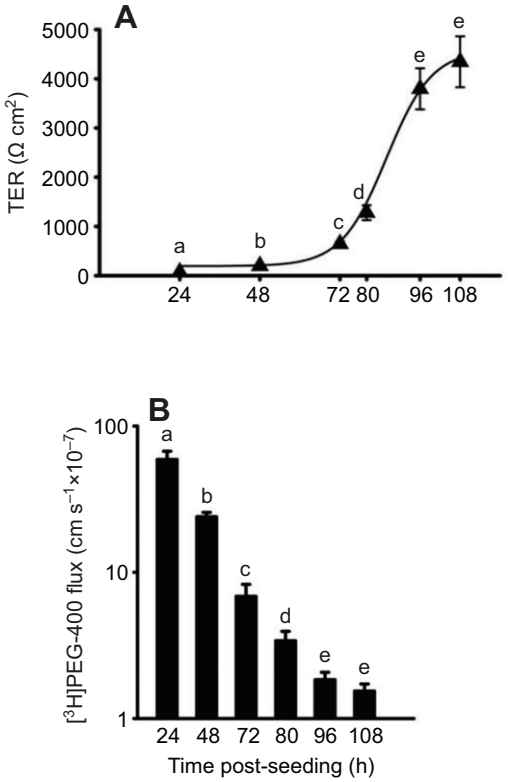

Fig. 5. Temporal change in the resistive properties and paracellular permeability of primary cultured rainbow trout gill cells. (A) Transepithelial resistance (TER) and (B) [^{3}H]PEG-400 flux across primary cultured single-seeded insert (SSI) preparations derived from rainbow trout gills. Measurements of TER and [^{3}H]PEG-400 flux were conducted at 24, 48, 72, 80, 96 and 108 h after seeding flask-cultured cells into cell culture inserts. Data are expressed as means ± s.e.m. (*N*=8). One-way analysis of variance on repeated measurements was performed to determine significant differences between time points. Significant difference is denoted by different lowercase letters.

markedly reduced (e.g. cldn-30 and cldn-32a). In addition, when epithelial cells were confluent in flasks, transcripts encoding three Cldn proteins (Cldn-10c, -10d and -33b) could not be detected (Fig. 3). The genes were also not detectable in insert-cultured gill epithelia composed of PVCs only, but *cldn-10c*, *-10d* and *-33b* were found to be expressed in insert-cultured preparations composed of gill PVCs and MRCs. This demonstrates that differences in the cellular composition of model gill epithelia can result in changes in TJ protein gene expression patterns and supports the idea that in the gill epithelium, select TJ proteins may be exclusively found in specific cell types, such as ionocytes. When comparing changes in epithelial TJ protein mRNA abundance during the first 120 h of flask culture with TJ protein mRNA abundance of insert-cultured epithelia (which were cultured for a comparable 108 h), it can be reasoned that over time, insert-cultured SSI preparations exhibit more conservative alterations in transcript abundance (see Figs 3, 4, 6, 7). This is despite the fact that TER of SSI epithelia increased from ~70 Ω cm^2 following 24 h in culture to ~4350 Ω cm^2 at 108 h in culture, and paracellular permeability (of [^{3}H]PEG-400) decreased by almost two orders of magnitude over the same culture period. Once insert-cultured epithelia reach a plateau in TER, they are not documented to exhibit any further increase in TER or decrease in paracellular permeability unless they are experimentally manipulated [e.g. by the addition of cortisol (Kelly and Wood, 2001)]. Therefore, if changes in TJ protein mRNA abundance are conservative during the first 108 h of culture, it seems unlikely that marked changes would occur during an extended plateau phase. Nevertheless, the possibility that some additional changes in TJ protein mRNA abundance in insert-cultured gill epithelia may take place if the 108 h culture period was extended cannot be ruled out. All the same, a progressive increase in *cldn-8b*, *-8d*, *-27b* and *-28b* mRNA levels was observed during the 108 h development period of SSI preparations (see Fig. 6). Given that these same mRNAs increase substantially during the process of confluence formation in flask-cultured cells (see Fig. 3), it can be reasoned that temporal changes in the abundance of the four TJ proteins would be useful molecular end points of model gill epithelium integrity and could also be potential markers of gill tissue integrity.

TJ protein gene expression profiles in discrete rainbow trout tissues

Gene expression patterns of Cldn TJ protein isoforms generally varied in the different rainbow trout tissues (see Fig. 1). This is consistent with distinctive gene expression patterns of Cldn TJ proteins in all vertebrates studied to date (reviewed by Günzel and Yu, 2013), including other fish species (reviewed by Kolosov et al., 2013). The differential distribution pattern of Cldn proteins in vertebrate tissues is generally believed to impart the diverse paracellular permeability properties of vertebrate epithelia (Günzel and Yu, 2013), and the abundant *cldn* mRNAs in tissues of rainbow trout were found to be similar to those in the same tissues of other fish species. For example, *cldn-3a* in kidney, *cldn-5a* in brain and eye, and *cldn-31* in brain, eye and gill have previously been observed to be abundant in the same tissues of species such as *Fugu rubripes* (Loh et al., 2004), *Tetraodon nigroviridis* (Bagherie-Lachidan et al., 2008; Bui et al., 2010; Bui and Kelly, 2011), *Tetraodon biocellatus* (Duffy et al., 2011), *Danio rerio* (Clelland and Kelly, 2010; Kumai et al., 2011; Jin et al., 2005; Jeong et al., 2008; Zhang et al., 2010; Xie et al., 2010; Hyoung Kim et al., 2011) and *Carassius auratus* (Chasiotis and Kelly, 2012). In contrast, gene expression patterns of the scaffolding TJ proteins Cgn and ZO-1

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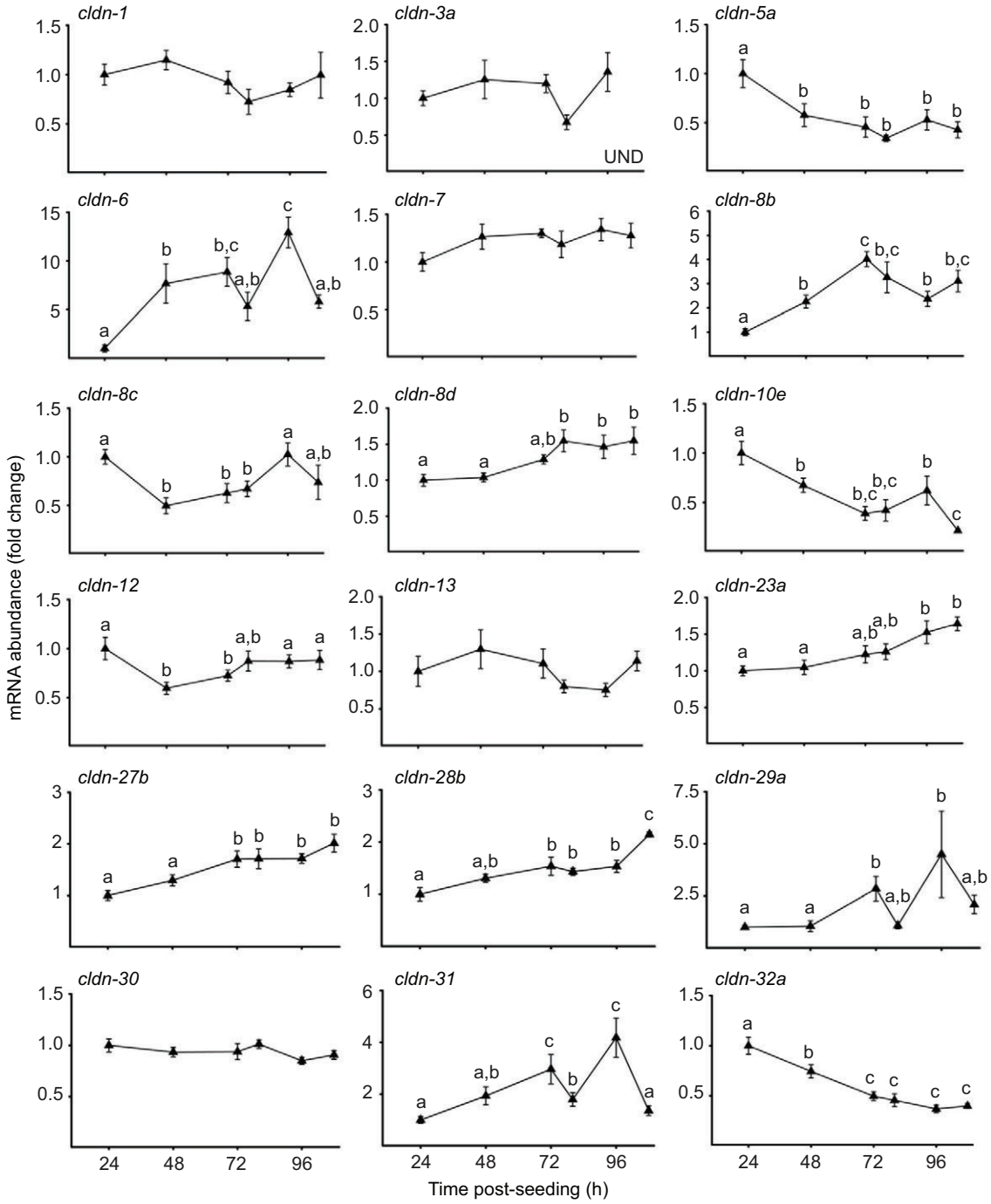

Fig. 6. Temporal alterations in the mRNA abundance of claudin (Cldn) tight junction (TJ) proteins in primary cultured single-seeded insert (SSI) preparations derived from rainbow trout gills. SSI epithelia were harvested at time points that correspond to the measurements of TER in Fig. 5 (i.e. 24, 48, 72, 80, 96 and 108 h post-seeding in cell culture inserts). TJ protein mRNA abundance was normalized to that of EF-1α, and expressed relative to values at 24 h, which were assigned a value of 1.0. Data are expressed as means ± s.e.m. (*N*=6). Different lowercase letters denote significant differences between groups of inserts collected at different time points. UND, undetected.

show that these proteins can be found in all tissues examined, and are often fairly consistent in abundance between most tissues. This is not unexpected because scaffolding proteins such as Cgn and ZO-1 are an essential component of TJ complex architecture in all epithelia, and a similar distribution pattern has previously been reported for goldfish ZO-1 mRNA (Chasiotis and Kelly, 2012). Indeed, even transmembrane TJ proteins that are integral to epithelia have been reported to be broadly and quite consistently expressed in discrete fish tissues (Kolosov and Kelly, 2013).

TJ protein gene expression profiles of different rainbow trout tissues revealed a number of *cldns* that were particularly abundant in the gill relative to other tissues examined. For example, mRNA encoding Cldn-8b, -8d, -10c, -10d, -10e, -27b, -28b and -33b were at least one order of magnitude more abundant in the gill than in other tissues. In addition, TJ protein genes such as *cldn-8d*, *-10c*, *-10d* and *-33b* mRNAs were relatively abundant but appeared in few other tissues (see Fig. 1). This suggests that these proteins play particularly important roles in the physiology of only a few discrete

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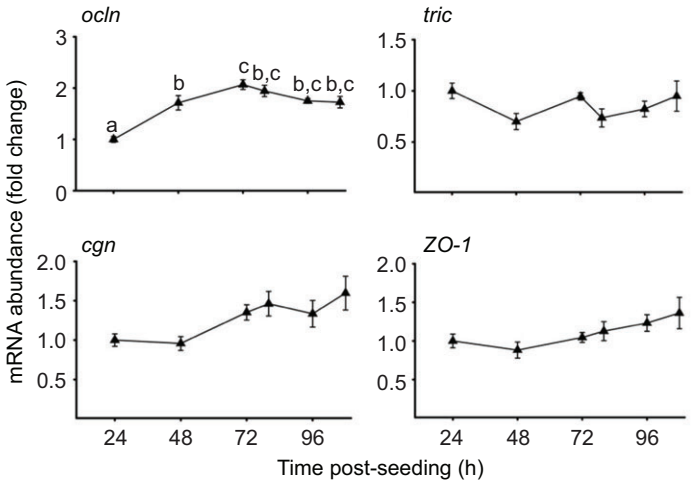

Fig. 7. Temporal alterations in the mRNA abundance of occludin (*ocln*) tricellulin (*tric*), cingulin (*cgn*) and zonula occludens 1 (*zo-1*) in primary cultured single-seeded insert (SSI) preparations derived from rainbow trout gills. SSI epithelia were harvested at time periods corresponding to the measurements of transepithelial resistance (TER) as seen in Fig. 5 (i.e. 24, 48, 72, 80, 96 and 108 h post-seeding in cell culture inserts). TJ protein mRNA abundance was normalized to that of EF-1α, and expressed relative to values measured at 24 h, which were assigned a value of 1.0. Data are expressed as means ± s.e.m. (*N*=6). Different lowercase letters denote significant differences between groups of inserts collected at different time points.

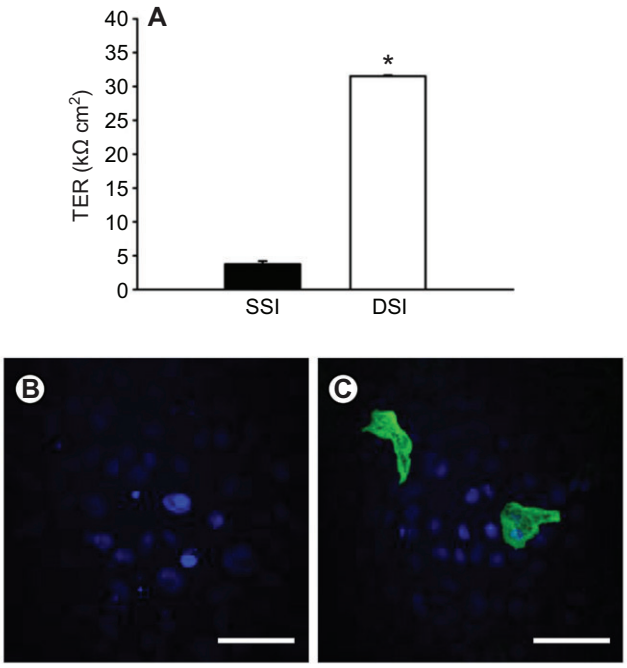

Fig. 8. Comparison of the properties of single-seeded insert (SSI) and double-seeded insert (DSI) preparations derived from rainbow trout gills. (A) Transepithelial resistance (TER) and (B,C) Na^+/K^+-ATPase immunoreactivity in (B) SSI and (C) DSI preparations. Na^+/K^+-ATPase immunoreactive (green) cells are absent from SSI epithelia but present in DSI preparations. In B and C, DAPI staining of nuclei can be seen in blue. Scale bars: 50 µm. The asterisk denotes significant difference in TER between SSI and DSI preparations as determined by Student's *t*-test.

tissues. Transcript encoding Cldn isoforms such as Cldn-8d and -27b have previously been reported to be particularly abundant in the gill tissue of species such as goldfish (Chasiotis and Kelly, 2012) and the puffer fish *Tetraodon nigroviridis* (Bagherie-Lachidan et al., 2009).

Flask-cultured gill epithelium model: establishment of confluence and temporal changes in TJ protein transcript abundance

When seeded into cell culture flasks, isolated gill epithelial cells attached to the solid substratum and after 24 h exhibited a patch-like morphology of cells that were loosely in contact (Fig. 2A). At 48 h very few gaps were present in the cell layer, but although many of the cells appeared to be confluent, cell-to-cell contact remained relatively loose in appearance (Fig. 2B). By 72 h, cells were fully confluent and began to exhibit definitive cell-to-cell boundaries which intensified over the remaining period of culture (Fig. 2C–F). As a consequence, by 72 h in culture (and onwards) the gill cells appeared as a mosaic-like epithelium sheet. This type of morphology is typical for many cultured vertebrate epithelia and overall, temporal differences in the appearance of cultured cells indicate that flask-cultured gill epithelia have two distinct stages of development: (1) attainment of confluence (i.e. cell-to-cell contact and establishment of polarity) during the first 48–72 h and (2) development of resistive properties (Freshney, 2005). During the first stage (i.e. first 72 h) epithelial cells establish a monolayer, and proliferation is likely to stop by contact inhibition (Freshney, 2005). Once the epithelial cell sheet is completely confluent, the second stage of developing resistive properties would begin, culminating in the attainment of a typical cobblestone mosaic pattern of cells with cell-to-cell borders clearly visible as dark lines. However, determining this by the measurement of epithelial resistance while cells are grown on a non-permeable solid substrate is not possible. Nevertheless, in addition to the observed changes in morphology, temporal alterations in TJ protein transcript abundance support the view that cells exhibit two stages of development in flask culture. Specifically, mRNA abundance of *cldn-3a*, *-7*, *-8b*, *-8d*, *-13*, *-23a*, *-27b*, *-28b*, *-30*, *ocln*, *cgn* and *zo-1* significantly increased in gill cells during the first 72 h of flask culture, indicating that they are important in establishing cell-to-cell contact in a gill epithelium, and thus significant in establishing gill epithelium integrity. In this regard, *cldn-3a*, *-7*, *-8d*, *-27b*, *-28b*, *-30*, *ocln* and *zo-1*, have already been suggested to enhance the barrier properties of the gill epithelium based on observed changes in mRNA and/or protein abundance in whole gill tissue following or during alterations in environmental conditions or upon exposure to permeability altering endocrine factors (reviewed by Chasiotis et al., 2012a). Indeed, it has also been shown that Ocln knockdown in a cultured gill epithelium model results in an increase in epithelium paracellular permeability (Chasiotis et al., 2012b). However, to the best of our knowledge there have been no studies that have examined a role for *cldn-13*, *cldn-23a* or *cgn* in gill epithelium integrity. Therefore, observations made in this study provide some evidence that these genes may be of significance in the development and maintenance of gill barrier properties. Nevertheless, elevated levels of only *cldn-8b*, *-8d*, *-27b* and *-28b* mRNA are maintained, whereas transcripts encoding Cldn-3a, -7, -23a, -30, Ocln, Cgn and ZO-1 drop back to levels equivalent to (or lower than) those observed 24 h after first seeding cells into flasks (Fig. 3). Therefore, although a reasonable number of TJ proteins exhibit elevated mRNA abundance during confluence formation in a model gill epithelium, it would appear that relatively few need to maintain elevated levels of transcript abundance as the model epithelium matures.

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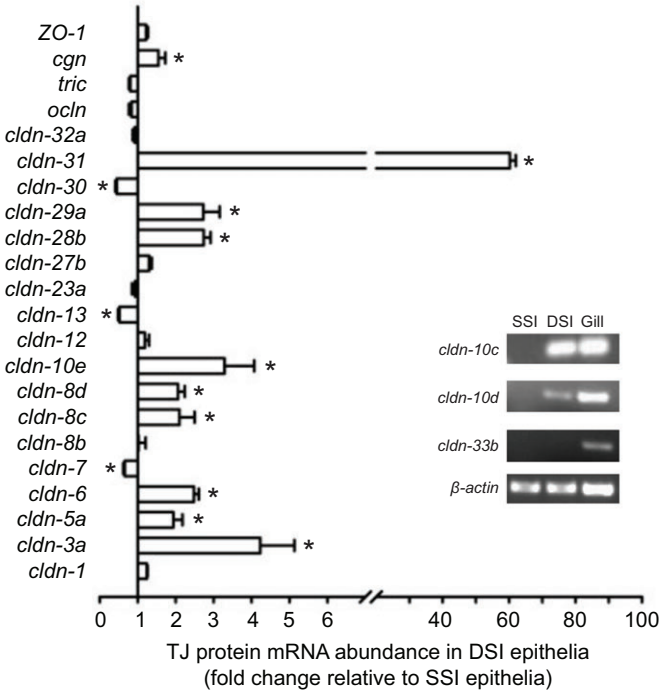

Fig. 9. Fold change in tight junction (TJ) protein mRNA abundance in rainbow trout gill cell double-seeded insert (DSI) preparations versus single-seeded insert (SSI) preparations. TJ protein mRNA abundance was normalized to that of EF-1α, and mRNA abundance of a TJ protein in DSI epithelia was expressed relative to the abundance measured in SSI preparations assigned a value of 1.0. Data are expressed as means ± s.e.m. (*N*=6). An asterisk denotes significant differences between SSI and DSI preparations. The inset shows images of agarose gel electrophoresis following RT-PCR amplification, to show the qualitative difference of a TJ protein transcript absent from SSI epithelia but present in DSI preparations. SSI, DSI and whole gill tissue sample were run side by side in the same gels.

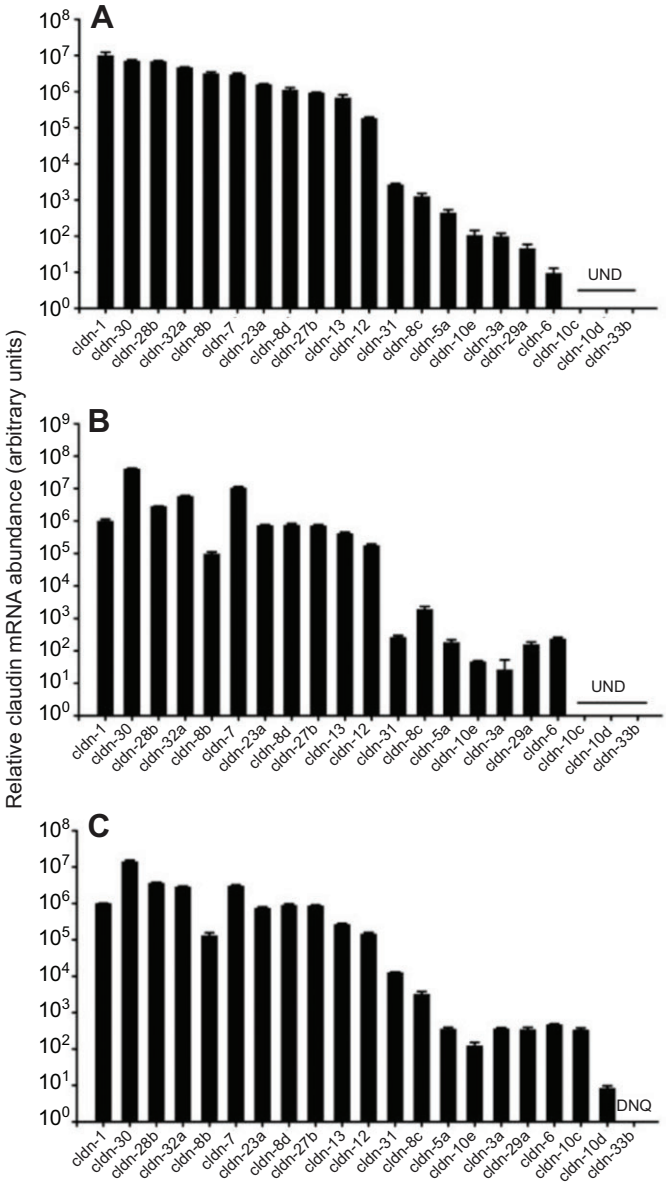

Fig. 10. Relative abundance of claudin (Cldn) TJ protein mRNA in different culture conditions. (A) Flask-cultured cells/epithelia, (B) single-seeded insert (SSI) epithelia and (C) double-seeded insert (DSI) preparations derived from rainbow trout gills. Note: *cldn-33b* was sporadically detected in DSI preparations, but was not quantifiable, and *cldn-10c* and *-10d* were detected in DSI preparations, but not in mature flask-cultured or SSI epithelia. mRNA abundance was normalized to that of EF-1α and expressed relative to *cldn-1*; mRNA abundance of claudins is expressed in arbitrary units. Data are expressed as means ± s.e.m. (*N*=6). UND, undetected; DNQ, detectable not quantifiable.

In contrast to the above, the mRNA abundance of several TJ proteins decreases over the flask culture period examined in this study. Specifically, the abundance of *cldn-5a*, *-8c*, *-10e*, *-29a*, *-31* and *-32a* mRNAs is lower at the end of the flask culture period than at 24 and/or 48 h after seeding into flasks (Fig. 3). In addition, transcripts encoding Cldn-10c, -10d and -33b were not detected at the end of the flask culture period (Fig. 3). Because flask-cultured epithelia are composed entirely of PVCs and *cldn-10c*, *-10d* and *-33b* are present in gill tissue of rainbow trout (see Fig. 1) it seems probable that these proteins are associated with other gill cell types. Transcript encoding Cldn-10d is also absent from cultured puffer fish (*Tetraodon nigroviridis*) gill epithelia composed entirely of PVCs (Bui et al., 2010; Bui and Kelly, 2011), and recently it has been shown that this Cldn is associated exclusively with gill ionocytes (Bui and Kelly, 2014). In puffer fish, Cldn-10e is also absent from primary cultured gill PVC epithelia and associated exclusively with ionocytes in gill tissue (Bui et al., 2010; Bui and Kelly, 2011). Therefore, it is possible that in trout gill tissue, *cldn-10c* and *-10d* may be associated with ionocytes and this would explain their absence in PVC epithelia in flask cultures. Indeed, transcript abundance of *cldn-10e* in flask-cultured trout gill PVC epithelia is detectable at the end of the 240 h experimental period, but only at very low levels. Therefore, the possibility that this gene is also strongly associated with gill ionocytes should not be ruled out. Further studies will be required to reveal why *cldn-5a*, *-8c*, *-29a*, *-31* and *32a* exhibit a decline in mRNA abundance over the flask culture period. In the case of *cldn-5a*, *-8c*, *-29a*, and to a lesser extent *cldn-31*, it is possible that they may be abundant in other gill cells, as their relative abundance in flask cells indicates comparatively low constitutive PVC expression (i.e. versus other *cldn* transcripts; see Fig. 10). In the zebrafish, *cldn-5a* has been associated with the development and maintenance of blood–brain and blood–retina barriers (Abdelilah-Seyfried, 2010). Therefore, the relative abundance of *cldn-5a* in trout gill tissue versus many other tissues (see Fig. 1) may be explained by the presence of higher levels of *cldn-5a* in the extensive vasculature of the gill. In contrast,

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cldn-32a remains one of the most plentiful in flask-cultured gill epithelial cells (see Fig. 10) despite a significant reduction in mRNA abundance during the culture period (Fig. 3). Notably, *cldn-32a* mRNA does not start to decline until at least 72 h following the introduction of cells into culture flasks, therefore it could still be playing an important role in establishing the barrier properties of the model gill epithelium, but once the epithelium is formed and subsequently begins to mature (i.e. tighten), a role for *cldn-32a* either diminishes or is significant only in its reduction (i.e. reduced levels of Cldn-32a contribute to a reduction in epithelial permeability).

Insert-cultured gill epithelium model: development of resistive properties and temporal changes in paracellular permeability and TJ protein transcript abundance

Flask-cultured epithelial cells that are seeded in cell culture inserts form a confluent epithelium by 24 h after their introduction. This can be observed when unattached material is rinsed out of the inserts at this time. Over time in culture, the TER of SSI preparations of trout gill epithelia exhibited a sigmoidal curve, which is consistent with previous observations of trout SSI epithelia (Wood and Part, 1997) and SSI preparations derived from other teleost fish species such as goldfish (Chasiotis and Kelly, 2011b) and tilapia (Kelly and Wood, 2002). During the first 72 h of insert culture there was a lag phase in development of SSI epithelia, which was followed by a log phase between 72 and 96 h, and a plateau phase from 96 h onwards (Fig. 5A). In conjunction with the lag phase of epithelial development (i.e. the first 72 h), [^{3}H]PEG-400 permeability decreased approximately one order of magnitude (Fig. 5B), and mRNA encoding Cldn-6, -8b, -27b, -28b, -29a, -31 and Ocln significantly increased. In the case of *cldn-8b*, *-27b*, *-28b* and *ocln*, significant elevation during the early period of SSI culture is consistent with the significant elevations observed during the early stages (i.e. first 72 h) of flask culture (see Fig. 3). These observations, together with our current knowledge of the aforementioned *cldn* genes in fishes (reviewed by Chasiotis et al., 2012a) as well as the observations made of flask-cultured cells (see previous discussion), reinforce the notion that *cldn-8b*, *-27b*, *-28b* and *ocln* play an important role in maintaining and/or enhancing the barrier properties and integrity of rainbow trout gills and possibly other teleost fish gill epithelia. Furthermore (and also consistent with observations of flask-cultured epithelia), *cldn-32a* mRNA abundance decreased in SSI preparations over the duration of the culture period, and this time it can be seen that reduced transcript abundance occurs in association with decreased [^{3}H]PEG-400 permeability (i.e. decreased paracellular permeability). In support of the idea that a reduction in *cldn-32a* occurs in association with reduced gill epithelium permeability and, therefore, may play a role in enhancing its barrier properties are observations of a cortisol-induced reduction in *cldn-32a* mRNA abundance in cultured PVC epithelia derived from the puffer fish (*Tetraodon nigroviridis*) (Bui et al., 2010). Cortisol has been documented to reduce the paracellular permeability of model gill epithelia derived from trout (Kelly and Wood, 2001; Wood et al., 2002; Chasiotis et al., 2010), tilapia (Kelly and Wood, 2002) and goldfish (Chasiotis and Kelly, 2011b). Therefore, these new observations, together with the limited distribution of *cldn-32a* (see Fig. 1) suggest that this TJ protein will be an interesting target for further study in fish gill epithelia.

As TER increases during the log phase of epithelial development and [^{3}H]PEG-400 permeability continues to decline (albeit at a reduced rate), *cldn-8d* and *-23a* mRNA significantly increase (Fig. 3). In SSI preparations, these are the only genes that exhibit this trend. As previously discussed, a barrier-forming role for Cldn-8d in the gill epithelium of fishes has already been proposed (see Chasiotis et al., 2012b), and the changes observed in SSI epithelia would be consistent with this. In addition, it is unclear what role Cldn-23a plays in the gill epithelium of fishes, but the observations of *cldn-23a* mRNA in SSI preparations would further support a barrier-forming role for this protein.

A number of TJ protein transcripts (i.e. *cldn-1*, *-3a*, *-7*, *13*, *-30*, *tric*, *cgn* and *zo-1*) did not alter in abundance over the SSI culture period, despite the changes in paracellular permeability. In the case of *cldn-30*, this is a curious observation because Cldn-30 has been proposed to be an important determinant in the maintenance of reduced gill epithelium permeability by virtue of the fact that significant changes in mRNA abundance have previously been associated with changes in environmental salinity (Tipsmark et al., 2008; Chasiotis and Kelly, 2011a; Chasiotis and Kelly, 2012; Chasiotis et al., 2012b) and corticosteroid-induced reductions in gill epithelium permeability (Chasiotis and Kelly, 2011a; Kelly and Chasiotis, 2011). Furthermore, the absence of *cldn-30d*/Cldn-30d in zebrafish (known as *cldn-b*) has been suggested to result in an increase in diffusive Na^+ loss across the integument (Kwong and Perry, 2013) and Cldn-30 has been proposed to reduce cation permeability across the gill epithelium of Atlantic salmon (Engelund et al., 2012). However, despite an apparent lack of contribution to changes in the permeability of primary cultured rainbow trout SSI epithelia, *cldn-30* is actually the most abundant *cldn* in this preparation (see Fig. 10). This suggests that *cldn-30* may assume a key role in the maintenance of gill epithelium integrity and that its modulation of ion movement, as observed in other systems, may be at least partly attributable to fundamental changes in epithelium integrity. This may also be the case for *tric*, which does not alter in SSI preparations despite substantial reductions in paracellular permeability. In this regard, it has recently been shown that removal of Tric from SSI preparations significantly compromises epithelial integrity and increases paracellular permeability (Kolosov and Kelly, 2013). The absence of any change in *cldn-1* transcript abundance in SSI preparations may not be surprising, despite its abundance in flask- and insert-cultured PVCs (see Fig. 10). This is because research using mammalian cell culture models has demonstrated repeatedly that *cldn-1* has low membrane mobility, indicating a low turnover rate, and, hence no need for large transcription rate changes (Cording et al., 2013).

Transcript encoding Cldn-10e decreased throughout SSI development and was the only *cldn-10* isoform examined in this study that was expressed in solid substrate preparations, albeit at a fraction of the level present in the gill. Previous studies have associated changes in *cldn-10e* mRNA abundance with acclimation to seawater (SW), smoltification and elevated levels of cortisol (Tipsmark et al., 2008; Bui et al., 2010; Bui and Kelly, 2011). In an SSI preparation derived from the gills of *T. nigroviridis*, *cldn-10e* was found to be absent (Bui et al., 2010), and it was subsequently shown that in gill tissue of this species Cldn-10e localizes exclusively to mitochondria-rich cells (Bui and Kelly, 2014). The presence of cldn-10e in rainbow trout PVCs therefore suggests that species-specific differences in the presence of 'ionocyte-specific' Cldn proteins may be expected, but that does not necessarily mean that a major role of proteins such as Cldn-10e may not be associated with ionocytes. For example, it is conceivable that the observed decrease in *cldn-10e* mRNA abundance in SSI preparations (and in flask culture) is due to the lack of MRC-associated binding partners, and thus a reduced need for *cldn-10e* transcription.

The abundance of *cldn-12* mRNA transiently decreased during the initial stages of epithelial development in SSI preparations, but returned to initial values quickly thereafter (Fig. 6). This also appeared to occur in flask-cultured cells (Fig. 3). Previous studies have suggested that Cldn-12 may be a barrier-forming protein in rainbow trout (Chasiotis and Kelly, 2011a; Kelly and Chasiotis, 2011), but there is no evidence to suggest that this may be the case in goldfish (Chasiotis and Kelly, 2011a; Chasiotis et al., 2012b). Assigning a specific role to Cldn-12 in the regulation of epithelial permeability is ambiguous as it has been associated with both 'leaky' and barrier-forming scenarios in different tissues (see Günzel and Fromm, 2012). Broad distribution of *cldn-12* in rainbow trout suggests that this TJ protein may also have tissue-specific roles in fishes.

A comparison of gill models that contain or do not contain MRCs

Cultured DSI epithelia included Na^+/K^+-ATPase (NKA)-rich cells (Fig. 8). Abundant NKA immunoreactivity is a characteristic typically exhibited by gill MRCs, which not only contain numerous mitochondria but also a rich complement of ionomotive enzymes (Perry, 1997). DSI epithelia also exhibited a greater TER and lower [^{3}H]PEG-400 permeability than SSI preparations (Fig. 8), and this is consistent with the observations of Fletcher et al., who first reported methods for the preparation of cultured DSI epithelia (Fletcher et al., 2000). The presence of MRCs in a cultured rainbow trout gill epithelium model resulted in a number of changes in TJ protein gene expression patterns as well as TJ protein mRNA abundance. One of the most significant observations was that once MRCs are introduced into the cultured gill epithelium, a 'return' of *cldn-10c*, *cldn-10d* and *cldn-33b* can be observed. All three of these genes are expressed in gill tissue but absent in model epithelia composed of PVCs only (see Fig. 9). As previously discussed, it seems very probable that *cldn-10c* and *-10d* may be associated exclusively with gill ionocytes as there is evidence to support the exclusive presence of Cldn-10 isoforms (i.e. Cldn-10d and -10e) in the MRCs of the euryhaline puffer fish *Tetraodon nigroviridis* (Bui and Kelly, 2014). It has been proposed that in gill tissue Cldn-10d and -10e are involved in SW-induced mechanisms of salt secretion because their abundance is greater in SW than in freshwater (FW), and CLDN-10 in mammalian epithelia has been reported to be pore-forming and selective for Na^+ movement (Breiderhoff et al., 2012; Günzel et al., 2009). Therefore, the association of Cldn-10 isoforms with gill MRCs (and *in vivo* possibly also accessory cells), together with the elevated levels of transcript and protein abundance that follow SW acclimation (see Bui et al., 2010; Bui and Kelly, 2014) may indicate their contribution to the organization of a gill Na^+ shunt, which is necessary for salt secretion in SW fishes.

At this stage in our understanding of TJ proteins in vertebrate epithelia, and in fish in particular, a rationale as to why *cldn-33b* expression occurs in association with the presence of MRCs and PVCs in DSI preparations but does not occur in PVC models is not entirely clear. This is mainly because *cldn-33b* is intermittently expressed and the transcript abundance is very low in DSI epithelia. It is tempting to suggest the expression of *cldn-33b* in DSI epithelia occurs because of the presence of MRCs, but *cldn-33b* is very abundant in gill tissue (see Fig. 9). Because the MRC:PVC ratio in DSI epithelia is approximately the same as that found in the gill (see Fletcher et al., 2000), the low abundance and intermittent presence of *cldn-33b* in an *in vitro* model that mirrors the *in vivo* MRC:PVC ratio seems inconsistent. Therefore, it is possible that *cldn-33b* is present in another type of cell from gill tissue, such as those of the vasculature or neuroepithelial cells. Because DSI epithelia are prepared by double seeding cells directly onto cell culture inserts, the second round of seeding may sporadically trap a very small number of other cell types in this preparation.

In DSI epithelia mRNA abundance of *cldn-7*, *-13* and *-30* was significantly reduced when compared with SSI epithelia. Because *cldn-7* and *-30* have been associated with barrier formation in gill epithelia (Chasiotis et al., 2012b), and DSI exhibit reduced paracellular permeability compared with SSI preparations, it seems unlikely that changes in mRNA abundance are a reflection of TJ permeability. Instead, there are two possible explanations for this phenomenon that relate to cell composition differences. First, *cldn-7*, *-13* and *-30* may be strongly associated with PVCs (i.e. MRCs either lack or have reduced abundance of *cldn-7*, *-13* and *-30* mRNA) and because SSI preparations consist exclusively of PVCs, the introduction of MRCs decreases mRNA abundance accordingly. Second, *cldn-7*, *-13* and *-30* are strongly associated with PVC-to-PVC TJs, and even though transcripts of these claudins are present in MRCs, addition of MRCs to the preparation reduces the number of PVC-to-PVC TJs and thus reduces the measured transcript abundance. In isolated goldfish gill cells, transcript abundance of *cldn-B* (a *cldn-30* homologue) but not of *cldn-7*, has previously been shown to be higher in PVCs than in MRCs (Chasiotis et al., 2012b). In addition, Cldn-30 has been suggested to have a limited association with MRCs in the gill epithelium of Atlantic salmon (Engelund et al., 2012). However, cldn-13 has been reported to be present in puffer fish PVCs (Bui and Kelly, 2011), but there are currently no studies that indicate either a barrier or pore-forming role of Cldn-13 in fishes or provide insight into its presence in MRCs.

When comparing SSI and DSI epithelia, a significant elevation of *cldn-3a*, *-5a*, *-6*, *-8c*, *-8d*, *-10e*, *-28b*, *-29a*, *-31* and *cng* mRNA abundance was observed (Fig. 9). With regard to *cldn-6*, *-8d*, *-28b* and *cgn*, an increase in mRNA abundance in DSI versus SSI epithelia is difficult to delineate from differences observed between the permeability of the two preparations because transcripts encoding these proteins increased in accordance with reduced permeability in PVC-only preparations. In contrast, *cldn-3a*, *-5a*, *-10e*, *-29a* and *-31* levels either did not alter in association with changes in permeability of PVC-only preparations or in fact decreased. Therefore, the differences observed between these genes in SSI versus DSI epithelia seem more likely to relate to differences in cell composition. In the case of *cldn-10e*, MRCs are likely to possess a rich complement of this TJ isoform, as has been shown in other fish species (Bui and Kelly, 2014). Furthermore, an ~60-fold increase in *cldn-31* strongly supports the idea that this *cldn* is highly expressed in MRCs, although it is not absent from PVCs. This may also be the case for *cldn-3a*, the abundance of which has been reported to be higher in MRCs than in PVCs isolated from goldfish gills (Chasiotis et al., 2012b). Indeed, *cdn-8d* mRNA abundance was also found to be higher in isolated goldfish MRCs than PVCs (Chasiotis et al., 2012b), so there is some evidence to suggest that elevated *cldn-8d* levels in DSI epithelia could at least be partly attributable to the presence of MRCs. However, cldn-29a did not exhibit any difference in mRNA abundance between goldfish PVCs and MRCs (Chasiotis et al., 2012b), therefore the increase observed in the current study in DSI preparations will require further examination.

Conclusion and perspectives

This study provides a first look at the expression of 23 TJ protein genes in discrete rainbow trout tissues, and the gene expression profiles of 25 TJ proteins in primary cultured model gill epithelia.

Moreover, changes in the transcript abundance of TJ proteins have been examined during the development of primary cultured model gill epithelia, thus permitting insight into the importance of these proteins in the formation, development and integrity of gill epithelia. The teleost fish gill epithelium is a complex tissue that is simultaneously involved in respiration, iono/osmoregulation, acid–base balance and nitrogenous waste excretion (Evans et al., 2005). Therefore, it plays a central role in the physiology of fishes and this provides an impetus to broadly understand how it functions and to develop and characterize models that allow us to study its function *in vitro*. The primary cultured gill epithelium models discussed in this study have recently proved to be useful for studying the paracellular permeability properties of gill epithelia (Chasiotis et al., 2012a). Therefore, a more expansive characterization of the molecular physiology of the TJ complex of these models was warranted. This study now provides a foundation upon which to build future studies that not only continue to explore the permeability properties of these models, but also how the permeability characteristics and integrity of model epithelia are affected when they are used to examine other aspects of gill function (e.g. the effects of xenobiotics etc.). Moreover, the current study also reveals how useful these models can be to delineate the potential presence or absence of specific TJ proteins in select gill cell types. The idea that transepithelial transport proteins in the gill epithelium of fishes are heterogeneously distributed in association with different cell types has been broadly recognized for over 40 years by those interested in the function of transcellular transport proteins and the transcellular pathway. In contrast, the idea that paracellular transport proteins, such as TJ proteins, might be heterogeneously distributed in the cells of the gill epithelium has only recently come to light, and the current study shows that models that will help us to further explore this heterogeneity are available. Therefore, there is great potential for the development of knowledge in this realm.

MATERIALS AND METHODS

Experimental animals

Rainbow trout (*Oncorhynchus mykiss* Walbaum 1792) were obtained from a local supplier (Humber Springs Trout Hatchery, Orangeville, ON, Canada), and held under a constant photoperiod (12 h:12 h light:dark) in 600 l opaque polyethylene tanks supplied with flow-through dechlorinated fresh water (FW; approximate composition in μmol l^{-1}: Na^+ 590, Cl^- 920, Ca^{2+} 760, K^+ 43; pH 7.35). Fish were fed *ad libitum* once daily with commercial trout pellets (Martin Profishent, Elmira, ON, Canada). Animal husbandry and experimental procedures were conducted in accordance with an approved York University Animal Care Committee protocol, which conformed to the guidelines of the Canadian Council on Animal Care.

TJ protein gene identification and primer design

Previously published primer sequences for rainbow trout *ocln*, *tric*, *zo-1*, *cldn-3a*, *-7*, *-8d*, *-12*, *-28b*, *-30*, *-31* and *-32a* were used (see Chasiotis et al., 2010; Kelly and Chasiotis, 2011; Kolosov and Kelly, 2013). Expressed sequence tags (ESTs) from the rainbow trout genome similar to those of *Takifugu rubripes cldn -1*, *-5a*, *-6*, *-8b*, *-8c*, *-10c*, *-10d*, *-10e*, *-13*, *-23a*, *-27b*, *-29a* and *-33b* were sought using the NCBI database BLAST search engine. ESTs similar to those of *Danio rerio cingulin b* (GenBank accession no. NM_001044762) were also sought. Newly identified ESTs were confirmed to be protein encoding using a reverse BLASTχ. A reading frame was established using nBLAST alignment and ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/). Primers were designed based on the predicted *cldn*-encoding (or *cgn*-encoding) EST sequence using Primer3 software (v. 0.4.0) (Table 1). Putative *cldn* fragments were amplified using reverse transcriptase PCR (RT-PCR). Amplicon sizes were verified using agarose gel electrophoresis. Amplicons were then isolated and purified for sequencing using a QIAquick gel extraction kit (Qiagen Inc., Mississauga, ON, Canada). Sequencing was conducted at the York University Core Molecular Biology Facility (Department of Biology, York University, ON, Canada). Sequence identity for each amplicon was confirmed using a BLAST search. ClustalX software was used to align and assemble multiple sequenced segments. Full length or partial coding sequences were submitted to GenBank (see Table 1).

RNA extraction, cDNA synthesis and qRT-PCR

Total RNA was isolated from trout tissues using TRIzol Reagent® (Invitrogen Canada Inc., Burlington, ON, Canada) according to manufacturer's instructions. RNA concentration was determined using a Multiskan Spectrum UV/Vis microplate spectrophotometer (Thermo Fisher Scientific, Nepean, ON, Canada). A fixed quantity of RNA (2 μg) was treated with DNase I (Amplifications Grade, Invitrogen Canada, Inc.) and used for cDNA synthesis. First-strand cDNA was synthesized using SuperScript™ III reverse transcriptase and Oligo(dT)$^{12–18}$ primers (Invitrogen Canada, Inc.).

Expression profiles of TJ protein mRNA and qRT-PCR

Rainbow trout were net-captured, anaesthetized in 0.5 g l^{-1} tricaine methasulfonate (MS-222; Syndel Laboratories Ltd, Qualicum Beach, BC, Canada) and killed by spinal transection. Discrete tissues were removed, quick frozen in liquid nitrogen and stored at –80°C until further analysis. The following tissues were examined: brain, eye, gill, esophagus, pyloric caeca, anterior intestine, middle intestine, posterior intestine, kidney and skin. TJ protein transcript presence and abundance were determined by quantitative real-time PCR (qRT-PCR) using SYBR Green I Supermix (Bio-Rad Laboratories Canada Ltd, Mississauga, ON, Canada), a Chromo4™ Detection System (CFB-3240; Bio-Rad Laboratories Canada Ltd) and primers as outlined in Table 1. The following reaction conditions were used: 1 cycle for denaturation (95°C, 4 min), followed by 40 cycles of: denaturation (95°C, 30 s), annealing (see Table 1, 30 s) and extension (72°C, 30 s). To ensure that a single PCR product was synthesized during reactions, a dissociation curve analysis was carried out after each qRT-PCR run. Transcript abundance was normalized to that of rainbow trout β-actin (*actb*; see Table 1). The use of *actb* for gene of interest normalization in expression profile studies was validated by statistically comparing *actb* threshold cycle values between tissues to confirm that no statistically significant changes occurred (P=0.176).

Preparations of primary reconstructed rainbow trout gill epithelia

Primary cultured gill epithelia were generated using methods originally reported by Wood and Pärt (Wood and Pärt, 1997) and Fletcher et al. (Fletcher et al., 2000). The methodology of Wood and Pärt (Wood and Pärt, 1997) was used to generate flask- and insert-cultured gill epithelium preparations composed of PVCs only. The latter is referred to as a single-seeded insert (SSI) preparation. The methodology of Fletcher et al. allowed the preparation of insert-cultured gill epithelia that were composed of both PVCs and MRCs, and these are referred to as double seeded insert (DSI) preparations (Fletcher et al., 2000). Detailed description of the methods used to generate flask-cultured cells, as well as SSI and DSI preparations can be found in Kelly et al. (Kelly et al., 2000). Cell culture flasks and inserts were held in an air atmosphere at 18°C. Flask-cultured epithelia were allowed to develop for 10 days. They were harvested for RNA extraction at 24, 48, 72, 120, 192 and 240 h post-seeding.

SSI and DSI preparations were cultured in polyethylene terephthalate filter inserts (0.9 cm^2 growth area, 0.4 μm pore size, $1.6×10^6$ pores cm^{-2} pore density; BD Falcon TM, BD Biosciences, Mississauga, ON, Canada). Inserts were housed in companion cell culture plates (Falcon BD) and the apical and basolateral compartments of the culture system contained L15 medium. Transepithelial resistance (TER) was monitored in SSI and DSI preparations at the times indicated following cell seeding into inserts. TER was measured using chopstick electrodes (STX-2) connected to a custom-modified EVOM epithelial voltohmmeter (World Precision Instruments, Sarasota, FL, USA) and corrected by subtracting TER measured across a blank insert. All TER measurements are expressed as Ω cm^2. For SSI preparations, [^{3}H]PEG-400 (American Radio Chemicals, St Louis, MO, USA) flux was measured prior

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Table 1. Primer sets, PCR annealing temperatures and amplicon size, and gene accession numbers for rainbow trout tight junction proteins and elongation factor-1

Tight junction protein	Gene	Primer sequence (5′–3′)	Annealing temperature (°C)	Amplicon size (bp)	Accession number
Cingulin	*cgn*	F: CTGGAGGAGAGGCTACACAG R: CTTCACACGCAGGGACAG	56	156	BK008767
Zonula occludens-1[a]	*zo-1*	F: AAGGAAGGTCTGGAGGAAGG R: CAGCTTGCCGTTGTAGAGG	60	291	HQ656020
Occludin[b]	*ocln*	F: CAGCCCAGTTCCTCCAGTAG R: GCTCATCCAGCTCTCTGTCC	58	341	GQ476574
Tricellulin[c]	*tric*	F: GTCACATCCCCAAACCAGTC R: GTCCAGCTCGTCAAACTTCC	60	170	KC603902
Claudin-1	*cldn-1*	F: GAGGACCAGGAGAAGAAGG R: AGCCCCAACCTACGAAC	60	182	BK008768
Claudin-3a[a]	*cldn-3a*	F: TGGATCATTGCCATCGTGTC R: GCCTCGTCCTCAATACAGTTGG	60	285	BK007964
Claudin-5a	*cldn-5a*	F: CTCACCGTCATCTCGTGTG R: CATCCAGCAGAGGGGAAC	59	171	BK008769
Claudin-6	*cldn-6*	F: TGAAACCACGGGACAGATG R: TGAAACCACGGGACAGATG	60	245	KF445436
Claudin-7[a]	*cldn-7*	F: CGTCCTGCTGATTGGATCTC R: CAAACGTACTCCTTGCTGCTG	61	261	BK007965
Claudin-8b	*cldn-8b*	F: ACGACTCCCTCCTGGCTCT R: GAGACCCATCCGATGTAGA	56	185	BK008770
Claudin-8c	*cldn-8c*	F: GCTTGATGTGCTGCTCTC R: CCCAGAGGTCAGGAGGA	60	201	BK008771
Claudin-8d[a]	*cldn-8d*	F: GCAGTGTAAAGTGTACGACTCTCTG R: CACGAGGAACAGGCATCC	60	200	BK007966
Claudin-10c	*cldn-10c*	F: CCTGGTCTGCTCTACAATGC R: CCCGAAGAATCCCAAAATAA	58	223	BK008772
Claudin-10d	*cldn-10d*	F: CTGCTTTGTGTCGTGTCTGG R: AGCGAAGAACCCAAGGATG	60	253	BK008773
Claudin-10e	*cldn-10e*	F: CTGTCACCAACTGCCAAGAC R: CCAGAAAGCCACTGATGATG	54	216	KF445437
Claudin-12[a]	*cldn-12*	F: CTTCATCATCGCCTTCATCTC R: GAGCCAAACAGTAGCCCAGTAG	60	255	BK007967
Claudin-13	*cldn-13*	F: AGCGGCACTCTGGACAA R: CGGAAACCACACCTCTCC	59	231	BK008774
Claudin-23a	*cldn-23a*	F: ATCCTAAACCTCACAGCGACA R: CGGTCTTTCCAGCACCTTAC	60	270	BK008775
Claudin-27b	*cldn-27b*	F: GCCAACATCGTAACAGGACA R: CCAGAAGAGCACCAATGAGC	60	283	BK008776
Claudin-28b[a]	*cldn-28b*	F: CTTTCATCGGAGCCAACATC R: CAGACAGGGACCAGAACCAG	60	310	EU921670
Claudin-29a	*cldn-29a*	F: CTTTCATCGGCAATAACATC R: CAGCAATGGAGAGCAGG	60	201	BK008777
Claudin-30[a]	*cldn-30*	F: CGGCGAGAACATAATCACAG R: GGGATGAGACACAGGATGC	59	297	BK007968
Claudin-31[a]	*cldn-31*	F: TCGGCAACAACATCGTGAC R: CGTCCAGCAGATAGGAACCAG	61	311	BK007969
Claudin-32a[a]	*cldn-32a*	F: ATTGTGTGCTGTGCCATCC R: AGACACCAACAGAGCGATCC	60	321	BK007970
Claudin-33b	*cldn-33b*	F: GTCCACAGACCTCTTGCTCAC R: TCCTGCCACCTTCATAATCC	60	268	BK008778
Beta-actin[a]	*actb*	F: GGACTTTGAGCAGGAGATGG R: GACGGAGTATTTACGCTCTGG	58	355	AF157514
Elongation factor-1α	*ef-1a*	F: GGCAAGTCAACCACCACAG R: GATACCACGCTCCCTCTCAG	60	159	AF498320.1

F, forward; R, reverse.
[a]Kelly and Chasiotis, 2011; [b]Chasiotis et al., 2010; [c]Kolosov and Kelly, 2013.

to harvesting cells for RNA extraction at 24, 48, 72, 80, 96 and 108 h following seeding. [^{3}H]PEG-400 flux was determined according to calculations reported by Wood et al. (Wood et al., 1998).

Microscopy and immunocytochemistry

The development of confluence exhibited by flask-cultured gill cells was monitored using an inverted microscope coupled to an Olympus digital camera (Olympus, Markham, ON, Canada). Brightfield phase-contrast microscopy was used to capture images of growing epithelia at 24, 48, 72, 120, 192 and 240 h post-seeding. Procedures for the immunolocalization of Na^+/K^+-ATPase (NKA) in cultured gill epithelia were conducted as described previously (Chasiotis and Kelly, 2011b). In brief, cultured gill epithelia were fixed with 3% paraformaldehyde, permeabilized with ice-cold methanol, washed with Triton X-100 in PBS and blocked with antibody-dilution buffer (ADB). ADB was prepared according to the reference cited above (10% goat serum, 3% BSA and 0.05% Triton X-100 in PBS). NKA

α-subunit antibody (α5; Developmental Studies Hybridoma Bank, Iowa City, IA, USA) was used to examine NKA immunoreactivity. After washing with PBS, preparations were incubated with FITC-labeled goat anti-mouse antibodies (1:500 in ADB; Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA). After incubation with primary and secondary antibodies, epithelia were mounted on slides using mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI). Images were captured using differential interference contrast (DIC) and laser-scanning confocal microscopy with an Olympus BX-51 microscope 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.

Statistical analyses

All data are expressed as means ± s.e.m. (N), where N represents the number of fish or, when cultured gill epithelia were used, the number of cell culture flasks or cell culture inserts. Either a Student's t-test or one-way ANOVA followed by a Student–Newman–Keuls test was used to determine significant differences ($P<0.05$) between groups as appropriate. All statistical analyses were conducted using SigmaStat 3.5 software (Systat Software Inc., San Jose, CA, USA).

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Competing interests

The authors declare no competing financial interests.

Author contributions

D.K. and S.P.K. contributed to the study conception and experimental design and execution, as well as drafting and revising the article. H.C. contributed to study execution, interpretation of findings, and drafting/revising the article.

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