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Functional dynamics of claudin expression in Japanese medaka (*Oryzias latipes*): Response to environmental salinity

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ABSTRACT

Salinity regulation of 13 claudin paralogs was investigated in osmoregulatory organs of euryhaline Japanese medaka. They were identified by blast-search in the medaka genome database based on representation in osmoregulatory organs of other teleosts. Our hypothesis was that, because of their sequence similarities to mammalian orthologs previously characterized as barrier- and ion-selective channel-forming proteins, these paralogs would respond to salinity according to expected modulation of osmoregulatory function. *Cldn10c*, *-10d*, *-10e*, *-10f*, *-27a*, *-28a*, *-28b* and *-30c* had 4- to 100-fold higher expression in gill than other examined organs. Two splice variants of *cldn10b* were predominantly expressed in kidney, while *cldn15a*, *-15b* and *-25* were found mainly in intestine. In gills, *cldn27a*, *-28a*, *-28b* and *-30c* did not change between fresh water (FW) and seawater (SW)-acclimated fish, while *cldn10c*, *-10d*, *-10e*, and *-10f* were most abundant in SW. Short-term SW transfer induced up-regulation of *cldn10* gill paralogs after 1 day, decrease in *cldn28b* and no difference for *cldn27a*, *-28a* and *-30c*. The reverse pattern was observed after FW transfer of SW medaka. Intestinal *cldn15a* and *-25* did not differ between FW and SW fish. However, *cldn15b* was 10-fold higher in FW than SW, suggesting a role in functional modulation of the intestine related to water and salt transport. In kidney, *cldn10bs* were elevated in SW fish, suggesting a role in paracellular ion transport in the marine nephron. Based on in silico analysis, most gill Cldn10s were predicted to form cation pores, whereas Cldn27a, 28a, 28b and 30c may increase epithelial resistance.

1. Introduction

Euryhaline fishes generally keep their internal osmotic concentration at about the same level (300–350 mOsm kg^{-1}) after acclimating to fresh water (FW: about 1–10 mOsm kg^{-1}) or seawater (SW; about 1050 mOsm kg^{-1}; Watts et al., 2001; Evans et al., 2005; Brauner et al., 2013) — a feat accomplished by the concerted action of osmoregulatory organs, mainly the gill, intestine and kidney. Tight junction (TJ) strands make up the apico-lateral barrier, or gate, in between epithelial cells and regulate paracellular solute and water movement (Van Itallie and Anderson, 2006). In the gill, TJs between adjacent pavement cells (PVCs) and mitochondrion-rich cells (MRCs) are deep with multi-stranded connections (Sardet et al., 1979). In SW, shallow TJs connect neighboring MRCs and MRCs with adjacent accessory cells (ACs) suggesting a "leaky" paracellular pathway between these cells (Sardet et al., 1979). In the marine environment, branchial NaCl secretion involves transcellular chloride transport basolaterally by Na^+,K^+,$2Cl^-$ co-transport and apically via cystic fibrosis transmembrane conductance regulator anion channel. This transport is secondarily coupled to the activity of Na^+,K^+-ATPase in the extensive tubular network of the MRCs (Karnaky et al., 1976). This anion transport is thought to generate a serosal positive transepithelial potential triggering sodium extrusion via a paracellular pathway (Evans et al., 2005; Edwards and Marshall, 2012). Passive uptake of water in the gut driven by active ion uptake compensates for osmotic water loss, while the kidney's main function in SW appears to be the excretion of excess divalent ions (Flik et al., 1996; Varsamos et al., 2004; Marshall and Grosell, 2006). In FW, fish are potentially subject to extensive ion loss and osmotic water gain. The gill epithelium has no MRC complexes or ACs and is generally considered as tight with a higher electrical resistance (Sardet et al., 1979; Wilson and Laurent, 2002). The branchial epithelium actively absorbs ions and the intestinal tract also allows for some ion uptake (mainly from food uptake; Marshall and Grosell, 2006). Meanwhile, the water load is countered by renal production of a large volume of hypotonic urine (Hickman and Trump, 1969).

TJs are composed of proteins like claudins (Cldns), occludin, junctional adhesion molecules and tricellulin. All have four transmembrane spanning domains, two extracellular loops (ECLs), and a cytosolic carboxy-terminal linked to the cytoskeleton through adaptor molecules like those from the zonula occludens family (Gonzalez-Mariscal et al., 2008). Proteins belonging to the Cldn super-family

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determine the specific permeability and ion selectivity of TJs, as demonstrated by over-expression of exogenous Cldns in epithelial cell cultures (Furuse et al., 1998; Van Itallie et al., 2001). Furthermore, site-directed mutageneses have shown that the specific paracellular ion movement is influenced by charged amino acids (aas) of the first ECL (ECL1) and intercellular homo- or heterotypic Cldn–Cldn interaction (Colegio et al., 2003; Angelow et al., 2008). In this way, they form permeability-selective barriers critical to the function of a specific tissue. While 27 *cldn* genes have been described in mammals (Günzel and Yu, 2013), genomic and tandem gene duplication events have contributed to an expanded Cldn family in teleost including 54–56 different genes (Loh et al., 2004; Baltzegar et al., 2013). Cldns are often developmentally regulated and expressed in a tissue- and cell-specific manner (Loh et al., 2004; Tipsmark et al., 2008b).

In teleost gills, SW-acclimation induces elevated expression in *cldn10d* and *cldn10e* (Tipsmark et al., 2008b; Bui et al., 2010). These paralogs are interesting candidates potentially involved in the formation of a cation-selective pathway associated with Na^+ extrusion in SW, especially since the homolog in mouse was shown to form cation-selective pores (Cldn-10; Günzel et al., 2009). Mouse Cldn4 appears to share a common ancestor with 13 teleost Cldns (Baltzegar et al., 2013) of which a number are highly expressed in the gill (*cldn27a*, *cldn28a-b*, *cldn30c*) in FW or in both FW and SW (Tipsmark et al., 2008a, 2008b). Of these, Cldn30c has been shown to be involved in tightening of fish epithelia (Engelund et al., 2012), similar to the barrier-forming mammalian homologs (Van Itallie et al., 2001; Yu et al., 2003).

The Japanese medaka (*Oryzias latipes*) is a euryhaline teleost of the ricefish family. This fish species generally lives in FW (Sakamoto et al., 2001), but is able to acclimate to full strength seawater (Inoue and Takei, 2002, 2003; Kang et al., 2008). Its euryhaline capacity, combined with an evolutionary history diverging from the well-studied stenohaline zebrafish (*Danio rerio*), makes this species a particularly good model to study osmoregulation. Japanese medaka has the advantage of being easy to rear. Furthermore, its genome is fully sequenced and annotated, and relatively small compared to other teleosts (Tanaka, 1995). Medaka easily acclimates to salinity challenges in a manner similar to other euryhaline teleost and a recent study revealed the existence of three types of branchial ionocytes in FW and one in SW (Hsu et al., 2014). However to date, no information is available on the functional expression of TJ proteins in this species.

The objectives of the present study were first to identify Cldn paralogs involved in regulation of paracellular permeability in osmoregulatory organs of Japanese medaka, with special focus on the gill. Our driving hypothesis was that fish paralogs sharing sequence similarity in the ECL1 of mammalian orthologs with barrier or ion-selective properties would respond to salinity according to the expected changes in osmoregulatory function. Based on their representation in the gill, kidney and intestine of other teleosts, 13 paralogs were pinpointed for this study. The chosen paralogs' nomenclature was examined using phylogenetic analysis and their functional properties predicted based on in silico analysis. This was followed by determination of organ distribution and expression patterns in osmoregulatory organs of fully FW- or SW-acclimated fish. Expression of *cldns* with high gill levels was examined during the initial stages of salinity acclimation. This was done in two transfer experiments (from FW to SW, or SW to FW) with sampling after 1 and 3 days.

2. Materials and methods

2.1. Animals

Adult Japanese medaka (*O. latipes*, Temminck & Schlegel; size range: 25–35 mm, 250–350 mg) were obtained from the Aquatic Research Organisms (Hampton, NH, USA). They were maintained in 150 L tanks filled with aerated de-chlorinated tap water, mechanically and biologically filtered (0.34 mM Na^+, 0.64 mM Ca^{2+}, 0.09 mM Mg^{2+}, 0.03 mM K^+). They were kept under a 14 h light/10 h dark photoperiod, at 20 °C. Fish were fed 3 times per day with Tetramin Tropical flakes (Tetra, United Pet Group, Blacksburg, VA, USA) or frozen brine shrimp (San Francisco Bay Brand, Inc., Newark, CA, USA). Food was withheld during the short-term salinity transfer experiments from one day before and throughout the experiment. All experiments and handling procedures were approved by the Animal Care and Use Committee of the University of Arkansas (IACUC protocol number 11005).

2.2. Experiments and sampling

Three experiments were performed in order to examine the chosen *cldns* organ distribution and response to salinity. One experiment applied long-term FW- and SW-acclimated (28 ppt) fish to determine the organ levels of medaka *cldns* at the two conditions. Fish were acclimated for one month at their respective salinity prior to sampling. They were anesthetized using 100 mg L^{-1} of tricaine methanesulfonate (MS-222, buffered with $NaHCO_3$ for FW) and then killed by cervical dislocation. The gill, kidney, intestine, muscle and liver were dissected, immediately frozen on dry-ice and stored at −80 °C before further use. Samples of each organ from FW were used in order to determine the distribution of claudins ($N = 6$). Samples from the osmoregulatory organs (gill, kidney and intestine) were used to determine *cldn* expression according to salinity ($N = 6$).

The initial gill response to salinity was examined in two short-term transfer experiments: fishes long-term acclimated for at least one month were transferred from FW to SW or SW to FW and sampled after 1 and 3 days. As sham controls, fish were transferred from FW to FW or SW to SW.

2.3. Claudin nucleotide sequences, alignment and phylogenetic analysis

The claudin sequences in medaka were identified by BLAST search against known claudins of tiger pufferfish, *Takifugu rubripes* (*Fugu*), using the GenBank data-base (Table 1). The phylogenetic tree construction was based on maximum-likelihood analysis of putative aa sequences of medaka (Me), *T. rubripes* (Fu) and mammalian (house mouse, *Mus musculus*, Mo), and as out-group the clarin1 from mouse (Table 2). The maximum likelihood consensus tree was generated using SEQBOOT, PROML and CONSENSE, all programs in the PHYLIP package (Felsenstein, 1989). A neighbor joining consensus tree was generated using SEQBOOT, PROTDIST, NEIGHBOR and CONSENSE also programs in the PHYLIP package. Protein sequences were aligned using ClustalW2 pairwise alignment algorithm.

2.4. RNA isolation, cDNA synthesis and quantitative PCR (qPCR)

Organs were homogenized in Tri reagent (Sigma-Aldrich, St. Louis, MO, USA) using a VWR PowerMax 200 rotating knife homogenizer

Table 1
Quantitative PCR primers used to detect *cldn* transcript levels in Japanese medaka (*Oryzias latipes*).

Target name	NCBI acc. no.	Forward primer	Reverse primer
Me-cldn10b1	XM_004081778.1	gagatcgtggctttcgttgt	tcattggacgacactttcca
Me-cldn10b2	XM_004081779.1	cgccattattggtgctacag	ggcatttccagcacagtttt
Me-cldn10c	XM_004081941.1	cggatcaattttggtcttgg	gcgcagattcctcctacaag
Me-cldn10d	XM_004066669.1	ccaccgccaactactactcc	atgcaggaacaccggtagag
Me-cldn10e	XM_004066670.1	caaagtggcctggtactggt	tggatgttgaggtccacaga
Me-cldn10f	XM_004081942.1	atcaaggtggcctggtactg	gagaaccgcgtaatctctgc
Me-cldn15a	XM_004079825.1	tgtcctcaaagggaggattg	tgatgttgaaggcgtaccag
Me-cldn15b	XM_004076466.1	caacatcacgcagcagttct	aaacctccacagatggcaag
Me-cldn25	XM_004078759.1	caagaagggcggatactttg	aggctctggatgactctgga
Me-cldn27a	XM_004075237.1	catgtgcatcatctccatcc	gctttggacttctcgtggtc
Me-cldn28a	XM_004075235.1	accgaaagcccagagtatcc	ggccgctattgtaggacttg
Me-cldn28b	XM_004076181.1	ggtcacagccttcattggat	tgcatctgccctgtactctg
Me-cldn30c	XM_004076176.1	cagccctctcctgagtaacg	ctccaatcagcatcagagca