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Prolactin regulates transcription of the ion uptake Na^+/Cl^- cotransporter (*ncc*) gene in zebrafish gill

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ABSTRACT

Prolactin (PRL) is a well-known regulator of ion and water transport within osmoregulatory tissues across vertebrate species, yet how PRL acts on some of its target tissues remains poorly understood. Using zebrafish as a model, we show that ionocytes in the gill directly respond to systemic PRL to regulate mechanisms of ion uptake. Ion-poor conditions led to increases in the expression of PRL receptor (*prlra*), Na^+/Cl^- cotransporter (*ncc*; *slc12a10.2*), Na^+/H^+ exchanger (*nhe3b*; *slc9a3.2*), and epithelial Ca^{2+} channel (*ecac*; *trpv6*) transcripts within the gill. Intraperitoneal injection of ovine PRL (oPRL) increased *ncc* and *prlra* transcripts, but did not affect *nhe3b* or *ecac*. Consistent with direct PRL action in the gill, addition of oPRL to cultured gill filaments stimulated *ncc* in a concentration-dependent manner, an effect blocked by a pure human PRL receptor antagonist (Δ1-9-G129R-hPRL). These results suggest that PRL signaling through PRL receptors in the gill regulates the expression of *ncc*, thereby linking this pituitary hormone with an effector of Cl^- uptake in zebrafish for the first time.

1. Introduction

It has long been recognized that the endocrine system plays a central role in the homeostatic regulation of salt and water balance in vertebrates (McCormick and Bradshaw, 2006). Among the pituitary hormones, prolactin (PRL) has received considerable attention as an osmoregulatory hormone with conserved actions across vertebrate groups. In mammals, PRL influences solute and water transport across renal, intestinal, mammary and amniotic epithelial membranes (Bole-Feysot et al., 1998; Freeman et al., 2000). In teleost fishes, PRL is recognized as an important "freshwater-adapting hormone" regulating osmoregulatory functions within the gill, kidney and gastrointestinal tract by promoting ion conserving and water secreting processes (Hirano, 1986; Sakamoto and McCormick, 2006). The gill possesses a rich population of ionocytes that are capable of active Na^+, Cl^-, and Ca^{2+} uptake needed to support hydromineral balance in freshwater environments that are hyposmotic to body fluids (Kaneko et al., 2008). Varying and contending models have been proposed for the cellular mechanisms that regulate branchial ion-uptake in fresh water (Evans, 2011), and this incomplete understanding of the molecular mechanisms driving ion uptake in freshwater ionocytes has impeded progress in understanding how PRL promotes acclimation to freshwater environments.

The zebrafish (*Danio rerio*) has emerged as a new model organism for studying vertebrate physiology and is particularly well suited to detailed mechanistic and comparative studies of developmental endocrinology (McGonnell and Fowkes, 2006; Löhr and Hammerschmidt, 2011) and osmoregulation (Hwang, 2009). Zebrafish are regarded as being stenohaline and are naturally distributed in soft-water rivers and streams of the Indian subcontinent. Adult zebrafish can rapidly adapt to ion-poor conditions and can survive in deionized water for extended periods (Craig et al., 2007; Boisen et al., 2003). To persist in ion-poor waters, zebrafish have high capacity for Na^+ and Cl^- uptake (Boisen et al., 2003), despite strong opposing electrochemical gradients across gill epithelium. This capacity for ion-uptake, along with genetic and experimental accessibility, makes zebrafish particularly useful for studies aimed at elucidating how the endocrine system governs effectors of ion transport in vertebrates.

Zebrafish possess at least three distinct ionocyte sub-types characterized by the expression of specific integral membrane ion transporters/exchangers. Cells expressing the Na^+/Cl^- cotransporter (SLC12A10.2; NCC-cells) play a key role in Cl^- ion uptake, while H^+-ATPase-rich (HR-cells) and Na^+–K^+-ATPase-rich (NaR-cells) cells function in the uptake of Na^+ and Ca^{2+}, respectively (Pan et al., 2005; Esaki et al., 2007; Wang et al., 2009). NCC expression in the apical membrane of teleost ionocytes was first reported

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in Mozambique tilapia (*Oreochromis mossambicus*) (Hiroi et al., 2008); Horng et al. (2009) subsequently demonstrated that NCC-expressing cells actively absorb Cl^-. As in tilapia, NCC is also expressed in a subset of ionocytes in the zebrafish gill and is essential for Cl^- balance (Wang et al., 2009). In HR cells, a Na^+/H^+ exchanger (NHE3b; SLC9A3.2) provides the apical pathway for Na^+ uptake from the external environment to the ionocyte interior where it is then transported into circulation (Yan et al., 2007). NaR cells specifically express an epithelial Ca^{2+} channel (ECaC; TRPV6) that facilitates the active uptake of Ca^{2+} from the external environment (Pan et al., 2005; Lin et al., 2011). The ion-absorptive functions of these three distinct zebrafish ionocytes have been demonstrated in zebrafish yolk integument, and all three genes are expressed in the gill (Liao et al., 2009). This characterization of ion transporters and cell types helps establish zebrafish as a new teleost model to assess the environmental and hormonal control of ion uptake capacities and mechanisms (Tseng et al., 2009; Chou et al., 2011; Lin et al., 2011).

While considerable progress has been made in establishing the cellular machinery supporting the functions of distinct ionocyte sub-types, the hormonal mechanisms that directly regulate ionocyte function, and thus ionoregulation by the gill, remain a mystery. PRL is a likely regulator of ionocytes based on the expression of teleost PRL receptors in gill tissue (Edery et al., 1984; Sandra et al., 1995; Weng et al., 1997; Santos et al., 2001; Lee et al., 2006), and the important role PRL plays in the osmoregulation of teleosts inhabiting freshwater environments. PRL can directly regulate gene expression in responding cells by binding to transmembrane receptors that activate the JAK/STAT signaling pathway (Bole-Feysot et al., 1998). There is evidence that the two zebrafish PRL receptors (denoted PRLRa and PRLRb) can regulate the transcription of distinct target genes upon PRL binding, at least *in vitro* (Chen et al., 2011). In Mozambique tilapia and black porgy (*Acanthopagrus schlegeli*), the expression of *prlr1* and *prlr2* transcripts (orthologous to zebrafish *prlra* and *prlrb*, respectively) in the gill is highly plastic, and the two *prlrs* are differentially influenced by osmotic and endocrine stimuli (Huang et al., 2007; Fiol et al., 2009; Breves et al., 2011). Modulation of *prlr* expression may provide a mechanism to regulate the sensitivity of target tissues to endocrine signaling. In fact, dynamic *prlr* expression in the gill appears to be an important aspect of adaptive responses to osmoregulatory challenges in euryhaline teleosts (Fiol et al., 2009; Breves et al., 2011; Flores and Shrimpton, 2012).

Here we show that PRL acts on ionocytes in the zebrafish gill by regulating the transcription of the ion cotransporter *ncc*, as well as the expression of *prlra*. The coordinated up-regulation of *prlra* and *ncc* in the gill upon transfer to ion-poor water, as well as following acute PRL treatment both *in vivo* and *in vitro*, suggests that PRL may be the key hormonal regulator of Cl^- uptake mechanisms in zebrafish gill.

2. Materials and methods

2.1. Animals and rearing conditions

Sexually mature zebrafish (*Danio rerio*) were selected from stocks maintained at the University of Massachusetts, Amherst Zebrafish Facility. Fish were maintained in a recirculating system of dechlorinated reverse-osmosis municipal water (6.9 mM Na^+, 6.6 mM Cl^-, 0.12 mM Ca^{2+}; pH 6.2–6.6) maintained at 26–27 °C. Fish were fed a flake diet supplemented with brine shrimp and maintained under a photoperiod of 14 h light: 10 h dark. The Institutional Animal Care and Use Committee of the University of Massachusetts approved the housing and maintenance of animals, and all experimental protocols.

2.2. Tissue distribution of PRL receptors

Tissues were collected from 10 adult zebrafish (5 males, 5 females, 1–2 g) maintained in standard rearing conditions for >1 year. Fish were lethally anesthetized with buffered tricaine methanesulfonate (MS-222; 250 mg/l), and the following tissues were collected: whole brain (olfactory bulb, telencephalon, optic tectum, cerebellum, diencephalon, and medulla), pituitary, gill, liver, body kidney, esophagus, anterior intestine and posterior intestine. Tissues were homogenized immediately in Trizol Reagent (Invitrogen, Carlsbad, CA) and stored at −80 °C until RNA isolation.

2.3. Transfer to ion-poor (ddH_2O) water

Seven days prior to experimentation, adult zebrafish (1–2 g) maintained in standard rearing conditions for >1 year were distributed into six static aquaria (9 L; 8–10 fish/tank) maintained with filtration and aeration. At the time of transfer (0 h), fish from two aquaria were quickly netted and transferred directly to two additional aquaria containing ion-poor water (Millipore ddH_2O; 0.2 mM Na^+, 0.1 mM Cl^-, 0.04 mM Ca^{2+}; pH 7.0–7.2) with filtration and aeration. Control fish were netted and then returned to the same aquaria to control for potential handling effects. Fish were fed twice daily during the initial 7-day acclimation and then fasted for the duration of the transfer experiment. Water temperature was maintained at 26–28 °C. At the time of sampling, fish (n = 8–10) from one system water- and one ion-poor water-containing aquaria were netted and anesthetized with a lethal dose of MS-222. Fish were sampled at 0, 2 and 7 days after transfer. Fish were rapidly decapitated and filaments from all branchial arches were homogenized immediately in Trizol Reagent and stored at −80 °C until RNA isolation. White muscle was sampled from the caudal musculature and the water content was measured gravimetrically after drying overnight at 90 °C.

2.4. In vivo effects of oPRL

Purified ovine PRL (oPRL; NIDDK-oPRL-21) was obtained from the National Hormone and Peptide Program and delivered in saline vehicle (0.9% NaCl; 20 μl/g body weight injection volume). Adult zebrafish (1–2 g) were administered oPRL (5 or 50 μg/g body weight) by two intraperitoneal (IP) injections. Fish were lightly anaesthetized with MS-222 and given the first injection. Twenty-four hours later, fish were netted, anaesthetized, and given a second injection. Fish were then returned to aquaria and left undisturbed for 24 h, after which time gill tissue was sampled as described above. The doses of oPRL were selected based on previous studies employing IP-injection in teleosts (Herndon et al., 1991; Eckert et al., 2001; Jackson et al., 2005; Breves et al., 2010).

2.5. Gill culture conditions and in vitro effects of oPRL and Δ1-9-G129R-hPRL

Gill filaments were isolated from adult zebrafish (1–2 g) and cultured according to McCormick and Bern (1989) with modifications. Fish were lethally anesthetized and branchial arches were removed and rinsed in pre-incubation Dulbecco's Modified Eagle Medium (DMEM; high glucose, HEPES, no phenol red; 311 mOsm; Invitrogen) containing 50 U/ml penicillin and 50 μg/ml streptomycin (Invitrogen). Gill filaments from a single fish were severed from the arches at the septum and placed in a single well (24-well cell culture plate; Corning Inc., Corning, NY) containing pre-incubation medium for 3 h. Each sample/well was designated as an individual fish. After the pre-incubation period, medium

was replaced with freshly prepared control medium (DMEM + vehicle control: phosphate-buffered saline; PBS) or DMEM supplemented with oPRL (1 μg/ml) dissolved in PBS. For the initial time-course experiment, filaments ($n = 6$) were incubated at 29 °C in a humidified chamber under atmospheric air for 4, 8, 12 and 24 h in either vehicle- or oPRL-supplemented DMEM. Gene expression was compared to pre-incubated filaments (0 h). For the subsequent concentration-response experiment, the effects of oPRL (0.01–10 μg/ml) were tested after 8 h of incubation. The recombinant human PRL receptor antagonist Δ1-9-G129R-hPRL was produced as described by Bernichtein et al. (2003). In an 8 h culture, Δ1-9-G129R-hPRL was used at concentrations ranging from 5 to 45 μg/ml in combination with 0.5 μg/ml oPRL. Gill cultures were terminated by removing the filaments from the culture wells and placing them in Trizol Reagent; filaments were homogenized immediately and stored at −80 °C until RNA isolation.

2.6. RNA extraction, cDNA synthesis and quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from individual tissues using Trizol Reagent according to the manufacturer's instructions. First strand cDNA was synthesized with a High-Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Relative amounts of mRNA were determined by qRT-PCR using the MxPro3000P system (Stratagene, La Jolla, CA). Primers for all genes are provided in Table 1, and when not previously reported were designed using Primer3. All primers were tested for non-specific product amplification and primer–dimer formation by melting curve analyses and gel electrophoresis. Assays were performed in a 10 μl reaction containing 1 μl cDNA template, 5 μl FastStart Universal SYBR Green Master (ROX) kit (Roche Diagnostics Corp., Indianapolis, IN), forward and reverse primers at 400 nM and nuclease-free water. The following cycling conditions were employed for all assays: 1 cycle of 95 °C for 15 min, 40 cycles of 95 °C for 15 s, 60 °C for 30 s, 72 °C for 30 s and 1 cycle of 95 °C for 1 min, 55 °C for 30 s and 95 °C for 30 s. Three transcripts (*β-actin*, *ef1α* and *18s rRNA*) were assessed for use as normalization genes in every experiment, a single gene was selected based upon its stable expression across treatments and time. qRT-PCR data were analyzed using the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001). Standard curves were prepared from serial dilutions of untreated gill cDNA and included on each plate to calculate the PCR efficiencies for target and normalization genes. Relative gene expression is reported as a fold-change from controls. Intra-assay coefficients of variation ranged from 0.04 to 0.12.

Table 1
Specific primer sequences for qRT-PCR.

Name	Primer sequence (5′–3′)	GenBank ID/reference
β-actin	F: CACCTTCCAGCAGATGTGGA R: AAAAGCCATGCCAATGTTGTC	Liao et al. (2007)
ef1α	F: CTGGTGTCCTCAAGCCTGGTA R: ACTTGACCTCAGTGGTTACATTGG	Walpita et al. (2007)
18s	F: TCGCTAGTTGGCATCGTTTATG R: CGGAGGTTCGAAGACGATCA	McCurley and Callard (2008)
prlra	F: AGGCAGTTCAATGCAGCACGA R: GCACAGCGGCGGAAATCCTCAT	EU517718.1
prlrb	F: GGATATCGTGCAGCCTCATCCTCCA R: GGTTACCCATCCGGAGCGCG	NM_001113500.1
ncc	F: GCCCCCAAAGTTTTCCAGTT R: TAAGCACGAAGAGGCTCCTTG	Wang et al. (2009)
nhe3b	F: GTTTTCTGCAGACAGCGCCTCT R: ATCCACACCAGCTCCAGTCGTGT	NM_001113479.1
ecac	F: TTTGCAAGTCTTGTTGGTCTCGGT R: TGCTGAAGGCGGAACCGCATT	NM_001001849.1
b2m	F: GCCTTCACCCCAGAGAAAGG R: GCGGTTGGGATTTACATGTTG	McCurley and Callard (2008)
gapdh	F: CGCTGGCATCTCCCTCAA R: TCAGCAACACGATGGCTGTAG	Tang et al. (2007)

2.7. In situ hybridization

An antisense digoxigenin probe was generated from PCR product against *ncc* for the region spanning nucleotides 639-2162 as in Liao et al. (2009). Whole mount *in situ* hybridization was performed as previously described (Karlstrom et al., 1999) using NBT/BCIP as the chromogenic substrate (Roche Ltd., Basel, Switzerland). Colorimetric reaction times were identical for all samples. Following completion of the labeling reaction, filaments were cleared in 75% glycerol and examined using a dissecting microscope.

2.8. Statistical analyses

The tissue expression experiment (Fig. 1) was analyzed by two-way ANOVA (analysis of variance) with tissue and sex as main effects. A significant effect of tissue was followed up with Tukey's honestly significant difference (HSD) test. The transfer experiment (Fig. 2) was analyzed by two-way ANOVA with treatment and time as main effects. Significant main effects of treatment or time were followed up by Student's *t*-test or Dunnett's test, respectively. Group comparisons for the *in vivo* injection (Fig. 3) and *in vitro* concentration-response (Fig. 5) experiments were conducted with Tukey's HSD. When data were not normally distributed, a nonparametric ANOVA was performed on ranked data, followed by Tukey's HSD to determine differences between groups. For the *in vitro* time-course (Fig. 4) and PRL receptor antagonist experiments (Fig. 6), two-way ANOVA was followed by a Student's *t*-test and Tukey's HSD test. All analyses were conducted using GraphPad Prism 5.0 (San Diego, CA, USA). Significance for all tests was set at $P < 0.05$.

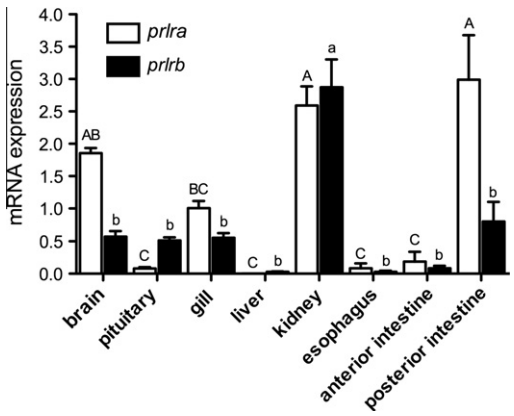

Fig. 1. Gene expression of *prlra* (open bars) and *prlrb* (solid bars) in the tissues of adult zebrafish maintained in normal fresh water. Data were normalized using *ef1α* as a reference gene and are presented relative to the amount of *prlra* mRNA in gill. Means ± SEM ($n = 10$; 5 males, 5 females). For a given transcript, denoted by upper- or lower-case letters, groups not sharing the same letter are significantly different (ANOVA, Tukey's HSD, $P < 0.05$).

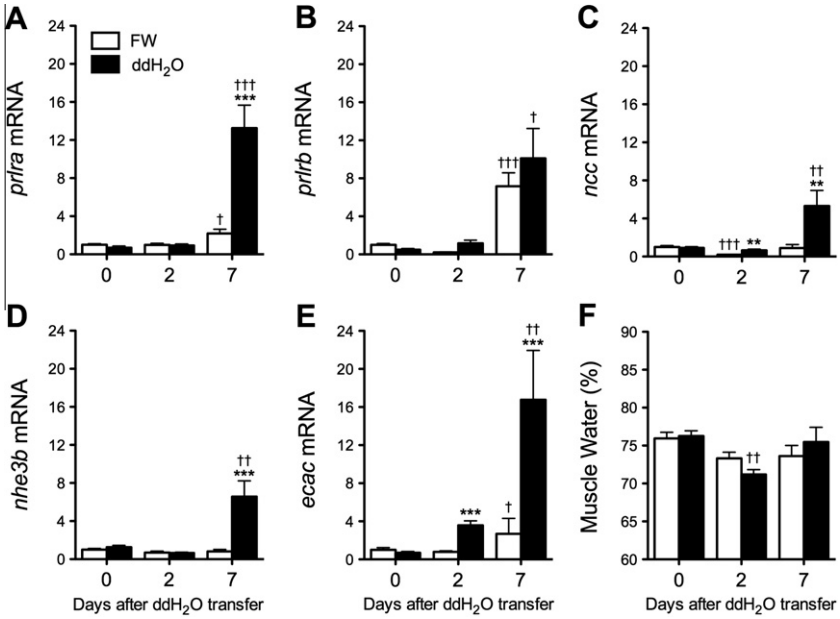

Fig. 2. Changes in branchial *prlra* (A), *prlrb* (B), *ncc* (C), *nhe3b* (D) and *ecac* (E) gene expression and muscle water content (F) at 0, 2 and 7 days after transfer of zebrafish adults from fresh water (FW) to ion-poor (ddH_2O) water (solid bars). Means ± SEM (n = 8–10). Time-matched control fish were maintained in FW (open bars). Gene expression is presented as fold-change from FW controls at time 0. Differences among groups were evaluated by two-way ANOVA. **,***Significantly different from time-matched FW controls at $P < 0.01$ and 0.001, respectively, by Student's t-test. †,††,†††Significantly different from time 0 at $P < 0.05$, 0.01 and 0.001, respectively, by Dunnett's test.

3. Results

3.1. PRL receptor gene expression in osmoregulatory tissues

We first verified that the two known zebrafish PRL receptor transcripts (*prlra* and *prlrb*) are expressed in the gill and established the relative amounts of expression across zebrafish tissues relative to gill *prlra* expression (Fig. 1). *prlra* mRNA was highly expressed in brain, gill, kidney and posterior intestine, with lower expression in other tissues. *prlrb* was also highly expressed in the kidney with lower expression in other tissues. In the gill, the relative expression of *prlra* versus *prlrb* was comparable, while in other tissues such as brain, pituitary, and posterior intestine, *prlra* and *prlrb* were expressed at distinct amounts. The expression of *prlra* and *prlrb* was not significantly different in males versus females (data not shown), thus data from both sexes were pooled (Fig. 1).

3.2. Transfer to ion-poor water led to an increase in PRL receptor and ion transporter/exchanger gene expression

To determine whether transfer to ion-poor conditions affects expression of PRL receptor genes, as well as genes known to be involved in ion transport, we performed qRT-PCR on gill tissues 0, 2 and 7 days following transfer of adult fish to ddH_2O (Fig. 2A–E). Transfer to ddH_2O did not overtly affect adult zebrafish; there were no mortalities and no detectable changes in muscle water content (Fig. 2F). *prlra* gene expression remained unchanged after 2 days in ddH_2O, but was increased 6-fold from FW-controls following 7 days in ddH_2O (Fig. 2A). In contrast, *prlrb* was unchanged relative to FW controls after 2 and 7 days in ddH_2O (Fig. 2B). *prlrb* expression increased variably at 7 days, but this increase was seen irrespective of treatment. Expression of *ncc* mRNA increased approximately 3-fold from time-matched FW controls after 2 days in ddH_2O, and nearly 6-fold after 7 days (Fig. 2C). Expression of *nhe3b* mRNA was relatively unchanged after 2 days in ddH_2O, but was increased over 8-fold from time-matched controls after 7 days (Fig. 2D). Similar to *ncc*, *ecac* was increased 4.6- and 6.2-fold from time-matched controls after 2 and 7 days in ddH_2O, respectively (Fig. 2E).

3.3. Increased PRL levels induced gene expression of ncc and prlra in vivo

We next examined whether increased systemic PRL levels could affect the expression of the same genes that were induced by ion-poor water conditions. Adult zebrafish were IP-injected two times at t = 0 and t = 24 h with 5 or 50 μg/g of purified oPRL. At t = 48 h, 50 μg/g of oPRL led to a ~2-fold increase in branchial expression of *ncc* mRNA (Fig. 3A). oPRL injections did not affect *nhe3b* or *ecac* expression (Fig. 3B and C), suggesting PRL effects on gill gene expression are specific. Similar to *ncc*, a 2-fold increase in *prlra* expression occurred following injection of 50 μg/g oPRL (Fig. 3D). There was no clear effect of oPRL on the expression of *prlrb*. While *prlrb* expression was significantly different between saline- and oPRL-injected fish (50 μg/g), there were no significant differences between unhandled and oPRL-injected fish (Fig. 3E). Since high oPRL levels may bind teleost growth hormone (GH) receptors (Prunet and Auperin, 1994), we tested whether oGH influenced the expression of these genes in the gill. IP injection of oGH did not alter branchial *prlra* or *ncc* gene expression at doses up to 50 μg/g (data not shown).

3.4. PRL directly induced ncc and prlra gene expression in cultured gill

To determine whether oPRL acts directly on gill tissue to regulate expression of ionoregulatory genes, we cultured zebrafish gills in the presence or absence of 1 μg/ml oPRL and assayed gene expression by qRT-PCR and *in situ* hybridization. As expected, *ncc*, *nhe3b*, and *ecac* declined during the culture period (Fig. 4A–C) consistent with the need for systemic signals to maintain expression of these ionoregulatory genes. Expression of *prlra* and *prlrb* also gradually declined under these culture conditions, but with distinct kinetics (Fig. 4D and E). Importantly, the amount of *β-actin* (Fig. 4F), *β-2-microglobulin* (*b2m*) and *glyceraldehyde 3-phosphate dehydrogenase* (*gapdh*) mRNA was unchanged through the culture period (data not shown), revealing the overall stability of gene expression within the cultured tissue. The supplementation of medium with oPRL resulted in markedly higher (~10-fold)

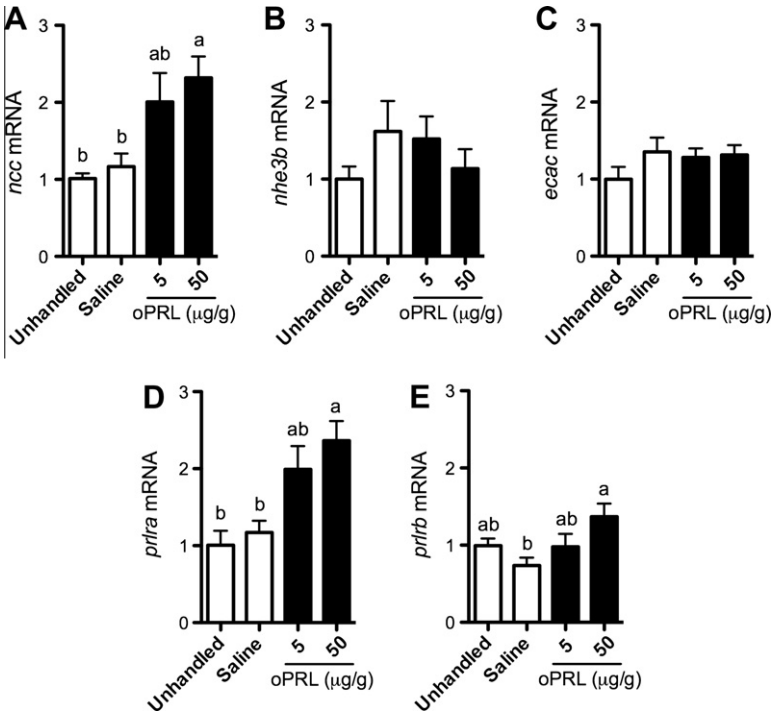

Fig. 3. Branchial gene expression of *ncc* (A), *nhe3b* (B), *ecac* (C), *prlra* (D) and *prlrb* (E) following intraperitoneal (IP) injection of oPRL. Means ± SEM (n = 6–10). Fish were maintained in system water and administered two IP injections (20 μl/g body weight) of saline or oPRL (5 and 50 μg/g) (solid bars) separated by 24 h. Fish were sampled 48 h after the first injection. Unhandled and saline-injected fish were included as controls (open bars). Gene expression is presented as fold-change from unhandled fish. Means not sharing the same letter are significantly different (ANOVA, Tukey's HSD, $P < 0.05$).

expression of *ncc* mRNA at 4, 8, 12 and 24 h versus time-matched controls (Fig. 4A). In contrast, *nhe3b* and *ecac* expression was unaffected by oPRL (Fig. 4B and C). *prlra* mRNA was elevated from time-matched controls at 4, 8 and 12 h by oPRL treatment (Fig. 4D) while *prlrb* mRNA was unaffected by oPRL (Fig. 4E). In the absence of PRL in culture, *ncc*-positive cells disappeared by 4 h (Fig. 4G). oPRL treatment led to the maintenance of *ncc* expression in discrete cells located along the length of the gill filaments.

This effect of oPRL on the maintenance of *ncc* expression in culture was concentration-dependent, further supporting the specific nature of this effect. Treatment with 0.1, 0.5, 1 and 10 μg/ml of oPRL for 8 h induced 8.4-fold, 9.5-fold, 13.0-fold and 11.9-fold higher *ncc* expression from controls, respectively (Fig. 5A). Again, there were no effects of oPRL on either *nhe3b* or *ecac* (Fig. 5B and C). There was a modest effect of oPRL at 0.5 μg/ml on *prlra* mRNA with a 2.5-fold increase from controls (Fig. 5D). *prlrb* was unaffected by oPRL treatment at all doses (Fig. 5E). We confirmed that addition of oGH had no effect on *ncc* expression *in vitro* (data not shown).

3.5. The PRL receptor antagonist Δ1-9-G129R-hPRL blocked the effects of oPRL on gill gene expression

To further test whether oPRL specifically affects *ncc* and *prlra* expression via PRL receptor mediated signaling, we took advantage of a modified human PRL peptide that antagonizes signaling by blocking PRL binding and subsequent PRL receptor activation (Bernichtein et al., 2003). As in the previous experiment (Fig. 5A), *ncc* expression was maintained in the presence of 0.5 μg/ml oPRL after 8 h in culture. Co-incubation with Δ1-9-G129R-hPRL blocked this effect on *ncc* in a concentration-dependent manner (Fig. 6A). Similarly, addition of Δ1-9-G129R-hPRL blocked the effect of oPRL on *prlra* expression (Fig. 6B). Importantly, incubation with Δ1-9-G129R-hPRL alone had no significant effect on *ncc* and *prlra* expression (Fig. 6A and B).

4. Discussion

4.1. PRL as an evolutionarily conserved osmoregulatory hormone

PRL has been identified as a freshwater-adapting hormone in fish through its actions on water permeability and ion retention in the gill, gut, kidney and integument (Hirano, 1986; Sakamoto and McCormick, 2006). However, there is little direct evidence of PRL stimulating ion uptake across branchial epithelia, and limited information on the actual ion-transport pathways regulated by PRL (Zhou et al., 2003). Here we present the first *in vivo* and *in vitro* evidence in a stenohaline freshwater teleost that PRL directly regulates branchial expression of *ncc*, a gene encoding the Na^+/Cl^- cotransporter that is central to the maintenance of Cl^- balance (Hiroi et al., 2008; Horng et al., 2009; Wang et al., 2009). Combined with our recent work showing that pituitary-derived PRL regulates ionocyte *ncc* expression in a euryhaline cichlid, the Mozambique tilapia (Breves et al., 2010), our data suggest that *ncc* may represent a conserved transcriptional target of PRL in fishes that employ NCC-dependent Cl^- uptake pathways.

We show that the two zebrafish *prlr* genes (*prlra* and *prlrb*) are robustly expressed in the gill (Fig. 1). This expression is in agreement with reports of radiolabeled-PRL binding and *prlr1*/*prlr2* expression in branchial tissue of tilapia, sea bream (*Sparus aurata*) and goldfish (Dauder et al., 1990; Prunet and Auperin, 1994; Tse et al., 2000; Santos et al., 2001; Pierce et al., 2007; Fiol et al., 2009) and strongly suggests gill tissue is competent to respond to PRL signaling. In teleosts, multiple GH and PRL receptor family genes have been retained following genome duplication events (Fukamachi and Meyer, 2007). These multiple forms have distinct expression patterns (Huang et al., 2007; Fiol et al., 2009; Breves et al., 2011) and capacities to activate intracellular signaling pathways (Huang et al., 2007; Fiol et al., 2009; Chen et al., 2011). The idea that PRL receptor proteins are functional in the gill is further supported by our finding that *prlra* expression is positively

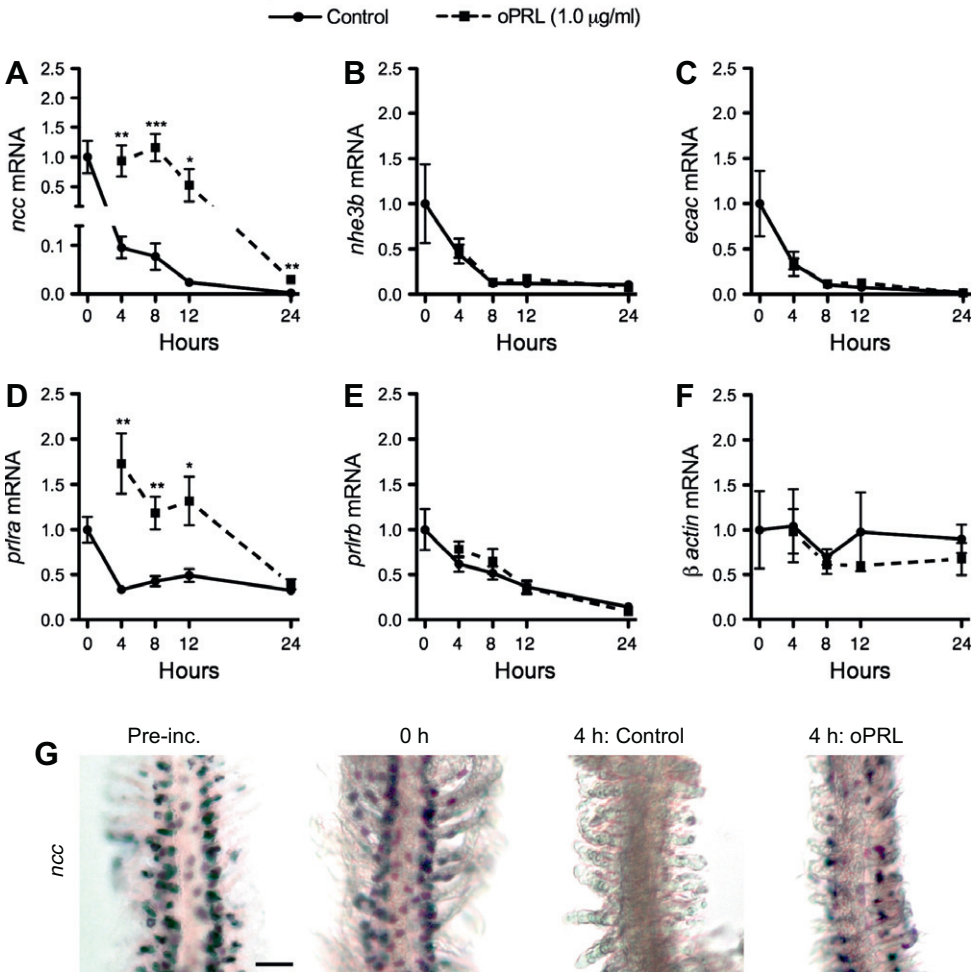

Fig. 4. Effects of incubation time and oPRL on *ncc* (A), *nhe3b* (B), *ecac* (C), *prlra* (D), *prlrb* (E) and *β-actin* (F) gene expression in cultured gill filaments. Solid line, control; dashed line, oPRL (1.0 μg/ml). Gene expression is presented as fold-change from 0 h. Means ± SEM ($n = 6$). Filaments were dissected from anesthetized fish and pre-incubated for 3 h prior to treatment with oPRL-supplemented medium. Effects of hormone treatment and time were evaluated by two-way ANOVA. For clarity, only the results of follow-up analyses for a significant main effect of hormone treatment are presented. *,**,***Significantly different from the time-matched controls at $P < 0.05$, 0.01 and 0.001, respectively, by Student's *t*-test. Whole-mount *in situ* hybridization showing *ncc* expression in gill filaments cultured for 4 h in the presence or absence of oPRL as compared with pre-incubated and 0 h control filaments (G). Scale bar = 50 μm.

regulated by oPRL (Fig. 3D) and by the fact that plasma PRL levels seemingly regulate transcription of clade 1 PRL receptors (*prlr1*) in other systems (Pierce et al., 2007; Breves et al., 2010).

Our finding that *prlra* expression increases in the zebrafish gill following transfer to ddH_2O (Fig. 2A) is the first evidence for this response in a stenohaline teleost. The ability of zebrafish to rapidly acclimate to dramatic reductions in environmental ion concentrations may allow them to adapt to natural variations in their native habitat, freshwater streams in the Indian subcontinent with significant seasonal fluctuations (Boisen et al., 2003). In euryhaline species including tilapia and rainbow trout (*Oncorhynchus mykiss*), branchial *prlr1* gene expression is similarly enhanced in parallel with freshwater acclimation responses (Pierce et al., 2007; Fiol et al., 2009; Breves et al., 2011; Flores and Shrimpton, 2012). Combined, these data suggest an evolutionarily conserved, PRL-mediated low salinity (freshwater) acclimation response.

Zebrafish can tolerate transfer to ion-poor water without apparent distress or severe perturbations of plasma ion levels (Fig. 2F; Boisen et al., 2003; Craig et al., 2007; Liao et al., 2009). To maintain hydromineral balance in such dynamic conditions, gill tissue rapidly modulates the transcription of genes encoding effectors of ion transport (Fiol and Kültz, 2007). NCC was previously shown to be a key effector of Cl^- uptake in at least a subset of teleost species (Hiroi et al., 2008), including zebrafish (Wang et al., 2009), with inward Cl^- currents detectable in the immediate vicinity of NCC-expressing tilapia ionocytes (Horng et al., 2009). Reduced *ncc* function in larval zebrafish significantly impacted Cl^- influx and tissue Cl^- content (Wang et al., 2009), confirming that NCC is a key component of physiological responses underlying Cl^- balance.

Our findings that PRL is (1) sufficient to upregulate gill *ncc* expression *in vivo* (Fig. 3A) and (2) required for maintenance of *ncc* expression in cultured gill tissue (Figs. 4–6) strongly suggest that PRL signaling is central to appropriate Cl^- regulation. The observation that increased *ncc* expression appears to precede an increase in *prlra* expression by several days following low ion exposure *in vivo* (Fig. 2A and C) suggests that basal *prlra* expression is sufficient to mediate the *ncc* response to PRL, with increased receptor expression helping maintain or induce additional osmoregulatory responses. It is also certainly the case that other hormones help trigger and modify adaptive responses to hyposmotic conditions *in vivo*. To confirm our findings and begin to uncover the kinetics of individual hormonal responses to ionoregulatory challenges, it will thus be important to develop sensitive radioimmunoassays for PRL, GH and other putative osmoregulatory hormones to determine plasma levels in zebrafish.

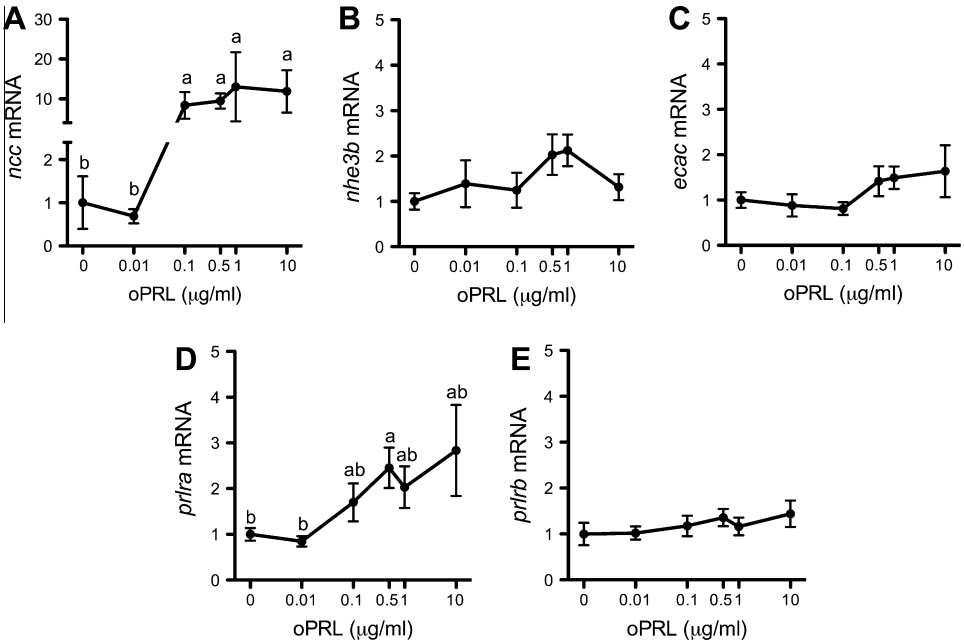

Fig. 5. Effects of oPRL concentration on *ncc* (A), *nhe3b* (B), *ecac* (C), *prlra* (D) and *prlrb* (E) gene expression in cultured gill filaments. Means ± SEM ($n = 6$). Filaments were pre-incubated for 3 h, and then incubated with oPRL-supplemented (0.01–10 μg/ml) medium for 8 h. Gene expression is presented as fold-change from the 0 oPRL group. Groups not sharing the same letter are significantly different (ANOVA, Tukey's HSD, $P < 0.05$).

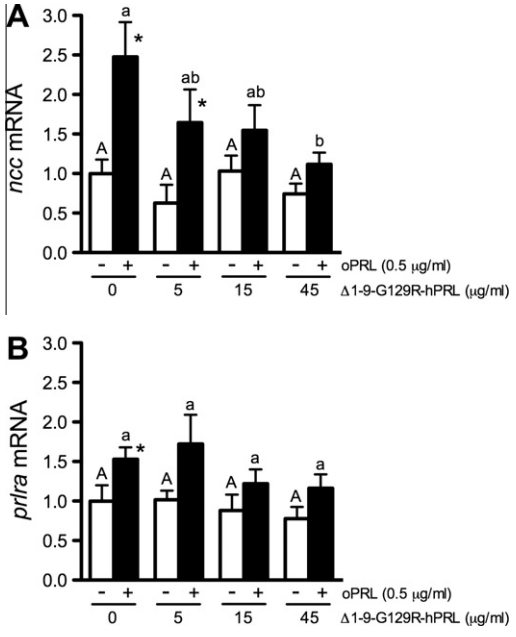

Fig. 6. Effects of Δ1-9-G129R-hPRL concentration on basal (open bars) and oPRL-stimulated (0.5 μg/ml; solid bars) *ncc* (A) and *prlra* (B) gene expression in gill filaments cultured for 8 h. Means ± SEM ($n = 6$). Gene expression is presented as fold-change from the 0 oPRL and 0 Δ1-9-G129R-hPRL group. Main effects of oPRL treatment and antagonist concentration were evaluated by two-way ANOVA. *Significantly different from dose-matched 0 oPRL controls at $P < 0.05$ by Student's *t*-test. Within a given series, denoted by uppercase or lowercase letters, means not sharing the same letter are significantly different (Tukey's HSD, $P < 0.05$).

4.2. Cellular mechanisms of PRL action in the gill

The filament culture system is particularly useful in uncovering cellular responses in the gill that do not require input from the whole animal, with gill-autonomous cellular functions being maintained for up to 4 days (McCormick and Bern, 1989; Kiilerich et al., 2007). We found that expression of *prlra*, as well as several ionoregulatory genes (*ncc*, *nhe3b*, and *ecac*) declined within 4 h of culture, as would be expected for endocrine regulated genes (Fig. 4). Addition of oPRL maintained the expression of *prlra* and *ncc* for up to 12 h in a concentration-dependent fashion (Fig. 4A and D), but did not affect *nhe3b* and *ecac* expression (Fig. 4B and C). Consistent with highly specific hormonal action on ionocytes, recent work by others showed that the *nhe3b* and *ecac* genes are regulated by distinct hormonal signals including stanniocalcin, isotocin, and/or cortisol (Tseng et al., 2009; Chou et al., 2011; Lin et al., 2011; Kumai et al., 2012).

In situ hybridization confirmed that *ncc* is expressed in discrete cells of the gill filaments, both at the beginning of the culture period as well as in cultures exposed to oPRL (Fig. 4G). NCC ionocyte number and distribution appeared normal in oPRL treated cultures, strongly suggesting that under these conditions PRL acts to maintain *ncc* expression in existing ionocytes rather than to induce expression in other cells of the gill. We are now developing the tools needed to determine PRL receptor expression in relation to differentiated ionocytes, and ionocyte precursors *in vivo* (reviewed by Chang and Hwang, 2011), in order to gain further insight on which cells directly respond to PRL during salinity acclimation.

Direct action of PRL through transmembrane PRL receptors in the gill is further supported by our experiments employing a specific PRL receptor antagonist (Δ1-9-G129R-hPRL) that binds PRL receptors with high affinity and prevents the receptor dimerization required for the activation of JAK/STAT signaling (Bernichtein et al., 2003). Δ1-9-G129R-hPRL is a variant of the human PRL sequence with a glycine to arginine substitution at position 129 and the deletion of nine residues at the N-terminus (Bernichtein et al., 2003). Glycine 129, which lies within the second receptor-binding site, is conserved from zebrafish to humans (Huang et al., 2009), which suggested Δ1-9-G129R-hPRL could act as an antagonist in zebrafish. Addition of Δ1-9-G129R-hPRL eliminated the ability of PRL to maintain *ncc* expression in a dose dependent manner, with 45 μg/ml Δ1-9-G129R-hPRL eliminating *ncc* gene expression seen

when gills were maintained in 0.5 μg/ml oPRL (Fig. 6A). This dose relationship is remarkably similar to that reported for the murine PRL receptor (Bernichtein et al., 2003). The lack of an effect of Δ1-9-G129R-hPRL alone on *ncc* expression confirmed findings from mammalian models that Δ1-9-G129R-hPRL does not operate as an agonist at high doses.

Our *in vivo* studies revealed that *prlra*, *ncc*, *nhe3b* and *ecac* gene expression increased following transfer to ion-poor conditions or PRL injections, and that these changes occurred on a multi-day time scale (Figs. 2 and 3). This slower response may point to more complex regulation within the whole organism, and/or may suggest that cell number regulation may also provide a mechanism for maintaining ion balance. Recent studies provide evidence that osmoregulatory hormones can act upon ionocyte progenitor populations to induce cell differentiation events that contribute to detectable changes in the expression of the ion transporters/exchangers that define mature ionocytes (Chou et al., 2011; Cruz et al., 2013). PRL signaling may thus work at both the transcriptional level and the level of cell differentiation to regulate immediate and long-term responses to osmotic challenges, respectively.

4.3. Conclusions

Taken together our data indicate that PRL is an endocrine signal that is necessary and sufficient to regulate expression of *ncc* in the zebrafish gill, and may thus be key to adaptive ionoregulatory physiology. This work helps establish zebrafish as a model for investigating the molecular and cellular mechanisms underlying this physiological response, including the regulatory networks in the hypothalamus and pituitary that confer osmoresponsive PRL expression and release (Liu et al., 2006; Hoshijima and Hirose, 2007). PRL binding has been documented in other key osmoregulatory tissues in both fish and mammals, including the kidney and intestine (Bole-Feysot et al., 1998). Given its power as an embryological model, the zebrafish promises to greatly facilitate the study of the interplay between endocrine system ontogeny and the onset of extra-branchial (renal and gastrointestinal) processes that underlie hydromineral balance. Finally, given the remarkable level of conservation in these regulatory mechanisms, this work in zebrafish promises to help uncover how defects in PRL-regulated ion and water metabolism might underlie hydromineral imbalances associated with a wide variety of human diseases.

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