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***In vitro* Effects of the Prolactin, Growth Hormone and Somatolactin on Cell Turnover in Fish Esophagus: Possible Mode of Opposite Osmoregulatory Actions of Prolactin and Growth Hormone**

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1. Introduction

Growth hormone, prolactin and somatolactin belong to the same hormone family and have a similar structure in teleost fishes [1]. Each of these three hormones appear to have opposite or specific functions in electrolyte balance in teleosts [2]. Teleost fish in freshwater environment face two primary challenges: preventing the loss of ions to the external hypoosmotic environment and preventing the influx of water. Prolactin plays a central role in these activities during the adaptation of fish to fresh water, as evidenced by its ability to increase plasma ion concentrations (primarily Na^+ and Cl^-) and decrease the permeability of osmoregulatory organs, such as gill, kidney and intestine [3]. In seawater environment, in contrast, diffusive water loss is counteracted by drinking seawater and actively taking up Na^+ , Cl^- and water across the gastrointestinal tract, and the gill actively secretes Na^+ and Cl^- through chloride cells. Growth hormone activates these gill chloride cells with ion transporters (e.g., Na^+ , K^+ -ATPase and the Na^+ , K^+ , 2Cl^- cotransporter) involved in secretion of Na^+ and Cl^- across the branchial epithelium [3], and appears to stimulate the intestinal absorption [4]. On the other hand, somatolactin is proposed to be involved in regulation of acid-base, calcium and phosphate levels in several species [2, 3, 5-7], but the role of somatolactin in the intestine is unknown. Although the presence of receptors for these hormones in ion-transporting organs has been reported in a variety of teleost species [8-14] and several studies have investigated the direct effects of the teleost hormones on osmoregulatory organs [5, 15-18], the modes of action

of the hormones are still unclear. In particular, the opposite *in-vitro* effects of growth hormone and prolactin have not been reported, and there is little information on the cellular and biochemical mechanisms of the osmoregulatory actions of these hormones.

In our *in-vivo* studies on esophagi from euryhaline fish, prolactin stimulated cell proliferation in the epithelium [14, 19]. This effect seems to be related to increased cell proliferation for the stratified epithelium to reduce permeability in fresh water [20]. We have also found the increased apoptosis for the simple epithelium to give high permeability in seawater [14, 19]. A large number of reported effects of prolactin are associated with cell proliferation and/or apoptosis [21]. Thyroid-hormone induced apoptosis of the metamorphosing amphibian tail is directly prevented by prolactin and enhanced by glucocorticoid [22]. Using recently developed techniques for the culture of esophagus from the euryhaline medaka, *Oryzias latipes*, we have demonstrated that cortisol directly induced both cell proliferation and apoptosis [23]. In this study, we compared the effects of prolactin, growth hormone and somatolactin in this system, and found the first evidence that prolactin and growth hormone have opposite effects on the osmoregulatory organ *in vitro*.

2. Materials and methods

2.1. Animals

Adult medaka (*Oryzias latipes*, 0.1-0.2 g in weight) of both sexes were kept in indoor freshwater tanks at $27 \pm 2^\circ\text{C}$ and fed on Tetrafin flakes (Tetra Werke, Melle, Germany) daily for more than two weeks. Osmoregulation, differentiation of gill chloride cells, hormonal status during adaptation to different salinities, and the *in-vivo* effects of osmoregulatory hormones in this species have been described in our previous reports [23, 24]. Fish were exposed to 0.1% Fungizone (Amphotericin B, Invitrogen, Tokyo, Japan) for two days without food to avoid contamination before isolation of the esophagus. All fish were handled, maintained, and used in accordance with the Guidelines for Animal Experimentation established by Okayama University in accordance with international standards on animal welfare and in compliance with national regulations.

2.2. Tissue culture

As previously described [23], esophagi were gently sliced open along the long axis and cut into halves. Each explant was placed in a individual well of 96-well culture plates containing preincubation medium (MEM with Hanks' salts, 25 mM HEPES, 5 mg/ml BSA, 250 U/ml penicillin G, and 250 $\mu\text{g}/\text{ml}$ streptomycin sulfate, adjusted to pH 7.8 at 25°C). After several hours, the medium was replaced with MEM containing Earle's salts, 4 mg/ml BSA, 292 $\mu\text{g}/\text{ml}$ L-glutamine, 50 U/ml penicillin G and 50 $\mu\text{g}/\text{ml}$ streptomycin sulfate adjusted to pH 7.8 when saturated with 99% O_2 / 1% CO_2 . The medium osmolarity was adjusted to 300 mOsm/kg with NaCl. Explants were randomly assigned to control and treatment groups ($N = 4-6$), and incubated at 27°C in an airtight humidified chamber and gassed daily. Chum salmon prolactin, growth hormone and somatolactin was (1, 10, 100 ng/ml) or was not

added to the culture medium. The concentrations were chosen based on the published effective physiological doses [5, 15-17, 25-27], plasma hormone concentrations [14, 28-30], and our preliminary studies. These salmonid hormones have approximately 60% amino-acid identities to the medaka counterparts (NCBI accession no. or Ensemble medaka genomic database browser ID: medaka growth hormone: ENSORLP00000024332, prolactin: AAP33052, somatolactin: AAT58046; [1, 13]) and have high specificities for their respective receptors in fish, unlike mammalian hormones which bind equally to other hormone receptors in fish [13, 31, 32]. The use of these salmonid hormones to study the specific physiological roles in many teleost species has been well established [25, 27, 29, 33, 34]. All the culture medium was replaced with freshly prepared medium daily. Although explants were occasionally found to adhere to the bottom of the plate well, they typically remained unattached during culture. The tissue culture maintained structural integrity for 8 days based on the presence of intact nuclei and cell-to-cell borders [23]. The cultured esophageal explants were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) at 4°C for 4 h for histological analysis ($N = 3-5$) or snap frozen in liquid nitrogen for quantification of apoptosis.

2.3. Cell proliferation assay

At given time points, cultured explants were pulsed with an oxidation-reduction indicator WST-1 (10% vol/vol, Roche, Tokyo, Japan) for 4 h and color development ($A_{450\text{ nm}}-A_{600\text{ nm}}$) was quantified to measure the activity of mitochondrial dehydrogenases. This activity is proportional to the number of viable cultured cells and is expressed as the cell proliferating index [35]. The result after pre-incubation (on day 0) was used to correct for differences in initial tissue content per esophageal slice, and also for quantification of apoptosis.

2.4. Quantification of apoptosis

DNA internucleosomal fragmentation in the esophagus was assessed using a cell death detection ELISA kit (Roche, Tokyo, Japan) according to the manufacturer's instructions. This kit uses a quantitative sandwich ELISA that specifically measures the histone region (H1, H2A, H2B, H3, and H4) of mono- and oligonucleosomes [36] in teleosts [37] that are released during apoptosis, but not during necrosis [38]. After a 10-minute reaction, color development ($A_{405\text{ nm}}-A_{492\text{ nm}}$) was quantified using an MTP-300 microplate reader (Corona, Ibaragi, Japan).

2.5. Proliferating cell nuclear antigen (PCNA) immunohistochemistry

To label proliferating cells in the esophagus, we used a mouse monoclonal antibody (clone PC10; Sigma, Tokyo, Japan) against proliferating cell nuclear antigen (PCNA), as described previously for teleosts [14, 19, 33]. In our previous study on the teleost esophagus [19], the level of PCNA immunoreactivity was in agreement with the uptake of [^3H]-thymidine. Slides were immersed in 0.3% H_2O_2 in methanol at 20°C for 30 min to inactivate endogenous peroxidase activity. After washing in PBS, the sections were placed in 5% normal goat serum in PBS at room temperature for 1 h to block non-specific binding. Sections were subsequently incubated at 4°C overnight with the primary antibody diluted 1:100 in a

solution containing 0.5% Triton X-100 and 1% BSA (Sigma, Tokyo, Japan) in PBS. Sections were then washed 3 times in PBS, incubated with peroxidase-labeled goat anti-mouse secondary antibody (Sigma, Tokyo, Japan) diluted 1:70 in PBS containing 0.5% Triton X-100 and 1% BSA at room temperature for 1 h, and then developed for 5 min with DAB substrate solution (Roche, Tokyo, Japan). Controls omitting the PCNA primary antibody were performed and yielded no immunoreactivity.

2.6. *In situ* 3'-end labeling of DNA (TUNEL)

Nuclei of apoptotic cells were detected by the TUNEL method [39] using an *in situ* cell death detection kit (Roche, Tokyo, Japan; [14, 19, 33]). The TUNEL procedure produces results that are similar to those obtained with analysis of internucleosomal DNA fragmentation in the esophagus using gel electrophoresis, and appears to discriminate apoptosis from necrosis [19]. Fixed tissue samples were dehydrated through graded alcohol concentrations and embedded in Paraplast. Sections were cut at 5 μm and attached to 3-aminopropyltriethoxysilane-coated slides. The sections were then treated with 20 $\mu\text{g}/\text{ml}$ proteinase K (Roche, Tokyo, Japan) at 20°C for 30 min, washed in PBS for 15 min, and immersed in 0.3% H_2O_2 in methanol at 20°C for 30 min to inactivate endogenous peroxidase activity. After washing in PBS, the sections were incubated with TdT and fluorescein-labeled dUTP at 37°C for 1 h in a humidified chamber. The reaction was terminated by transferring the slides to PBS for 15 min. The sections were then incubated with peroxidase-labeled anti-fluorescein antibody at 37°C for 30 min and then for 5 min with DAB substrate solution (Roche, Tokyo, Japan). Omission of TdT gave completely negative results.

2.7. Statistical analysis

The significance of differences between the means for cell proliferation were analyzed using two-way repeated measures analysis of variance (ANOVA), with time within groups (after application of treatment) as one factor and treatment among or between groups as the other factor. Since there was a significant interaction between treatment and time, each time was analyzed separately to identify differences among the treatments using the appropriate post-hoc test. The significance of differences among means for concentration-response relationships for apoptosis was also tested using ANOVA followed by a post-hoc test. All data were checked for normality and equal variances. Where assumptions of normality or equal variances were not satisfied, equivalent non-parametric tests were used. Results were considered significant for $P < 0.05$.

3. Results

The effects of growth hormone, prolactin and somatolactin (1, 10 and 100 ng/ml) on esophageal cell proliferation for 8 days in culture are shown in Figures 1 and 2. Addition of prolactin at 10 ng/ml to the culture medium significantly ($P < 0.001$) enhanced cell proliferation after 4 and 8 days (Figs. 1A and 2). Significant ($P < 0.05$) induction of cell proliferation was also

observed at 1 day after treatment with 10 ng/ml growth hormone. In contrast, the esophagus did not respond significantly ($P > 0.05$) to treatment with somatolactin at any concentration. Similar results were obtained from six separate trials. Based on the highly significant results for prolactin treatment in the cell proliferation assays, the localization of the proliferating cells was examined in esophagi cultured for 8 days with or without 10 ng/ml prolactin (Fig. 1B). In control esophagi, labeling with an antibody against PCNA revealed few proliferating cell nuclei in the epithelium. In contrast, esophagi treated with prolactin had many PCNA-labeled nuclei in the epithelium and mucus cells in the epithelium were seemed to be more abundant than those in control tissue. No obvious longitudinal regionalization of the epithelial structure, including localization of proliferating cells, was observed.

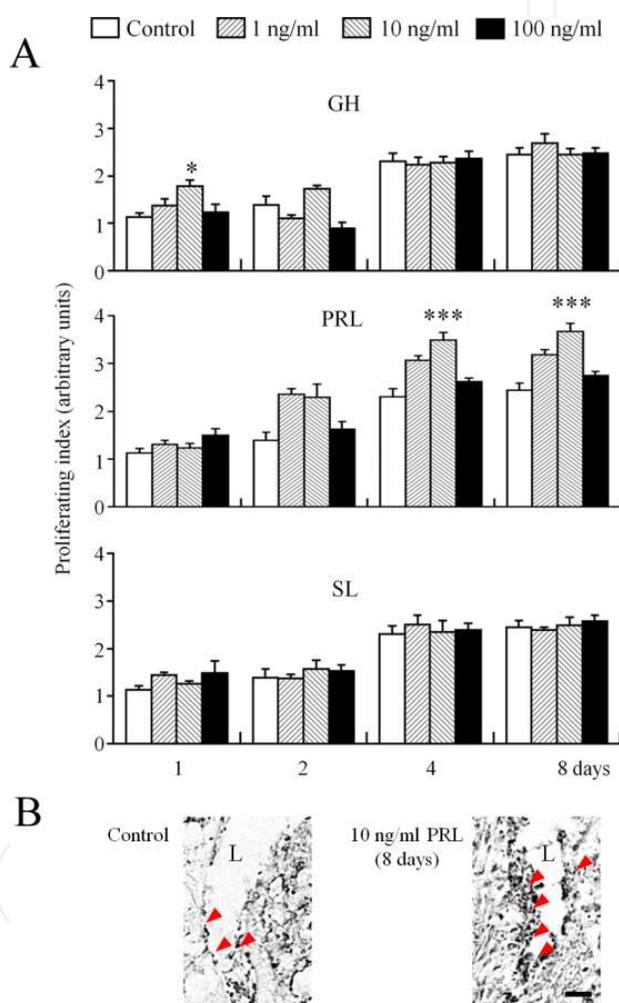


Figure 1. Effects of 1-100 ng/ml growth hormone (GH), prolactin (PRL) and somatolactin (SL) on cell proliferation of esophageal explants cultured for 1-8 days (A). Values are means \pm SEM ($N = 4-6$) and are expressed in arbitrary units normalized to the results on day 0 (initial tissue content). * $P < 0.05$, *** $P < 0.001$ vs. the control on the same day. Proliferating cell (dark nuclei, arrowheads) based on labeling by PCNA immunocytochemistry in the esophageal epithelium after 8 days in culture (B). A few PCNA-positive nuclei were detected in the epithelium in control esophagi, whereas many labeled nuclei appeared in the epithelium in esophagi treated with 10 ng/ml prolactin. L: lumen. Representative results are shown. Scale bar = 50 μ m.

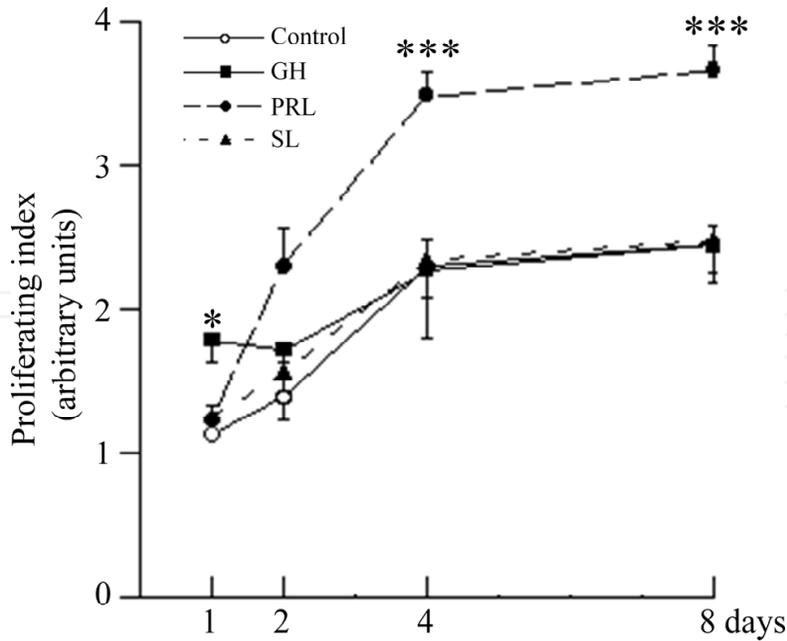


Figure 2. Effects of 10 ng/ml growth hormone (GH), prolactin (PRL) and somatolactin (SL) on cell proliferation of esophageal explants cultured for 1-8 days (redrawn from Fig. 1). Values are means \pm SEM ($N = 4-6$) and are expressed in arbitrary units normalized to the results on day 0 (initial tissue content). * $P < 0.05$, *** $P < 0.001$ vs. the control on the same day.

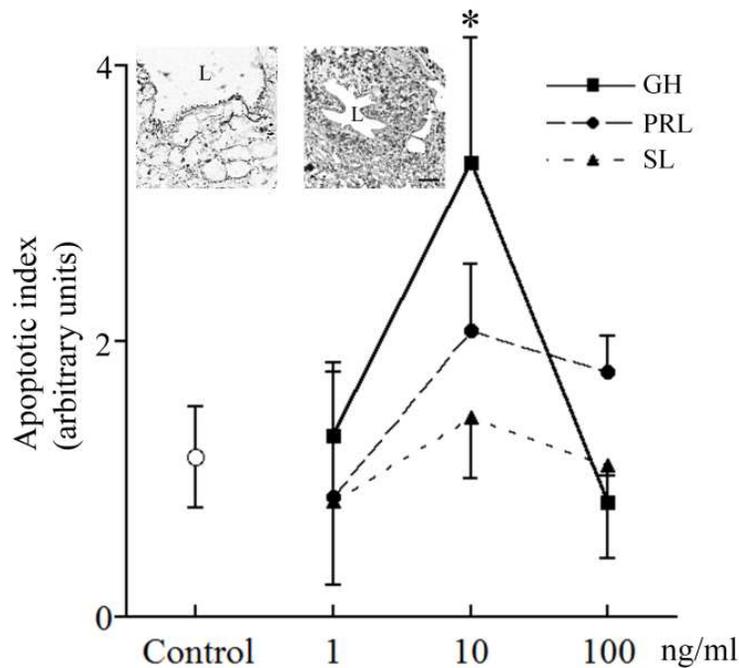


Figure 3. Effects of growth hormone (GH), prolactin (PRL) and somatolactin (SL) on esophageal apoptosis after 8 days of culture. Values are means \pm SEM ($N = 4-5$) and are expressed in arbitrary units normalized to the initial tissue content. * $P < 0.05$ vs. control. Apoptosis in the esophageal epithelium detected in a TUNEL assay after 8 days in culture (left and right insets). A few TUNEL-positive nuclei were observed in the epithelium in control esophagi (left inset), whereas numerous labeled nuclei appeared in the epithelium of esophagi treated with 10 ng/ml growth hormone (right inset). L: lumen. Representative results are shown. Scale bar = 50 μm .

We examined the effects of growth hormone, prolactin and somatolactin (1, 10 and 100 ng/ml) on esophageal apoptosis after 8 days of culture, since esophageal apoptosis has been shown to be induced significantly 5-10 days after salinity acclimation and hormonal treatment of fish [14, 19, 33]. Addition of growth hormone (10 ng/ml) to the culture medium significantly induced esophageal apoptosis ($P < 0.05$), whereas prolactin and somatolactin showed no significant effects at any doses (Fig. 3). Similar results were obtained from three separate trials. Based on these results, the localization of apoptotic cells was examined in esophagi cultured for 8 days with or without 10 ng/ml growth hormone. In control esophagi cultures, there were few apoptotic cells based on TUNEL staining of DNA fragments (Fig. 3, left inset), whereas a large number of apoptotic cells were observed in esophageal epithelia treated with 10 ng/ml growth hormone for 8 days (Fig. 3, right inset). No obvious longitudinal regionalization of apoptotic cells was observed.

4. Discussion

Prolactin is an important hormone for freshwater adaptation in teleost species, whereas growth hormone is involved in seawater adaptation in several euryhaline fishes [3]. In accord with the greater permeability of the esophagus in seawater-acclimated euryhaline fish than in freshwater-acclimated fish [20], we have previously shown that apoptosis throughout the esophageal epithelium occurs for the simple columnar epithelium in seawater and that cell proliferation is induced for the stratified epithelium, which is composed of numerous mucus cells, in fresh water [14, 19]. We have also shown that injection of prolactin stimulates cell proliferation in the esophageal epithelium [33], but neither the mode nor specificity of the action of prolactin is clear. The present study shows that the esophagus of medaka is responsive to prolactin and growth hormone after several days in culture, with induction of cell proliferation and apoptosis, respectively. This is the first demonstration of opposite *in-vitro* effects of prolactin and growth hormone on the teleost osmoregulatory organ. The 10-ng/ml concentrations of prolactin and growth hormone required for these specific responses were similar to the physiologically increased levels observed in the plasma of several teleost fishes during acclimation to different salinities [14, 29, 30]. The time course is also similar to those for esophageal cell proliferation and apoptosis as well as for plasma prolactin and growth hormone in euryhaline fishes during salinity acclimation and after *in-vivo* hormonal treatment [14, 19, 33]. The *in-vitro* response to prolactin in the esophagus is consistent with the localization of prolactin receptors in proliferating cells of esophageal epithelia found in our previous study [14]. The growth hormone receptor is also expressed in the gastrointestinal tracts of fishes, which accounts for the ability of growth hormone to act on these organs [8, 11, 40-42]. Taken together, these results suggest that the osmoregulatory esophagus is one of the primary targets for the actions of prolactin and growth hormone during acclimation of euryhaline fishes to fresh water and seawater, respectively.

The *in-vitro* effects of prolactin on the permeability of trout gill epithelia [16, 43] may also be associated with direct stimulation of cell proliferation by prolactin, as proposed above for the medaka esophagus. Prolactin has also been shown to induce cell proliferation in jejunal

explants from fetal rat [44], and several studies have shown direct effects of prolactin on cell proliferation throughout vertebrates [21]. In teleosts, prolactin induces proliferative responses in cultured salmonid leukocytes [25, 26], and promotes osteoblastic activities in goldfish scales *in vitro* [27]. However, the epithelium appears to be the major target, as in human keratinocytes and prostate epithelial cells [3, 45, 46], and it is likely that a primary function of prolactin in teleost osmoregulation is direct stimulation of cell proliferation in osmoregulatory epithelia. On the other hand, prolactin has also been shown to stimulate apoptosis in newt spermatogonia and rat luteal tissues [47, 48]. In addition, we suggested that the inhibitory effect of prolactin on osteoclastic activity in goldfish scales is mediated in part through osteoclast apoptosis [27]. Further studies of intracellular signaling pathway will elucidate how prolactin regulates these cell turnover. At any rate, one of prolactin's primary functions may be control of cell proliferation/apoptosis. Indeed, the prolactin receptors belong to the large superfamily of class 1 "cytokine" receptors.

There are few reports on the *in vitro* actions of growth hormone on teleost osmoregulatory organs, although growth hormone appears to be an important hormone for seawater adaptation. Direct regulatory roles of growth hormone on the gill Na^+, K^+ -ATPase and heat-shock protein 70 in climbing perch and silver sea bream have been described [15, 17, 18]. Our experiment reveals direct effects of growth hormone on esophageal cell turnover. Induction of cell proliferation was observed 1 day after addition of 10 ng/ml growth hormone, whereas epithelial apoptosis was stimulated after 8 days. The direct action of growth hormone on cell proliferation may occur through locally produced insulin-like growth factor I (IGF-I), since IGF-I has been suggested to mediate the direct proliferative effects of growth hormone in mammalian gastrointestinal tracts [49, 50]. On the other hand, the stimulation of apoptosis by long-term growth hormone exposure is at variance with the commonly reported protective role of the growth hormone /IGF-I axis against cell death [51]. However, increases in myocyte apoptosis are associated with high levels of growth hormone in patients with acromegaly [52] and in 9-month-old transgenic mice overexpressing growth hormone [53]. Furthermore, coho salmon implanted with growth hormone for 2 weeks showed stimulated Na-dependent proline absorption in the intestine [4], which may reflect increased permeability of the apoptotic intestinal epithelia in seawater.

At 100 ng/ml prolactin or growth hormone, the above significant effects on esophageal cell turnover were disappeared. Very high doses of these hormones may also activate receptors for the other hormones even in homologous systems. It is reported that prolactin can bind to growth hormone receptor in tilapia (*Oreochromis mossambicus*) [54], and that growth hormone has an ability to bind to somatolactin receptor in salmon (*Oncorhynchus masou*) [13]. In addition, our results may also be related to the functional distinction of multiple prolactin receptor isoforms in teleost fish [55]. These receptors have different sensitivities to prolactin and may have the distinct physiological functions as described above [27, 49, 50, 55-57].

In our previous study using this esophagus culture system, low levels of cortisol stimulate epithelial apoptosis through glucocorticoid receptors in seawater, whereas high levels of cortisol induce epithelial cell proliferation also via glucocorticoid receptors in freshwater [23]. Interactions of prolactin/growth hormone with glucocorticoid may play an important

role in the cell turnover in osmoregulatory esophageal epithelia during acclimation to different salinities. In the gill, cortisol is suggested to promote the differentiation of the ion-secretory chloride cell (seawater-form) with growth hormone, and also expedite the differentiation of ion-uptake chloride cell (freshwater-form) by interacting with prolactin [3, 58]. In the amphibian metamorphosis, on the other hand, thyroid hormones with glucocorticoid signaling induce apoptosis in the regression of tadpole tail, whereas prolactin prevents this apoptosis [22](Fig. 4B). Although apoptosis by glucocorticoid appear to be conserved throughout the vertebrates, thyroid hormone has no significant effect on esophageal cell proliferation or apoptosis in euryhaline fish [33]. Therefore, we hypothesize that thyroid hormones are involved only in irreversible metamorphosis and/or developmental processes.

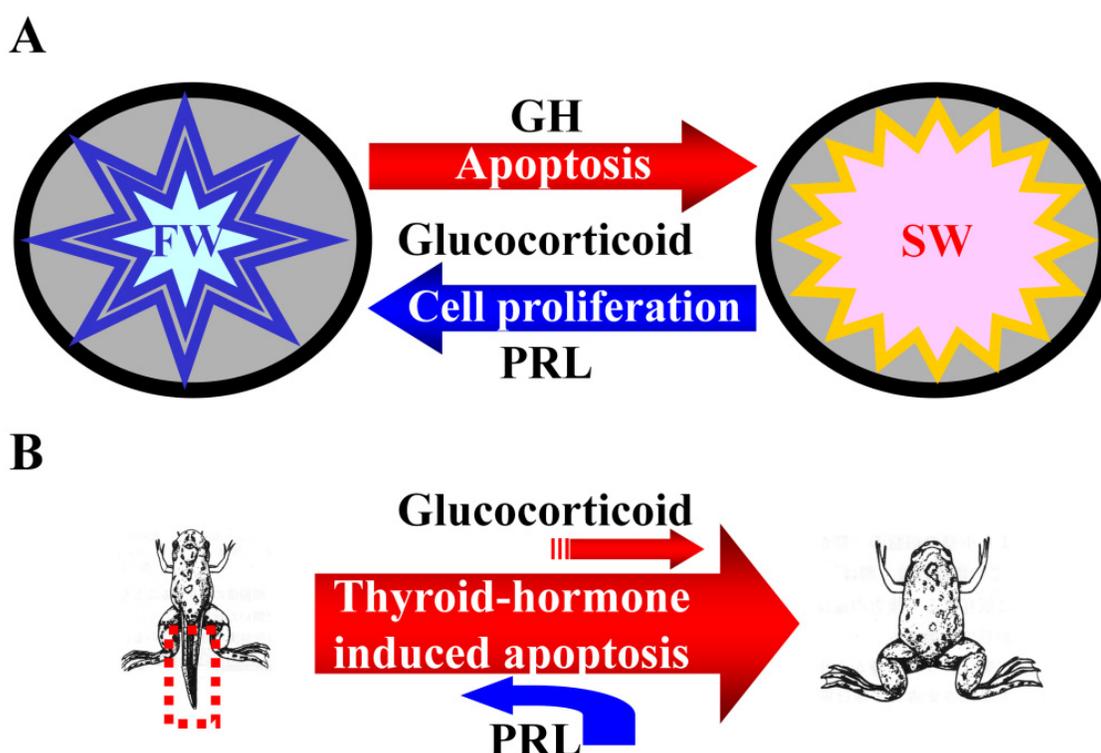


Figure 4. The summary representation of the epithelial differentiation in the esophagus of an euryhaline teleost during adaptation to different salinities. During freshwater (FW) adaptation, glucocorticoid signaling (cortisol-glucocorticoid receptor) and prolactin (PRL) stimulate epithelial cell proliferation for the stratified epithelium, resulting in the low permeability. During seawater (SW) adaptation, growth hormone (GH) and glucocorticoid signaling induce epithelial apoptosis for the simple epithelium with the high permeability (A). In the amphibian metamorphosis, thyroid hormones with glucocorticoid signaling induce apoptosis in tadpole tail whereas prolactin inhibits the apoptosis induced by thyroid hormones in regression of tadpole tail (B).

5. Conclusions

Our *in vitro* study in medaka esophagi indicates that prolactin directly induces the epithelial cell proliferation for the stratified epithelium and this response appears to be important in

the freshwater adaptive process. Furthermore, growth hormone directly stimulates the apoptosis for the simple epithelium and seems to be a key factor in seawater acclimation (Fig. 4A). To clarify the mechanism of prolactin/growth hormone actions in osmoregulation, future investigations using molecular tools are required to examine the relationship among the esophageal proliferating/apoptotic cells and important gene/protein expression patterns (e.g. prolactin/ growth hormone receptors, IGF-I and IGF-I receptor as well as transporters/pumps and intercellular junctions such as $\text{Na}^+, \text{K}^+, \text{ATPase}$, $\text{Na}^+, \text{K}^+, 2\text{Cl}^-$ cotransporter, aquaporins., tight junctions, gap junctions and claudins. A further study is also required determine if the apoptosis induced by growth hormone in medaka esophagus is also characteristic of other species.

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