Metabolic hormones regulate basal and growth hormone-dependent *igf2* mRNA level in primary cultured coho salmon hepatocytes: effects of insulin, glucagon, dexamethasone, and triiodothyronine

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Abstract

Igf1 and Igf2 stimulate growth and development of vertebrates. Circulating Igfs are produced by the liver. In mammals, Igf1 mediates the postnatal growth-promoting effects of growth hormone (Gh), whereas Igf2 stimulates fetal and placental growth. Hepatic Igf2 production is not regulated by Gh in mammals. Little is known about the regulation of hepatic Igf2 production in nonmammalian vertebrates. We examined the regulation of *igf2* mRNA level by metabolic hormones in primary cultured coho salmon hepatocytes. Gh, insulin, the glucocorticoid agonist dexamethasone (Dex), and glucagon increased *igf2* mRNA levels, whereas triiodothyronine (T₃) decreased *igf2* mRNA levels. Gh stimulated *igf2* mRNA at physiological concentrations (0.25×10^{-9} M and above). Insulin strongly enhanced Gh

stimulation of *igf2* at low physiological concentrations (10^{-11} M and above), and increased basal *igf2* (10^{-8} M and above). Dex stimulated basal *igf2* at concentrations comparable to those of stressed circulating cortisol (10^{-8} M and above). Glucagon stimulated basal and Gh-stimulated *igf2* at supraphysiological concentrations (10^{-7} M and above), whereas T₃ suppressed basal and Gh-stimulated *igf2* at the single concentration tested (10^{-7} M). These results show that *igf2* mRNA level is highly regulated in salmon hepatocytes, suggesting that liver-derived Igf2 plays a significant role in salmon growth physiology. The synergistic regulation of *igf2* by insulin and Gh in salmon hepatocytes is similar to the regulation of hepatic Igf1 production in mammals.

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Introduction

The insulin-like growth factors (Igf1 and Igf2) regulate growth in vertebrates (Humbel 1990, Stewart & Rotwein 1996). These mitogenic peptide hormones are produced throughout the body, and are present at high levels in the blood. Circulating Igfs are mainly derived from the liver. Both Igfs act through the same membrane receptor, the type 1 Igf receptor (Igf1r). The activity of both Igfs is modulated by a family of Igf-binding proteins (Igfbps), which bind strongly to the Igfs and may inhibit or enhance their activity. In mammals, the functions of the Igfs are divided by developmental stage. Igf2 regulates embryonic growth, and regulates the growth of the placenta, but Igf2 is not thought to regulate growth in postnatal mammals. Igf1, in contrast, is a principal regulator of postnatal growth in mammals, and disruption of Igf1 signaling results in severe postnatal growth deficits (Efstratiadis 1998). Both liver-derived endocrine Igf1 and locally produced Igf1 contribute to the regulation of growth. In mice, the contributions of endocrine and local Igf1 are roughly equal (Stratikopoulos *et al.* 2008).

Research on the role of endocrine Igf1 in growth regulation has been guided by the somatomedin hypothesis, which states that Gh secreted by the pituitary gland stimulates the liver to produce Igf1 (Humbel 1990). Igf1 mediates the growth-promoting effects of Gh, and feeds back at the pituitary and hypothalamus to inhibit Gh secretion, completing the Gh/Igf1 endocrine axis. The basic framework of the somatomedin hypothesis is widely accepted; however, it is now clear that multiple levels of regulation occur downstream from Gh secretion. At the liver, the sensitivity of hepatocytes to stimulation by Gh is modulated by physiological state. During catabolic states such as fasting or disease, liver Igf1 production becomes insensitive to stimulation by Gh, a phenomenon termed Gh resistance (Thissen *et al.* 1994, Rodgers 1996). After birth, Igf2 circulates at levels comparable to those of Igf1 in most mammalian species, except in rodents (Humbel 1990, Stewart & Rotwein 1996). However, in contrast to Igf1, liver Igf2 production is not stimulated by Gh in adult mammals. Unlike Igf1, plasma Igf2 levels do not respond to physiological status or relate to growth rate (Holly 1998). For these reasons, Igf2 is not

considered part of the Gh/Igf1 endocrine axis proposed in the somatomedin hypothesis. The function of endocrine Igf2 in mammalian postnatal life remains enigmatic (Humbel 1990, Holly 1998). In contrast, important roles for locally produced Igf2 in cell growth and differentiation in the muscle and other tissues have been described recently (Chao & D'Amore 2008).

All components of the Gh/Igf1 axis are present in teleost fishes. Major functional relationships, such as stimulation of liver Igf1 production by Gh, modulation of liver Gh sensitivity by physiological state (Gh resistance), and modulation of Igf actions by Igfbps, are well conserved (Kelley *et al.* 2002, Reinecke *et al.* 2005, Wood *et al.* 2005). Variation in individual fish plasma Igf1 level can explain 50–60% of variation in growth rate in fishes (Picha *et al.* 2008a). Teleost fishes differ from mammals in that liver *igf2* gene expression is stimulated by Gh (Shamblott *et al.* 1995, Tse *et al.* 2002, Vong *et al.* 2003, Carnevali *et al.* 2005, Peterson *et al.* 2005, Gahr *et al.* 2008, Moriyama *et al.* 2008, Ponce *et al.* 2008, Devlin *et al.* 2009). Liver *igf2* gene expression responds to fasting and metabolic status in fishes (Peterson *et al.* 2004, Gabillard *et al.* 2006, Ayson *et al.* 2007, Terova *et al.* 2007, Picha *et al.* 2008b), and plasma Igf2 levels are decreased by fasting (Gabillard *et al.* 2006, Wilkinson *et al.* 2006). These findings suggest that Igf2 plays a role in the Gh/Igf1 (henceforward Gh/Igf) axis in fishes. However, the roles of hormones associated with metabolic status in the regulation of liver *igf2* gene expression have not been studied in detail.

Gh reliably stimulates *igf1* gene expression in primary cultured fish hepatocytes (Duan *et al.* 1993, Shamblott *et al.* 1995, Schmid *et al.* 2000, Pierce *et al.* 2004, 2005a, Leung *et al.* 2008) and liver tissue incubation (Moriyama *et al.* 2006). In coho salmon (*Oncorhynchus kisutch*) hepatocytes, glucocorticoids strongly reduced Gh stimulation of *igf1* mRNA levels, suggesting that glucocorticoids mediate the development of Gh resistance in this species (Pierce *et al.* 2005a). Surprisingly, insulin also reduced Gh stimulation of coho salmon hepatocyte *igf1* mRNA levels, the inverse of the synergy found in mammals and birds (e.g. Tollet *et al.* 1990, Houston & O'Neill 1991). Gh stimulates *igf2* gene expression in rainbow trout (*Oncorhynchus mykiss*) primary hepatocytes (Shamblott *et al.* 1995) and in tissue incubation of eel (*Anguilla japonica*) liver slices (Moriyama *et al.* 2008). We hypothesized that metabolic hormones may modulate the effect of Gh on liver Igf2 production in fishes. To test this hypothesis, we examined basal and Gh-dependent effects of insulin, glucagon, dexamethasone (Dex), and triiodothyronine (T_3) on *igf2* gene expression in primary cultured coho salmon hepatocytes. Our results show that both basal and Gh-stimulated *igf2* levels are highly regulated in salmon hepatocytes. In conjunction with our previous studies (Pierce *et al.* 2004, 2005a), our results suggest that hepatic Igf2 and Igf1 production is inversely regulated by insulin and glucocorticoids in salmon.

Materials and methods

Animals

Coho salmon were raised in 1.3-m diameter cylindrical tanks with recirculated fresh water at 11–12 °C under simulated natural photoperiod at the Northwest Fisheries Science Center (Seattle, WA, USA). Fishes were fed BioOregon-grower (use of trade names does not imply endorsement of NOAA, US Department of Commerce) pellets at a ration of 0.6% body weight per day. Two-year-old coho salmon were used. Fishes were fasted for 1 day prior to cultures. Data shown are from the following cultures: 12/07/02, pooled cells from two immature male fishes, 146 and 179 g for Fig. 1; 10/12/02, independent cultures from three immature female fishes, 143, 149, and 157 g for Fig. 2A and B; 7/10/04, pooled cells from two immature male fishes, 155 and 169 g for the fourth replicate in Fig. 2B; 6/12/04, pooled cells from two immature male fishes, 262 and 275 g for Fig. 3; 8/13/05, independent cultures from three immature fishes, one male, 349 g, and two females, 292 and 411 g, for Fig. 2C; 9/29/08, pooled cells from three immature male fishes, 155, 150, and 120 g for Fig. 4; and 10/06/08, pooled cells from two immature male fishes, 120 and 135 g for Fig. 5.

Hepatocyte culture and RNA isolation

Hepatocytes were isolated and cultured as described previously (Pierce *et al.* 2004). Cells were cultured in Falcon Primaria 24-well plates at 15 °C in modified RPMI 1640 medium (Gibco BRL; 20 mM HEPES, 5 mM NaHCO_3 , and 0.2% BSA) under plain air. Hepatocytes were plated at a density of $1\text{--}2 \times 10^6$ cells/ml, 0.5 ml/well, and were allowed to adhere for 24 h, and then the medium was changed to the test medium containing hormones. Cultures were stopped 18 h later by adding Tri Reagent (MRC, Cincinnati,

Figure 1 Effect of Gh (coho salmon) concentration on *igf2* mRNA level in primary cultured salmon hepatocytes. Error bars show S.E.M.; points not sharing a superscript letter differ significantly (Student–Newman–Keuls test, $P < 0.05$, $n = 5\text{--}6$ wells per point). Data shown is from a single cell preparation and culture.

OH, USA) to the wells. This time point was determined previously to be appropriate for the measurement of *igf1* gene expression in salmon primary hepatocyte culture (Pierce *et al.* 2004). We assumed that the 18-h time point would also be appropriate for *igf2* gene expression. Please see

Duan *et al.* (1993) for illustrations showing the morphology of cultured salmon hepatocytes. RNA was isolated following the MRC protocol, with bromochloropropane as the phase separation reagent and two 70% ethanol washes. RNA was quantified by spectrophotometry (260:280 ratios 1.8–2.0), and was diluted to 20 ng/μl.

Hormones

Native coho salmon Gh was purified by HPLC. Recombinant salmon/trout (*Oncorhynchus spp.*) Gh was purchased from GroPep Ltd (Adelaide, SA, Australia). T₃, water-soluble Dex (cyclodextrin encapsulated), native bovine insulin, and native bovine glucagon were purchased from Sigma.

Real-time quantitative RT-PCR

Hepatocyte *igf2* mRNA levels were quantified using a TaqMan probe quantitative RT-PCR (qPCR) assay as described previously (Campbell *et al.* 2006). The amplicon for the *igf2* qPCR assay was in the region of the transcript encoding the leader peptide and covering the predicted exon 2/exon 3 junction. Gene expression data were normalized to the expression level of acidic ribosomal phosphoprotein P0 (*arp*, listed as *rplp0* in the Zfin Database) using an efficiency-corrected relative expression technique (Pierce *et al.* 2004). Primer and probe sequences for qPCR assays are listed for reference in Table 1. First-strand cDNA was synthesized in 10-μl reverse transcription (RT) reactions with 2 μl RNA template, 2.5 U/μl SuperScript II reverse transcriptase (Invitrogen), 5 μM random hexamer primers, 500 μM dNTPs, 0.4 U/μl RNase inhibitor (Applied Biosystems, Inc. (ABI), Foster City, CA, USA), 10 mM dithiothreitol, and 1 × RT buffer, and was incubated at 25 °C for 10 min, 48 °C for 60 min, and 95 °C for 5 min. PCR assays were run in 96-well format on an ABI 7700 qPCR machine using the standard cycling conditions recommended by the manufacturer (50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 1 min). Wells contained 25 μl PCR mixture, prepared from ABI Universal PCR Master Mix, with 200 nM probe, 900 nM forward and reverse primers, and 2 μl cDNA template. As a standard, a fivefold serial dilution from a coho salmon liver (highest concentration of 100 ng/ml RNA input to RT reaction, four standards, three replicate wells per standard) was run on each

Figure 2 Effects of metabolic hormones on basal and Gh-stimulated *igf2* mRNA levels in primary cultured salmon hepatocytes. (A) Effects of dexamethasone (Dex, cyclodextrin encapsulated), triiodothyronine (T₃), and Gh (coho salmon). (B) Effects of insulin (Ins, bovine) and Gh. (C) Effects of glucagon (Glu, bovine) and Gh. Error bars show S.E.M.; points or bars not sharing a superscript letter differ significantly (Student–Newman–Keuls test, $P < 0.05$). Data shown are combined from three, four, and three independent cultures respectively, four wells per treatment per culture. For each experiment, data from replicate cultures were normalized to the average response to Gh within that experiment.

Figure 3 Effect of dexamethasone concentration on *igf2* mRNA level in primary cultured salmon hepatocytes. Error bars show S.E.M.; points not sharing a superscript letter differ significantly (Student–Newman–Keuls test, $P < 0.05$, $n = 5$ –6 wells per point). Data shown is from a single cell preparation and culture.

plate. A single replicate of each PCR was run. TaqMan PCRs for target and reference genes were run from the same RT reaction.

Data analysis

Gene expression levels were expressed relative to the control treatment for each hepatocyte preparation. Descriptions of increases or decreases in gene expression refer only to the comparison of control and treatment groups at the single time point used in this study. Gene expression data were $\log_2$ transformed prior to analysis to achieve homogeneity of variance. In the factorial experiments (Fig. 2), the pattern

Figure 4 Effect of insulin (bovine) concentration on basal and Gh-stimulated (GroPep recombinant salmon/trout) *igf2* mRNA levels in primary cultured salmon hepatocytes. Error bars show S.E.M. The effect of Gh was significant at every insulin concentration; within the basal and Gh-stimulated treatment groups, points not sharing a superscript letter differ significantly (Student–Newman–Keuls test, $P < 0.05$, $n = 6$ –10 wells per point). Data shown are from a single cell preparation and culture.

of responses to different treatments was similar between hepatocyte preparations, but the magnitude of responses differed sometimes. Therefore, for each experiment, the data from each hepatocyte culture were normalized to the average Gh response level of all cultures within that experiment. Treatment and culture effects were examined by one- or two-way ANOVA, followed by the Student–Newman–Keuls test. Statistical analysis was conducted with PRISM 4 (GraphPad Software, San Diego, CA, USA). Results were considered statistically significant at $P < 0.05$.

Results

Gh increased *igf2* mRNA level in salmon hepatocytes in a concentration-dependent manner (Fig. 1). The increase in *igf2* mRNA became significant at 0.25 nM Gh and was maximal at 5 nM Gh (3.35-fold). Decreases from maximal *igf2* expression occurred at higher Gh concentrations; however, *igf2* mRNA levels were not significantly lower than the maximal level (Gh 500 vs 5 nM: $P = 0.0891$, Fisher's LSD).

Factorial experiments were conducted to assess basal and Gh-dependent effects of metabolic hormones on *igf2* gene expression (Fig. 2). At least three replicate cultures were tested in each experiment. All cultures responded to Gh with a significant increase in *igf2* mRNA level. After normalization to the average response to Gh, replicate cultures showed strong and highly significant effects of experimental treatments with metabolic hormones, and much weaker effects of culture and treatment $\times$ culture interaction in two-way ANOVA (Fig. 2A: treatment 85.61% of variation, $P < 0.0001$; culture 0.42% of variation, $P = 0.2089$; interaction 6.90% of variation, $P < 0.0001$; Fig. 2B: treatment 74.9% of variation, $P < 0.0001$; culture 0.96% of variation, $P = 0.3356$; interaction 8.82% of variation, $P = 0.002$; Fig. 2C: treatment 80.54% of variation, $P < 0.0001$; culture 6.82% of variation, $P < 0.0001$; interaction 8.59% of variation, $P < 0.0001$). Therefore, data are presented for combined replicate cultures.

T_3 (10^{-7} M) significantly decreased basal and Gh-stimulated *igf2* mRNA levels (Fig. 2A). The decrease was of a comparable magnitude (basal: 0.49; Gh-stimulated: 0.67 copies of *igf2* mRNA). In contrast, Dex (10^{-6} M) significantly increased basal and Gh-stimulated *igf2* mRNA levels. The increase was of a comparable magnitude (basal: 0.76; Gh-stimulated: 0.86 copies of *igf2* mRNA). Insulin (10^{-6} M) significantly and strongly increased basal and Gh-stimulated *igf2* mRNA levels (Fig. 2B). The increase was greater in the presence of Gh (basal: 1.19; Gh-stimulated: 2.27 copies of *igf2* mRNA). Glucagon (10^{-6} M) significantly and even more strongly increased basal and Gh-stimulated *igf2* mRNA levels (Fig. 2C). The increase was much greater in the presence of Gh (basal: 2.14; Gh-stimulated: 7.11 copies *igf2* mRNA). These findings suggest that glucagon and insulin may interact with Gh in the regulation of *igf2* gene expression in salmon hepatocytes, but provide no support for

Figure 5 Effect of glucagon (bovine) concentration on basal and Gh-stimulated (GroPep recombinant salmon/trout) *igf2* mRNA levels in primary cultured salmon hepatocytes. Error bars show S.E.M. The effect of Gh was significant at every glucagon concentration; within the basal and Gh-stimulated treatment groups, points not sharing a superscript letter differ significantly (Student–Newman–Keuls test, $P < 0.05$, $n = 4$ –10 wells per point). Data shown are from a single cell preparation and culture.

interactions between Gh and Dex or Gh and T_3 . Therefore, concentration–response studies were conducted with Dex by itself, and with insulin and glucagon in combination with Gh.

Dex increased *igf2* mRNA levels in salmon hepatocytes in a concentration-dependent and biphasic manner (Fig. 3). Dex concentrations as low as 10^{-12} M were tested because 10^{-12} M Dex significantly repressed Gh-stimulated *igf1* mRNA levels in a previous study (Pierce *et al.* 2005a). However, in the current study, Dex did not affect hepatocyte *igf2* mRNA levels at concentrations from 10^{-12} to 10^{-9} M. The *igf2* mRNA increase in response to Dex became significant at 10^{-8} M and was maximal at 10^{-7} M (3.16-fold). *igf2* mRNA was significantly reduced from maximal at 10^{-6} M Dex.

The effect of insulin on *igf2* mRNA levels, and potential interactions between insulin and Gh were assessed in a concentration–response study (Fig. 4). The effects of Gh, insulin, and the interaction term were significant in a two-way ANOVA (Gh 48.9% of variation, $P < 0.0001$; insulin 27.3% of variation, $P < 0.0001$; interaction 5.9% of variation, $P < 0.0001$). Gh (5 nM) significantly increased *igf2* mRNA levels at all concentrations of insulin (1.7-fold

with no insulin). Insulin increased basal *igf2* mRNA levels in a concentration-dependent manner. The increase became significant at 10^{-8} M and was maximal at 10^{-6} M (1.9-fold). There was a significant linear trend toward increased *igf2* mRNA with increasing insulin concentration from 10^{-10} to 10^{-6} M ($P < 0.0001$, $r^2 = 0.416$), but not with that from 0 to 10^{-10} M ($P = 0.97$). Insulin increased Gh-stimulated *igf2* mRNA levels in a concentration-dependent manner. The increase versus Gh alone became significant at 10^{-11} M. The steepest increase occurred from 10^{-12} to 10^{-10} M insulin, and maximal response occurred at 10^{-8} M (3.0-fold increase versus Gh alone; approximately fivefold versus basal). A nonsignificant decrease from maximal response was found at 10^{-6} M insulin (10^{-8} vs 10^{-6} M, $P = 0.0578$, Fisher's LSD).

Interactions between glucagon and Gh were assessed in a concentration–response study (Fig. 5). The effects of Gh and glucagon were significant in a two-way ANOVA; however, the interaction term was not (Gh 55.1% of variation, $P < 0.0001$; glucagon 18.1% of variation, $P < 0.0001$; interaction 1.1% of variation, $P = 0.5536$). Gh (5 nM) significantly increased *igf2* mRNA levels at all concentrations of glucagon (2.2-fold with no glucagon). Glucagon significantly increased basal *igf2* mRNA levels at 10^{-7} and 10^{-6} M; the increase was maximal at 10^{-6} M (1.8-fold). Similarly, glucagon increased Gh-stimulated *igf2* mRNA levels versus controls at 10^{-7} and 10^{-6} M, with maximal response at 10^{-7} M glucagon (1.7-fold versus Gh alone).

The C_t for *arp* did not vary between treatments in any experiment, indicating that treatments did not affect our reference gene.

Discussion

This study shows that *igf2* mRNA levels are highly regulated in primary cultured salmon hepatocytes. *igf2* mRNA levels were increased by Gh, Dex, insulin, and glucagon, and were decreased by T_3 . In tetrapods, in contrast, liver Igf2 production has not been shown to be regulated by hormones in adult animals (Humbel 1990, Stewart & Rotwein 1996, Holly 1998). This suggests that liver-derived endocrine Igf2 may play a greater role in growth regulation in sexually immature subadult salmon than in adult tetrapods.

Gh stimulated *igf2* gene expression in salmon hepatocytes (Fig. 1). Stimulation became significant at 0.25 nM Gh, and was maximal at 5 nM. Stimulation by Gh occurred reliably with 5 nM Gh (Fig. 2). Physiological concentrations of Gh range from < 50 pM to over 5 nM in salmonids (Gomez *et al.* 1996, Pierce *et al.* 2005b). Thus, the response of cultured hepatocytes occurred over the physiological range. *igf2* gene expression levels declined at Gh concentrations above 5 nM ($P = 0.0891$), suggesting that the response is probably biphasic. Previous *in vitro* studies in fishes found Gh stimulation of *igf2* gene expression in cultured rainbow trout hepatocytes (Shamblott *et al.* 1995) and in tissue

Table 1 Primer and probe sequences for coho salmon RT-PCR assays

Element	Sequence (5'–3')
Igf2 F primer	AACACCATCCGCAAGAACTG
Igf2 R primer	TGTCCAGAGCTGCATGCA
Igf2 probe	Fam-TGGAACAGGGTCCCTGCCACATTG-Tamra
Arp F primer	GAAAATCATCCAATTGCTGGATG
Arp R primer	CTCCACGCAAGGACAGA
Arp probe	Vic-CTATCCCAAATGTTTCATTGTCGGCGC-Tamra

incubation of eel liver slices (Moriyama *et al.* 2008). The response of *igf2* to Gh in salmon hepatocytes is quite similar to the response of *igf1* (Pierce *et al.* 2005a). *In vivo*, Gh treatment increases liver *igf2* gene expression in a diverse array of teleost species (Shamblott *et al.* 1995, Tse *et al.* 2002, Vong *et al.* 2003, Carnevali *et al.* 2005, Peterson *et al.* 2005, Gahr *et al.* 2008, Moriyama *et al.* 2008, Ponce *et al.* 2008, Devlin *et al.* 2009). These studies and the present study add to a growing consensus that Igf2 functions as a somatomedin in teleost fishes.

The glucocorticoid receptor agonist Dex increased *igf2* mRNA levels in cultured salmon hepatocytes (Fig. 2A). Dex stimulated *igf2* at 10^{-9} – 10^{-6} M, with maximal stimulation at 10^{-7} M and a decrease from maximal stimulation at 10^{-6} M (Fig. 3). The native glucocorticoid hormone in fishes is cortisol. Cortisol concentrations range from $\sim 10^{-9}$ to 10^{-6} M from basal to stressed respectively in salmonids (Norris & Hobbs 2006). Dex is five- to ten-fold stronger than cortisol (Mommensen *et al.* 1999). Thus, the response of *igf2* to Dex in cultured salmon hepatocytes occurred over the stressed range. In a study in gilthead sea bream (*Sparus aurata*), both Gh-injected and saline-injected fishes showed very high liver *igf2* gene expression (Duguay *et al.* 1996), possibly mediated by a cortisol response to the injection.

In mammals, glucocorticoid signaling plays a role in the development of liver Gh resistance in terms of Igf1 production (Thissen *et al.* 1994, Rodgers 1996). Glucocorticoid induction of Gh resistance in terms of Igf1 production appears to be conserved in fishes (e.g. Kajimura *et al.* 2003). In silver sea bream (*Sparus sarba*) hepatocytes, cortisol decreased basal *igf1* mRNA levels at physiological concentrations (Leung *et al.* 2008). In salmon hepatocytes, Dex did not affect basal *igf1*, but it strongly inhibited Gh-stimulated *igf1* over an extremely wide range of concentrations (10^{-12} to 10^{-6} M; Pierce *et al.* 2005a). These and other studies show that liver *igf1* gene expression and circulating Igf1 levels decrease during stress in fishes. The current study suggests that liver *igf2* gene expression and circulating Igf2 levels would be expected to increase during stress. Further studies are required to determine whether this inverse regulation occurs *in vivo*.

The most striking finding of the current study was the synergism between insulin and Gh in stimulation of hepatocyte *igf2* gene expression. Insulin stimulated basal *igf2* at 10^{-6} M, and the combination of insulin and Gh appeared greater than additive (Fig. 2B). A detailed concentration–response study revealed that insulin synergized with Gh at concentrations of 10^{-11} M and above (Fig. 4). The steepest increase occurred from 10^{-12} to 10^{-10} M insulin, with a decline from maximal stimulation at 10^{-6} M ($P=0.0578$). Insulin significantly stimulated basal *igf2* at 10^{-8} M and above. In salmonids, insulin concentrations in the hepatic portal vein are approximately fivefold higher than those in the general circulation (Plisetkaya & Sullivan 1989), suggesting that coho salmon hepatocytes experience insulin concentrations from $\sim 10^{-9}$ M during fasting to 10^{-7} M

postprandially *in vivo* (Shimizu *et al.* 2009). Salmon hepatocytes would be expected to respond more strongly to salmon insulin than the bovine insulin used in this study (Navarro *et al.* 2006). Therefore, the present study suggests that physiological concentrations of insulin are required for normal Gh stimulation of Igf2 production by the salmon liver.

We are not aware of any studies in fishes addressing the regulation of hepatic *igf2* by insulin. The only relevant mammalian hepatocyte culture studies used fetal hepatocytes. In fetal rat hepatocytes, *Igf2* gene expression was positively regulated by insulin, but it was not responsive to Gh (Goya *et al.* 1999, 2001). Based on this very limited dataset, basal insulin regulation of *igf2* in juvenile salmon hepatocytes is similar to insulin regulation of *igf2* in fetal rat hepatocytes. In contrast to the scarcity of information on *igf2* regulation, there are many studies in tetrapods on insulin regulation of liver *igf1* gene expression and Igf1 secretion. Insulin synergizes with GH to stimulate hepatocyte *Igf1* gene expression and IGF1 protein secretion in mammals and birds, and also stimulates basal *igf1* and IGF1 (Tollet *et al.* 1990, Boni-Schnetzler *et al.* 1991, Houston & O'Neill 1991, Phillips *et al.* 1991). The synergism is found over physiological concentrations of insulin. In rats, normal secretion of insulin into the hepatic portal vein is required to maintain liver IGF1 production, plasma IGF1 levels, and growth *in vivo* (Griffen *et al.* 1987). In catabolic lactating cows, insulin infusion is sufficient to restore liver GH sensitivity and plasma IGF1 levels (Butler *et al.* 2003). Thus, the regulation of salmon hepatocyte *igf2* by insulin and Gh found in the present study is similar to the regulation of hepatic Igf1 production by insulin and Gh in tetrapods.

Insulin regulation of *igf2* gene expression contrasts with insulin regulation of *igf1* gene expression in salmon hepatocytes. Insulin did not affect basal *igf1* in salmon hepatocytes, but it inhibited Gh-stimulated *igf1* (Pierce *et al.* 2005a). Although insulin can bind Igf1r with lower affinity than Igf1 (Wood *et al.* 2005), addition of 5 nM Igf1 to culture medium did not affect basal or Gh-stimulated *igf1* mRNA level, suggesting that these effects are not mediated by the Igf1r pathway (Pierce *et al.* 2004). In the present study, the significant increase in Gh-stimulated *igf2* mRNA level with 10^{-11} M insulin treatment is not consistent with an effect mediated by Igf1r, and also argues against an effect mediated by insulin-induced changes in hepatocyte metabolism. Plasma insulin increased strongly after a meal in fed salmon, whereas plasma Igf1 increased more weakly, and the response of Igf1 but not of insulin was abolished by fasting for 1–3 weeks (Shimizu *et al.* 2009). In the context of the present study, these findings predict that, *in vivo*, liver *igf2* gene expression and plasma Igf2 may increase more strongly than plasma Igf1 in response to postprandial increases in hepatic portal insulin. This could provide a mechanism for nutritional regulation of plasma Igf2. Plasma Igf2 levels are positively regulated by nutritional status in salmonids (Gabillard *et al.* 2006, Wilkinson *et al.* 2006). Further studies are required to investigate these relationships.

Glucagon increased basal and Gh-stimulated *igf2* in salmon hepatocytes (Fig. 2C). The combination appeared synergistic at 10^{-6} M glucagon and 5×10^{-9} M Gh. A concentration–response experiment showed that glucagon concentrations of 10^{-7} M and above increased basal and Gh-stimulated *igf2* (Fig. 5). However, the synergism between glucagon and Gh was not apparent in this experiment. Hepatic portal glucagon concentrations are around 10^{-10} M in salmonids (Plisetskaya & Mommsen 1996). Therefore, the effect of glucagon on *igf2* is probably not important in the normal physiological regulation of *igf2*. In our previous study, glucagon inhibited Gh-stimulated *igf1* at 10^{-6} M (Pierce *et al.* 2005a). At concentrations as low as 10^{-8} M, heterologous glucagons increase glycogenolysis and lipid catabolism in fish hepatocytes (Plisetskaya & Mommsen 1996). Regulation of the Igfs by high concentrations of glucagon in salmon hepatocytes suggests that the Igfs may respond to cellular concentrations of metabolic substrates. In fetal rat hepatocytes, both Igfs are stimulated by glucose (Goya *et al.* 1999). An indirect effect of glucagon through changes in hepatocyte metabolism might also explain the variability in magnitude of response found in the present study, since hepatocyte preparations would be expected to vary in stores of glycogen and lipids. The effect of high concentrations of insulin on basal *igf2* could also be mediated by an effect on hepatocyte metabolism. Further studies are required to address these possibilities.

T_3 (10^{-7} M) suppressed both basal and Gh-stimulated *igf2* in salmon hepatocytes (Fig. 2A). We are not aware of any vertebrate hepatocyte culture studies reporting the effects of thyroid hormones on *igf2*. In cultured tilapia (*Oreochromis mossambicus*) hepatocytes, thyroid hormones increased basal *igf1* (Schmid *et al.* 2003), and in silver sea bream hepatocytes, thyroid hormones increased basal *igf1* at high concentrations (Leung *et al.* 2008). In coho salmon hepatocytes, no effect of 10^{-7} M T_3 was found on basal or Gh-stimulated *igf1* (Pierce *et al.* 2005a). In anadromous salmonids such as coho salmon, thyroid hormones increase during smoltification, the series of preadaptive physiological, developmental, and behavioral changes that occur prior to migration to the ocean (Folmar & Dickhoff 1980). It is possible that suppression of liver *igf2* gene expression by T_3 plays a role in smoltification.

Most of the effects of hormonal treatment on *igf2* mRNA levels found in this study occurred at physiological concentrations. Furthermore, in other studies using identical culture conditions, we found differential and often inverse regulation of different transcripts. Gh increased *igf1* and *igf2* mRNA levels, but decreased *igfbp1* mRNA and protein levels (present study; Pierce *et al.* 2005a, 2006). Insulin increased basal and Gh-stimulated *igf2*, decreased Gh-stimulated *igf1*, and decreased *ghr* mRNA levels. Dex increased *igf2*, *igfbp1*, and *ghr* mRNA levels, but strongly decreased Gh-stimulated *igf1* mRNA levels. These considerations suggest that most of the effects found in this study cannot be due to nonspecific improvements in cell health due to hormonal treatment.

In conclusion, this study shows that metabolic hormones strongly regulate both basal and Gh-stimulated *igf2* mRNA

levels in salmon primary hepatocytes. This level of regulation suggests that liver-derived endocrine Igf2 must play a significant role in salmon growth regulation. Insulin strongly synergized with Gh to stimulate *igf2*, and this effect was found at low concentrations of insulin. This suggests that insulin is a major positive regulator of liver Igf2 production in salmon via enhancement of the effect of Gh, which is similar to the way liver Igf1 production is regulated in tetrapods. However, insulin suppresses hepatocyte *ghr* mRNA levels, suggesting that enhancement of Gh sensitivity in terms of *igf2* gene expression may occur downstream from Ghr (Pierce *et al.* 2005a). In combination with our previous studies (Pierce *et al.* 2004, 2005a), our findings suggest that liver Igf1 and Igf2 production is differentially regulated by insulin, glucocorticoids, glucagon, and thyroid hormones in salmon. This suggests that differential effects of the two Igfs may exist. All our data are at the level of *igf2* gene expression *in vitro*. It will be necessary to confirm these findings at the level of protein secretion and circulating Igf2 *in vivo*. However, as far as it is known, Igf2 is constitutively secreted; gene expression and secreted protein levels correlate well in mammalian hepatocyte culture studies (Goya *et al.* 1999, 2001), and liver *igf2* gene expression and Igf2 production drive circulating levels *in vivo* (Humbel 1990).

Declaration of interest

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

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Author contribution statement

ALP and WWD designed the studies, analyzed the results, and wrote the manuscript. ALP conducted the cell culture experiments and qPCR assays. PS provided native salmon Gh. JTD designed the *igf2* qPCR and managed the laboratory. LF ran qPCR assays. PS and WWD provided funding, laboratory facilities, reagents, and project oversight.

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