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The Liver-Enriched Transcription Factors HNF-1α, HNF-3β, and C/EBPβ Contribute to the Growth Hormone-Induced Transcription of the Progranulin a Gene in Zebrafish (*Danio Rerio*)

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Abstract

Progranulin (PGRN) is a secreted growth factor that has been implicated in diverse biological processes including wound healing, embryo development, morphogenesis and disease. We previously showed that PGRN is induced along with IGF-1 in tilapia liver upon GH administration. In the present study, we demonstrate that the co-induction of PGRN and IGF-1 by GH administration is a common regulation in fish also presented in zebrafish. To understand the regulatory mechanism of GH-induced PGRN expression, the zebrafish PGRN promoter was isolated and analyzed. We found that a region from -2400 to -3000 within PGRN promoter is essential for GH-induced PGRN expression. A promoter competition assay showed that HNF-3 β, HNF-1 α and C/EBP β binding motifs within the -2400/-3000 region of PGRN promoter contributed to GH-induced PGRN expression. The individual or concomitant transfection of HNF-3 β, HNF-1 α and C/EBP β into HepG2 cells showed that the three transcriptional regulators all participate in GH-induced PGRN expression with independent activities. These results demonstrated that the liver-enriched transcription factors HNF-3 β, HNF-1 α and C/EBP β are involved in GH-induced PGRN expression in liver. We expect that these results will be useful in understanding the regulatory relationship between PGRN and IGF-1 in response to GH regulation.

Keywords: Progranulin; Liver enriched transcription factors; Growth hormone

Introduction

Growth hormone (GH) is synthesized and secreted by cells called somatotrophs in the anterior pituitary gland. GH plays important roles in the control of several complex physiological processes, including growth, metabolism, immune function and aging [1-3]. The effects of GH on these physiological functions are mediated by multiple signaling pathways that are activated by the interaction between GH and growth hormone receptor (GHR). GH binds to its specific GHR, causing the dimerization of the GHR, activation of the GHR-associated protein JAK2, and the tyrosyl phosphorylation of both JAK2 and GHR. These events promote the recruitment and/or activation of a variety of signaling molecules, including mitogen-activated protein kinases (MAPKs), insulin receptor substrate-1 (IRS1), phosphatidylinositol-3-phosphate-kinase (PI3K) and signal transducer and activators of transcription (STAT) proteins. These signaling molecules contribute to the GH-induced changes in enzymatic activity, transport function, and gene expression that ultimately culminate in changes in growth and metabolism [4].

The signaling pathway by which GH mediates gene expression in the liver has been clarified, including the identification of STAT family members such as STAT5B and STATA5A as key transcription factors mediating the GH-induced regulation of the expression of Spi 2.1 [5], IGF-1 [6,7] and several other members of the GH/IGF-I axis in the liver [8,9]. IGF-1 is secreted by the liver into the circulation in response to the growth-stimulating actions of GH. Because the liver is the chief organ that is responsible for circulating IGF-1, the role of liver-specific and liver-enriched transcription factors in GH-induced gene expression have also been studied intensively. Thousands of genes expressed in the liver are regulated directly or indirectly by liver-specific and liver-enriched transcription factors including hepatocyte nuclear factor (HNF)-1 α, HNF-1 β, HNF-3 α, HNF-3 β, HNF-3γ, HNF-4 α, HNF-6, albumin D-element binding protein (DBP), and the CCAAT/enhancer- binding proteins (C/EBP) α and C/EBP β [10,11]. Thus, at least some genes that are expressed in the liver in response to GH may be regulated by these liver-specific and liver-enriched transcription factors. This model has been supported by the recent finding that HNF-1 α and HNF-3 α contributed to the GH regulation of IGF-1 expression [12,13]; C/EBP β participates in the GH-regulation of *c-fos* expression [14,15], HNF-3 β contributes to the GH regulation of P450 (CYP) 2A2, CYP4A2, and CYP2C11 expression [16], and HNF-3, HNF4 and HNF-6 contribute to the GH regulation of CYP2C12 expression [17-19] in the liver.

Progranulin (PGRN), an extracellular glycoprotein precursor consisting of multiple copies of the cystine-rich granulin motif, is considered to represent a family of epithelial growth factors (also known as granulin-epithelin precursor, proepithelin, acrogranin and PC cell-derived growth factor) that has been implicated in cell cycle progression and motility, embryonic development and liver morphogenesis, wound healing response, frontotemporal dementia and the progression of several cancers [20-22]. Our previous studies, which touched upon the growth-related genes involved in the growth hormone/insulin-like growth gactor-1 (GH/IGF-1) axis, have shown that the expression of PGRN and IGF-1 are co-induced by GH in the

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tilapia liver [23]. This result suggests that PGRN may be involved in GH-mediated physiological functions. While much is known about the regulatory mechanism of GH-stimulated IGF-1 expression, less is known about the regulatory activity of GH-induced PGRN expression. To clarify the liver-specific and liver-enriched transcription factors that may be involved in the regulation of PGRN gene expression by GH in the liver, we used zebrafish as a model to identify the PGRN promoter region that is responsible for the GH regulation of the PGRN gene and characterized the major liver-enriched transcription factors that contribute to the effects of GH-induced PGRN expression.

Materials and Methods

Injection of recombinant human Growth Hormone (hGH)

Adult AB strain zebrafish (*Danio rerio*) were maintained in a freshwater recirculating tank at the Institute of Cellular and Organismic Biology, Academia Sinica (Taipei, Taiwan) with a controlled light cycle of 14 h light/10 h dark at 28°C. After starvation for 2 days, the fish were anesthetized in 2-phenoxyethanol (Sigma; P-1126) and then given an i.p. injection of 10, 100, or 500 ng of human GH (GenWay, CA, USA) per gram of body weight or phosphate-buffered saline as a control (n=3 for each concentration of hGH). Liver tissue was sampled at 3 h after GH treatment for determining the dose response of PGRN and IGF-1. To determine the time course of PGRN and IGF-1 after GH treatment, liver samples were collected from three PBS injected zebrafish (control) and three GH-injected zebrafish at 0, 1.5, 3, 4.5, 6, 9 (h) after GH injection (n=3 at each time point). The 0 h indicated the liver samples were sampled immediately after GH injection. The RNA and protein samples were prepared to allow the estimation of IGF-1 and PGRN expression. The results were confirmed by three independent experiments.

cDNA synthesis and real-time PCR

Total RNA was extracted from 100 mg of liver tissue using the RNAzol™ B reagent (Tel-Test, Friendswood, TX) according to the manufacturer's protocol. Final RNA concentrations were determined by optical density measurement at 260 nm, and first-strand cDNA was synthesized in a 20 μl RT reaction from 2.5 μg of total RNA using SuperScript III (Invitrogen). Quantitative PCR was performed using Power SYBR Green PCR Master Mix in a LightCycler 480 System (Roche). Gene-specific primers for *igf-1* and *pgrn* were designed using Roche ProbeFinder software for real-time quantitative PCR. The transcript of the *ef-1α* gene was used as endogenous control. The primer sets used in quantitative PCR are listed in Table 1.

Recombinant DNA constructs

The reporter vector psiCHECK-2 (Promega, Foster City, CA, USA) was used for luciferase assays to determine the activities of each length of zebrafish PGRN promoter. Fragments of 5 kb, 4 kb, 3 kb, 2.4 kb, 1.5 kb and 1 kb of the 5'-flanking region of the PGRN gene containing the 5'-untranslated region (UTR) were amplified from zebrafish genomic DNA using specific PCR primers (Table 1) and inserted into psiCHECK-2 by *Not*I and *Mlu*I restriction to generate pPGRN5, pPGRN4, pPGRN3, pPGRN2.4, pPGRN1.5 and pPGRN1. The expression of a synthetic firefly luciferase (*hluc*+) gene located downstream of each *pgrn* promoter was used to evaluate promoter activity. The *Renilla* luciferase (*hRluc*) harbored in each pPGRN-CHECK plasmid was used as an internal control for normalization. Zebrafish HNF1 *α*, HNF3 β and C/EBP β cDNA were cloned from liver tissue by PCR using the gene-specific primers listed in Table 1. The PCR products of HNF1 *α*, HNF3 β and C/EBP β (1683 bp, 1230 bp and 843 bp, respectively) were inserted into the pCDNA3.1/ V5-His-TOPO (Invitrogen, Carlsbad, USA) vector by TA cloning. *E. coli* DH5 was used as a host strain for the cloning and maintenance of the plasmids. The sequence of each PGRN promoter fragment and the transcription factor genes were confirmed by sequencing the recombinant plasmids using an ABI3730 automated DNA sequences.

Primers name	Sequences (5' to 3')	Accession No.
zIGF(f)	CGACAGGATATGGACCTAGTTCAA	NM_131825
zIGF(r)	ACAATACATCTCGAGGCGCC	NM_131825
zPGRN(f)	AGTAGCACAGGGCCTTGCAT	AF375477
zPGRN(r)	CTTTTACTTTCCGTTTGACACACAA	AF375477
ef-1α (f)	TGCCTTCGTCCCAATTTCAG	L47669
ef-1α (r)	TACCCTCCTTGCGCTCAATC	L47669
pPGRN5-NotI (f)	CGGCCGCTTCTTACCT TCATCTCTGAAATCTTGCCAT	---
pPGRN5-MluI (r)	ACGCGTGTTGCTGTAGCTGTCAATCATTAACAGATG	---
pPGRN4-NotI (f)	CGGCCGCATGTTCTGG GCCACTGAACACAGGTT	---
pPGRN4-MluI (r)	ACGCGTGTTGCTGTAGCTGTCAATCATTAACAGATG	---
pPGRN3-NotI (f)	CGGCCGCATGCATATCAAATTTTGGTCTTCAATGGA	---
pPGRN3-MluI (r)	ACGCGTGTTGCTGTAGCTGTCAATCATTAACAGATG	---
pPGRN2.4-NotI (f)	CGGCCGCAACTACCTAAAACTTGCATTTGGATTCGTA	---
pPGRN2.4-MluI (r)	ACGCGTGTTGCTGTAGCTGTCAATCATTAACAGATG	---
pPGRN1.5-NotI (f)	CGGCCGCTTGTTTTCATTCTCATTTGACTCTTTCTGC	---
pPGRN1.5-MluI (r)	ACGCGTGTTGCTGTAGCTGTCAATCATTAACAGATG	---
pPGRN1-NotI (f)	CGGCCGCCTGAACAGATAATCAAAGTCTAAAAAGTTA	---
pPGRN1-MluI (r)	ACGCGTGTTGCTGTAG CTGTCAATCA TTAACAGATG	---
zHNF1α (f)	ATGGACGGAGGAGAGTCGAGGAGATCAGAA	AF244934
zHNF1α(r)	CTATTGTGCAGTGGAGACCATCTGTGCAGGAAT	AF244934
HNF3β(f)	ATGCTCGGTGCTGTCAAAATGGAGGG	NM130949
HNF3β(r)	TTAGGAAGAGTTCAGGATGGGCCTGG	NM130949
C/EBPβ(f)	ATGGAAGTGGCCGGTTTTTACG	NM131884
C/EBPβ(r)	TCAGCACTGGCCGGTGGCGGAG	NM131884

Table 1: Oligonucleotide primers used for PCR amplification of the PGRN promoter and specific genes.

Cell culture and transfection

Human hepatocellular liver carcinoma HepG2 cells (ATCC HB 8065) were used as the model for promoter activity assay. Cells were seeded at a density of 1×10^5 cells per well in 24-well microplates and cultured in 200 µl of α -modified essential medium (α-MEM) with 10% fetal bovine serum (FBS), 10 IU/ ml penicillin and 10 mg/ ml streptomycin at 37 °C in 5% CO_2. After 24 h of incubation, the original culture medium was discarded and replaced with 200 µl of α,-MEM without serum containing 1 µg of each pPGRN promoter construct and 4 µl PolyJet reagent (SignaGen, MD, USA) for transient transfection. After 24 h of transfection, fresh serum-free α -MEM supplemented with 10 IU/ml penicillin and 10 mg/ml streptomycin with or without 300 ng/ml hGH was added. The cells were harvested 3 hours later, washed in PBS buffer and incubated in lysis buffer for 15 min. Firefly and *Renilla* luciferase activity were measured according to the manufacturer's instructions for the dual-luciferase reporter assay system (Promega Corp., Madison, USA). Relative activity levels were expressed as firefly/Renilla ratios.

Promoter competition assay (PCA)

Promoter competition assays (PCA) were performed according to previously published protocols, with some modifications [24]. Briefly, HepG2 cells were transiently incubated with 0.5 µM double-stranded PCR oligonucleotide competitors consisting of a specific transcription binding element and the pPGRN3 construct. An exogenous oligonucleotide corresponding to nucleotides +54 to +254 β -actin cDNA (AF057040) was used as negative control. GH-treated HepG2 cells without competitor transfection were used as controls. The relative luciferase activity levels were expressed as firefly/Renilla ratios.

Western blot analysis of liver IGF-1 and PGRN protein

Liver samples were collected from three GH-treated or control zebrafish at various time intervals (0, 3, 6, 9, 12 h). The liver samples were washed twice in ice-cold phosphate-buffered saline (PBS) and then homogenized in a tissue lysis buffer containing proteinase inhibitor with a motorized Teflon pestle at 1000 rpm for 2 min. The lysate was centrifuged for 30 min at 16,000 *g*, and the supernatant was collected and kept at -80°C until used for immunoblotting. The Bradford assay (Bio-Rad, Hercules, CA) was used to measure protein concentration. A 20 µg aliquot of protein from each sample was separated on a Tricine SDS-PAGE gel and transferred to a polyvinylidene difluoride (PVDF) Immobilon membrane (Millipore, Bedford, MA, USA) at 20 V for 45 min in a buffer containing 192 mM glycine and 25 mM Tris-HCl. Membranes were blocked with 0.25% gelatin and 0.05% Tween-20 in Tris-buffered saline for 2 h at room temperature and then probed with anti-IGF1 or anti-PGRN antibody for 2 h at room temperature. After washing, membranes were incubated with horseradish peroxidase-linked anti-rabbit, anti-mouse or anti-rat antibodies for 2 h. The membranes were then incubated with a chemiluminescence reagent (Immobilon Western, Millipore) with gentle agitation and exposed to Hyperfilm ECL (GE Healthcare, Buckinghamshire, UK).

Results

GH mediates the induction of IGF-1 and PGRN expression in the zebrafish liver

The GH-mediated IGF-1 response is a well-known endocrine pathway that drives somatic growth in vertebrates. In our previous study, we showed that PGRN and IGF-1 expression in the tilapia liver are co-induced by GH administration in a dose-dependent manner [23]. To demonstrate that this phenomenon is also occurs in zebrafish, PGRN expression induced by GH administration was measured in this study. Adult zebrafish were fasted for 2 days prior to i.p. injection with various doses of hGH, and the mRNA expression levels of PGRN and IGF-I were measured by real-time PCR. The expression of PGRN and IGF-I mRNAs exhibited a dose-dependent increase in response to hGH at concentrations from 10 ng/g to 100 ng/g; however, the hGH-induced PGRN and IGF-1 responses declined in the presence of 500 ng/g hGH concentrations (Figure 1A). The levels of PGRN and IGF-1 protein expression corresponded to the mRNA levels and were also induced by GH administration. The magnitude of IGF-I response to GH was greater than that of PGRN (Figure 1B). The results of the time course of PGRN and IGF-1 mRNA and protein induction upon treatment with 100 ng/g hGH are shown in Figure 1C and D. The levels of PGRN and IGF-1 mRNA were induced by hGH with similar profiles; both reached a plateau at 4.5 h after hGH treatment and decreased thereafter. In the immunoblot analysis, increasing levels of both PGRN and IGF-1 were detected in zebrafish liver accompanying with the time of hGH treatment. In summary, the results confirmed that PGRN expression in zebrafish liver was induced by GH administration, similar to the results observed in tilapia.

The PGRN promoter region from -2.4k to -3k is responsible for GH- dependent activation of the PGRN expression

To understand the transcriptional regulation of GH-induced PGRN gene expression, a 5.0 kb region upstream of the PGRN gene was isolated and analyzed to identify putative transcription factor binding elements. We found several motifs that were predicted to be binding sites for C/EBP, GATA binding protein (GATA), HNFs, STATs and cyclic AMP response element binding protein (CRE-BP). To clarify the regulatory mechanism of GH-induced PGRN expression, the effect of GH on the PGRN promoter was studied in HepG2 cells transiently transfected with different pPGRN promoter constructs. The regions within the PGRN promoter that are most critical for GH-dependent activation of PGRN expression were identified by serial promoter deletion. After transfection, cells were incubated in the presence of 300 ng/ml of GH for 3 h, after which the cells were lysed and the luciferase activity in the lysate was measured. As shown in Figure 2, although the relative luciferase activity was diminished by approximately 26.8% ± 2.3% in the 3 kb PGRN promoter compared to the 5 kb PGRN promoter, there was little difference in the activities of the PGRN promoter that contained different portions of the sequence from -3 kb to -5 kb. A more noticeable change in promoter activity occurred when the length of the PGRN promoter was shortened to 2.4 kb; at this length, the relative luciferase activity declined significantly, to 15% ± 4.3% of the activity of PGRN promoters longer than 3 kb. These results suggested that the regulatory binding motifs for transcription factors within the PGRN promoter region from -2.4 kb to -3 kb are critical for the GH- mediated regulation of PGRN expression.

Characterization of transcriptional binding motifs within the -2.4 kb to -3 kb region of the PGRN promoter

The critical roles of the sequence between -2.4 kb and -3 kb of the PGRN promoter help illuminate the regulatory mechanism of GH-mediated PGRN expression. To characterize the transcriptional regulation of the PGRN gene, the regions from -2.4 kb to -3 kb upstream of the zebrafish PGRN gene were isolated, and the putative response motifs within this sequence were analyzed. In this promoter region, an HNF-3β binding motif was identified at

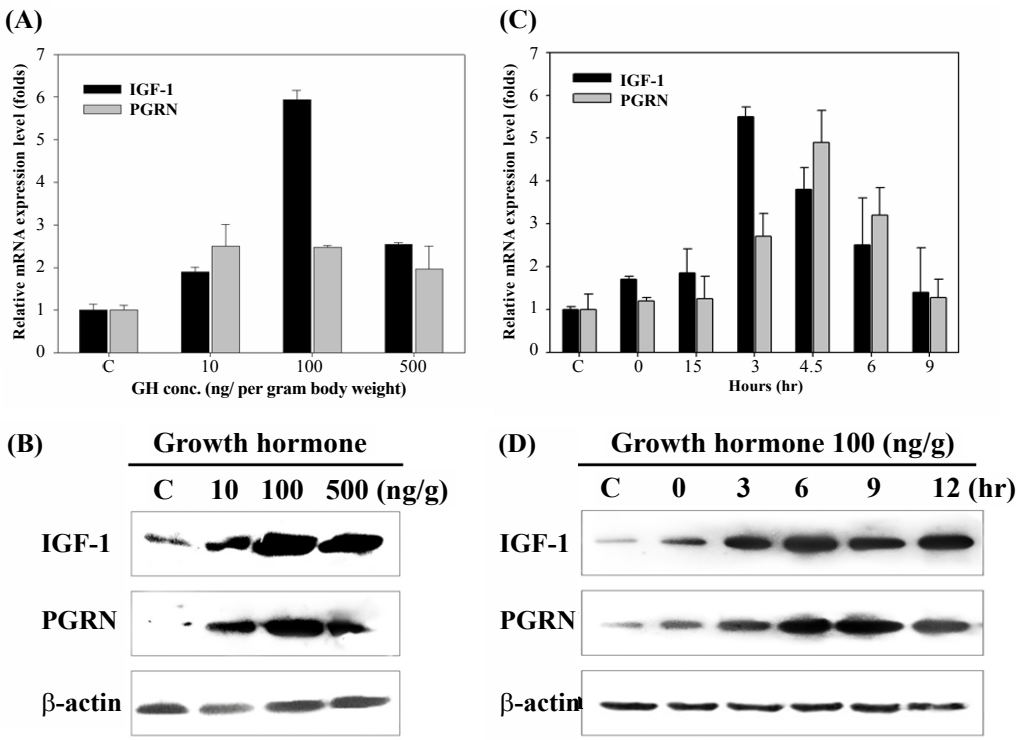

Figure 1: PGRN and IGF-1 were co-induced in the zebrafish liver by GH treatment. The dose response of PGRN and IGF-1 mRNA (A) and protein (B) after different doses of GH (10, 100 and 500 ng/g body weight) i.p. injection were determined by quantitative PCR and western blotting. Liver tissues were sampled at 3 h after GH treatment. The time course of PGRN and IGF-1 mRNA (C) and protein (D) expression after i.p. injection with 100 ng/g body weight of GH were determined by quantitative PCR and western blotting. β-actin was used as a loading control for normalization.

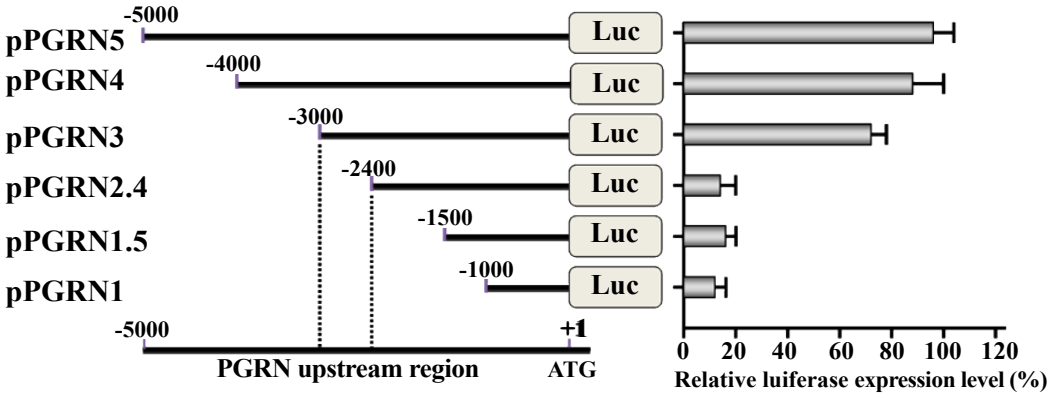

Figure 2: Schematic representation of each zebrafish pPGRN promoter construct and the effect of GH on the promoter activity of each pPGRN construct. The luciferase activity used to estimate the promoter activity of pPGRN promoters of different lengths was expressed luciferase/renilla. Renilla activity was used as internal control. The data represent the mean ± SD values of three independent experiments.

-2875/-2886 (AACCAAATATAT), an HNF-1α binding motif was found at -2670/-2686 (GCCTCATAATTAACCCA) and two C/EBP β motifs were found at -2648/-2662 (AGCTTTTGAAATTC) and -2416/-2429 (ATTTTTCTTAATAT), as shown in supplementary Figure S1. To identify the potential importance of each motif within the PGRN promoter, a promoter competition assay was carried out using oligonucleotides corresponding to each motif sequence as a competitor. As shown in Figure 3, the luciferase activity of a negative control that was transfected with a non-competing oligonucleotide consisting of β-actin sequence was identical to that in a control group without any competitor transfection under GH treatment. A CP1 competitor consisting of the HNF-3 β motif sequence caused a 27.8 ± 3.7% decrease in luciferase activity compared to the control group. The luciferase activities in experiments with CP2 and CP4 competitors,

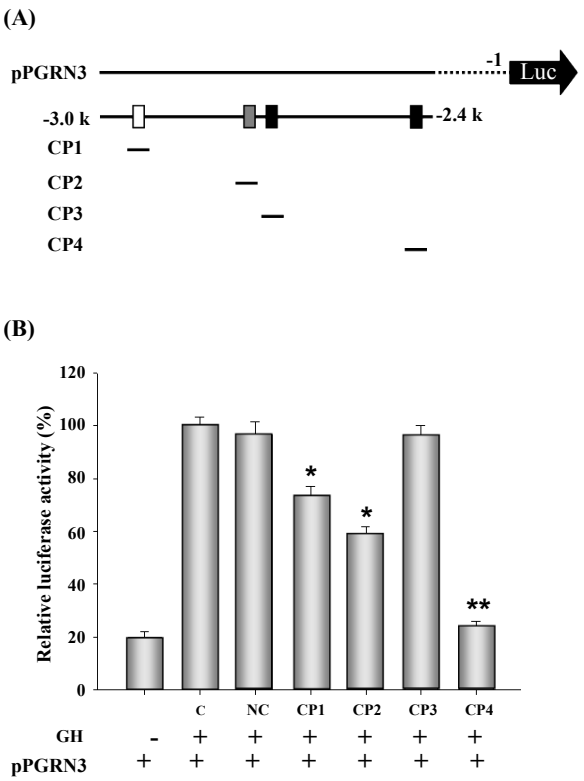

Figure 3: A promoter competition assay using double-strand oligonucleotide competitors. (A) Schematic representation of regulatory motifs within the PGRN promoter and each competitor. A pPGRN3 promoter construct comprising the region from -1 to -3000 was used for the luciferase assay. The regulatory motifs in the PGRN promoter region from -2.4 K to -3.0 K are represented as follows: White box, HNF-3β binding motif; gray box, HNF-1α; black box, C/EBPβ binding motif. The competitors used were 200 bp PCR oligonucleotides consisting of the HNF-3β binding motif (AACCAAATATAT) (CP1), HNF-1α binding motif (GCCTCATAATTAAC CCA) (CP2), or C/EBPβ binding motifs (AGCTTTTGAAATTC) (CP3) and (ATTTTT CTTAATAT) (CP4). (B) Twenty-four hours post-transfection, cells were lysed and luciferase activity was measured as described in the Materials and Methods. The luciferase activity of the NC (negative control) or each competitor (CP) compared to that in the control group was expressed as luciferase/renilla ratio. Renilla activity was used as an internal control. The data represent the mean ± SD of three independent experiments. Asterisks indicate significant differences. * indicates p <0.05.

which consisted of the HNF-1 α motif and C/EBP β motif sequence, respectively, were clearly decreased, to 41.4% ± 3.1% and 76.4% ± 2.4% compared to the control group. The lack of variation of luciferase activity in response to the CP3 competitor demonstrated that the C/EBP β motifs located at -2648/-2662 did not affect the GH regulation of PGRN expression (Figure 3). These results suggested that the HNF-3 β motif at -2875/-2886, HNF-1 α motif at -2670/-2686 and the C/EBP β motifs at -2416/-2429 potentially contributed to GH-induced PGRN expression.

GH activation of the PGRN promoter by the co-expression of HNF-1 α, HNF-3 β and C/EBP β

Efficient promoter activation often requires the collective effect of two or more transcription factors. Thus, we tested whether the simultaneous expression of HNF-1 α, HNF-3 β and C/EBP β resulted in GH-induced PGRN promoter activation to levels higher than those observed for each transcription factor alone. In the absence of GH, the pPGRN3 promoter construct was not activated in the presence of HNF-1 α, HNF-3 β or C/EBP β alone; however, in the presence of GH, the pPGRN3 promoter constructs were induced significantly by the presence of HNF-1 α, HNF-3 β or C/EBP β alone. These results again demonstrated that HNF-3 β, HNF-1 α and C/EBP β contributed to the GH regulation of PGRN expression. In the presence of GH, the combined addition of HNF-3 β and HNF-1 α resulted in 14.3-fold increase in the activation of the pPGRN3 promoter, almost equal to the sum of the increases stimulated by HNF-3 β alone and HNF-1 α alone. Similar activation status was also observed upon combination treatment with HNF-3 β and C/EBP β and with HNF-1 α and C/EBP β. To further understand whether the activities of HNF-3 β HNF-1 α and C/EBP β on GH-induced PGRN expression are independent or not, the combined effect of HNF-3 β HNF-1 α and C/EBP β was also evaluated. The combination of HNF-3 β HNF-1 α and C/EBP β leads to 29.4-fold increase in pPGRN3 promoter activity, approximately equal to sum of the increases induced by the individual transcription factors. These results demonstrated that HNF-3 β HNF-1 α and C/EBP β participated in GH-induced PGRN expression via independent mechanisms.

Discussion

The GH/IGF-1 axis acts an important endocrine modulator of postnatal growth and metabolism, and the regulation of growth by the GH/IGF-1 axis is well conserved in vertebrates. Reports have showed the structural and functional domains of GH appear to be highly conserved among vertebrates including fish [25,26]. These reports elucidated that exogenous growth hormones can potential reveal their biological function in teleost. For example, transgenic common carp (*Cyprinus carpio L.*) expressing human growth hormone showed a faster growth rate and feed utilization compare to that of control [27]. In our previous study, we showed that progranulin is induced along with IGF-1 in tilapia liver tissue by seabream GH administration. This

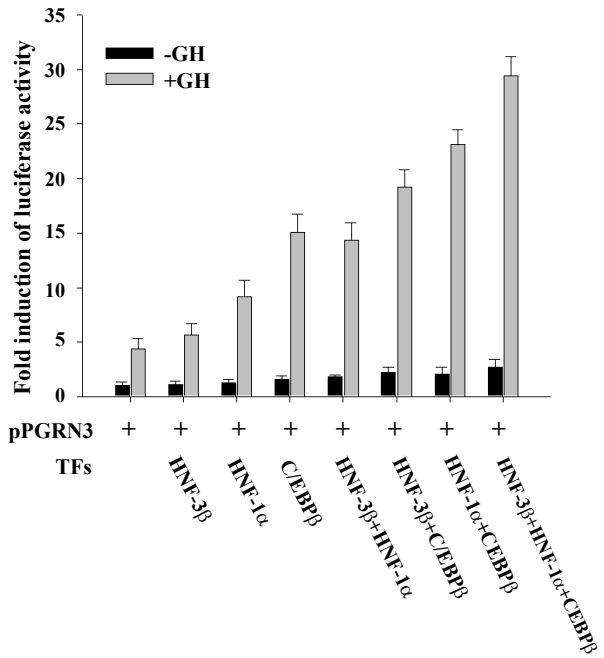

Figure 4: The effect of GH treatment in HepG2 cells transiently transfected with pPGRN3 construct and expression plasmids encoding HNF-3βHNF-1α and C/EBPβ. The basal luciferase activity of pPGRN alone (without GH treatment) was set as 1. The indicated values for the fold induction of luciferase activity represent the mean ± SD of three independent experiments.

result suggests that PGRN participates in GH/IGF-1 axis-regulated growth and may serve functions similar to those of IGF-1 signaling [23]. Similar to our results in tilapia, the mRNA and protein levels of PGRN and IGF-1 were also co-induced in the zebrafish liver by human GH administration, suggesting that this phenomenon may be a common regulatory mechanism in fish. Several transcriptional regulators involved in GH-mediated IGF-1 expression have been characterized in diverse cell lines; for instance, STAT5b participated in GH-stimulated IGF-1 expression in monkey kidney fibroblast (Cos-7) cells [6,8]; and the interaction of nuclear factor-kappa B (NF-kB) and STAT5b mediated GH-induced IGF-1 expression in chondrocytes [28]. Although increasing evidence clarifying the regulatory mechanisms of GH-stimulated IGF-1 expression has been published in recent years, little is known about the regulatory activity of GH-induced PGRN expression. In the present study, a 5.0 kb region upstream of the PGRN gene was isolated and analyzed to understand the mechanism through which GH regulates PGRN expression. There are several potential binding sites for transcription factors from the GATA family, AP-1, Sry, Oct-1, C/EBP, and HNF family within the promoter that may contribute to GH-induced PGRN expression. Serial deletion is a useful method for identifying promoter regions that are particularly important in driving gene expression. Although the results of the PGRN promoter deletion experiments here showed that luciferase activity was slightly decreased in the 3 kb promoter compared to the 5 kb promoter, the deletion of the region from -2400 to -3000 causes a much more significant loss of luciferase activity. This result demonstrated that the region from -2400 to -3000 in the PGRN promoter plays a critical role in GH-induced PGRN expression and suggested that certain regulatory motifs within this region might be responsible for the GH-induced PGRN expression. Putative binding motif analysis showed that one NHF-3 β, one NHF-1 α and two C/EBBP β binding sites exist within the -2400/-3000 PGRN promoter. To determine whether these motifs are involved in GH-induced PGRN expression, we used a promoter competition assay to interfere with the activities of each motif. The cotransfection of the pPGRN3 construct and individual competitors showed that GH-induced luciferase activity clearly decreased, indicating that all of these binding motifs participate in GH-induced PGRN expression. Several reports have shown that the liver-enriched transcription factors HNF-1, HNF-3 and C/EBP β influence the activation of GH-mediated gene expression. For instance, HNF-1 and HNF-3 control CYP2C13 gene expression in rat [29], and HNF-1 α and HNF-3 β mediated GH-induced IGF-1, CYP2A2, CYP4A2 and CYP2C11 expression [12,16]. C/EBP β controls a network of GH-regulated liver transcription factors and mediates the expression of multiple target genes, such as *c-fos*, *Cyr61* and *Btg2* [14,30]. These findings suggested that NHF-3 β, NHF-1 α and C/EBP β might participate in GH-regulated PGRN expression. In the present study, NHF-3 β, NHF-1 α and C/EBBP β expression constructs were individually co-transfected into HepG2 cells along with the pPGRN3 construct to further validate this model. The results revealed that the transfection of each transcription factor increased the luciferase activity, indicating that NHF-3 β, NHF-1 α and C/EBBP β are indeed involved in GH-induced PGRN expression. It has been shown that GH-mediated salmon IGF-1 transcription in human hepatoma-derived (Hep3B) cell line is synergistically regulated by HNF-1 α and STAT5 [12]. To determine whether NHF-3 β, NHF-1 α and C/EBBP β also contributed to GH-induced PGRN expression synergistically, we also transfected cells with a combination of the individual transcription factors and evaluated the subsequent luciferase activity. The results revealed that the luciferase activity driven by two transcription factors in combination was almost equal to sum of the luciferase activities driven by each transcription factor alone; a similar result was observed when all three transcription factors were combined. These results indicate that NHF-3 β, NHF-1 α and C/EBBP β participate in GH-induced PGRN through a collective (additive) effect rather than synergistic activity.

The present work shows that the phenomenon of PGRN and IGF-1 co-induction in the liver after GH administration is common in fish, including zebrafish and tilapia. The examination of PGRN promoters of distinct lengths revealed that the region from -2400 to -3000 plays a critical role in GH-induced PGRN expression. Promoter competition assays showed that the NHF-3 β, NHF-1 α and C/EBBP β binding motifs within the region from -2400 to -3000 of the PGRN promoter is involved in GH-induced PGRN expression. The effects of NHF-3 β, NHF-1 α and C/EBP β on stimulation of GH-induced PGRN expression were proven through collective action. We expect that the results presented in this study will be useful for understanding the relationship between the PGRN and IGF-1 regulatory mechanisms and signaling pathways in response to GH administration.

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