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Novel Mechanisms for IGF-I Regulation by Glucagon in Carp Hepatocytes: Up-Regulation of HNF1 α and CREB Expression via Signaling Crosstalk for IGF-I Gene Transcription

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Glucagon, a key hormone for glucose homeostasis, can exert functional crosstalk with somatotrophic axis via modification of IGF-I expression. However, its effect on IGF-I regulation is highly variable in different studies and the mechanisms involved are largely unknown. Using grass carp as a model, the signal transduction and transcriptional mechanisms for IGF-I regulation by glucagon were examined in Cyprinid species. As a first step, the carp HNF1 α , a liver-enriched transcription factor, was cloned and confirmed to be a single-copy gene expressed in the liver. In grass carp hepatocytes, glucagon treatment could elevate IGF-I, HNF1 α , and CREB mRNA levels, induce CREB phosphorylation, and up-regulate HNF1 α and CREB protein expression. The effects on IGF-I, HNF1 α , and CREB gene expression were mediated by cAMP/PKA and PLC/IP₃/PKC pathways with differential coupling with the MAPK and PI3K/Akt cascades. During the process, protein:protein interaction between HNF1 α and CREB and recruitment of RNA Pol-II to IGF-I promoter also occurred with a rise in IGF-I primary transcript level. In parallel study to examine grass carp IGF-I promoter activity expressed in α T3 cells, similar pathways for post-receptor signaling were also confirmed in glucagon-induced IGF-I promoter activation and the trans-activating effect by glucagon was mediated by the binding sites for HNF1 α and CREB located in the proximal region of IGF-I promoter. Our findings, as a whole, shed light on a previously undescribed mechanism for glucagon-induced IGF-I gene expression by increasing HNF1 α and CREB production via functional crosstalk of post-receptor signaling. Probably, by protein:protein interaction between the two transcription factors and subsequent transactivation via their respective cis-acting elements in the IGF-I promoter, IGF-I gene transcription can be initiated by glucagon at the hepatic level.

Keywords: IGF-I, glucagon, HNF1 α , CREB, signal transduction, gene expression, hepatocytes, grass carp

INTRODUCTION

Glucagon released from the pancreas by functional interaction with insulin is known to play a key role in glucose homeostasis (1), mainly through regulation of gluconeogenesis and glycogenolysis in the liver (2). At the hepatic level, glucagon is also involved in lipid metabolism (3), and recently, a hepato-pancreatic feedback loop has been proposed for regulation of amino acid turnover by glucagon (4). In humans, aberrant expression of glucagon can be associated with pathophysiological or disease conditions (e.g., diabetes, non-alcoholic fatty liver, and glucagon-producing tumor) (5). In mammals, positive correlation between circulating levels of glucagon and growth hormone (GH) (6) and glucagon-induced GH secretion are well-documented (7, 8), suggesting a functional crosstalk of the somatotrophic axis with glucagon. However, the effect of glucagon on hepatic expression of IGF-I, the downstream effector for GH, is controversial, as both stimulatory (9) and inhibitory effects (10) have been reported (e.g., in rat hepatocytes). Although the biological actions of glucagon are known to be mediated by the cAMP/PKA and/or PLC/IP₃/PKC pathways (11, 12), the signal transduction/molecular mechanisms for IGF-I regulation by glucagon are still unknown. In lower vertebrates, including fish species, glucagon, and its receptor are highly conserved in terms of their structure and functions (e.g., glucose production and cAMP coupling) (13, 14). However, little information is available regarding the comparative aspects for IGF-I regulation by glucagon. To date, there is only a single report in salmon hepatocytes showing that glucagon did not alter basal but reduced GH-induced IGF-I mRNA expression at the hepatic level (15). These findings suggest that, similar to mammals, glucagon may also play a role in IGF-I regulation in fish models.

In mammals, IGF-I expression can be activated at the transcriptional level through recruitment of STAT₅ (16), hepatic nuclear factor 1 α (HNF1 α) (17), hepatic nuclear factor 3 γ (HNF3 γ) (18), C/EBP β (19), and p300 to its 5' promoter (20), which forms a "transcriptional circuitry" mediating GH-induced IGF-I gene expression (21). Among the transcription factors involved in IGF-I promoter activation, the functional role of STAT₅ and HNF1 α appears to be well-conserved in vertebrates and their binding sites can be consistently identified within the promoter of IGF-I gene ranging from fish to mammals (22). In representative species of fish models, e.g., salmon (23, 24) and common carp (25), 5' deletion of the promoter region with STAT₅ or HNF1 α binding sites (as SBE and HBE, respectively) has been shown to reduce both basal and GH-induced IGF-I promoter activity. However, whether the expression of STAT₅ and HNF1 α can also serve as a target of regulation by hormonal signals to modify IGF-I expression is unclear and remains to

be investigated. In our recent study, the 5' promoter of grass carp IGF-I gene has been cloned. Consistent with the presence of a SBE site in the proximal promoter region, the carp IGF-I promoter was found to be highly responsive to GH activation of JAK₂/STAT₅ pathway and this stimulatory effect could be down-regulated by the negative feedback via type II SOCS expression (26). Interestingly, a binding site for cAMP response element binding protein (CREB) (as CRE site) sitting right next to a HBE site was identified downstream of the SBE site in the carp IGF-I promoter and the positions of CRE and HBE sites were also found to be well-conserved in the IGF-I genes of fish species (unpublished results, Wong 2019). This new information based on promoter sequence analysis raises the possibility that CREB may work with HNF1 α to trigger IGF-I gene transcription in the carp liver.

Since CREB is the downstream effector of the cAMP/PKA pathway (27, 28) and CREB activation by glucagon has been reported in fish model, e.g., goldfish (29), we hypothesize that HNF1 α and CREB may serve as the regulatory targets for glucagon and mediate its effect on IGF-I regulation at the hepatic level in carp species. Grass carp was used as the animal model in our study as (i) it is a representative of Cyprinids with a high market value in Asian countries (~4.6 million tons per year and equivalent to 15.6% of global finfish production; FAO yearbook of Fishery and Aquaculture Statistics 2011), and (ii) bony fish are lower vertebrates leading to the tetrapod lineage and their studies are supposed to provide novel insights on the evolution of endocrine structure/function. Since little/no information is available for HNF1 α in carp species, grass carp HNF1 α was cloned and characterized for its gene copy number, tissue expression, and bioactivity in stimulating HBE-containing promoter. Using primary culture of grass carp hepatocytes, the functional role of HNF1 α and CREB as the targets of regulation mediating the effect of glucagon on IGF-I gene expression was tested in the carp liver. For the signal transduction involved, the post-receptor signaling pathways and their functional interaction at the hepatic level in mediating glucagon regulation of IGF-I, HNF1 α , and CREB mRNA expression were examined using a pharmacological approach. Meanwhile, protein:protein interaction between HNF1 α and CREB and Pol-II recruitment to IGF-I promoter after glucagon induction were also investigated and correlated with the parallel changes in IGF-I primary transcript levels. The results obtained were further confirmed with transfection studies in α T3 cells to examine IGF-I promoter activation induced by glucagon as well as the functional role of CRE and HBE sites identified in the IGF-I promoter. Our studies for the first time unveil the signal transduction and transcriptional mechanisms for IGF-I regulation by glucagon through up-regulation of HNF1 α and CREB expression and transactivation of their respective cis-acting elements in the IGF-I promoter.

MATERIALS AND METHODS

Animals and Test Substances

One-year old grass carp (*Ctenopharyngodon idellus*), were obtained from local wholesale market and maintained in well-aerated water in 250l aquaria at 20°C under a 12-h

Abbreviations: IGF-I, Insulin-like growth factor I; GH, Growth hormone; HNF1 α , Hepatic nuclear factor 1 α ; CREB, cAMP response element binding protein; HBE, HNF1 α binding element; CRE, cAMP response element; RNA Pol-II, RNA polymerase II; ChIP, Chromatin immunoprecipitation; AC, Adenylyl cyclase; PKA, Protein kinase A; PLC, Phospholipase C; PKC, Protein kinase C; IP₃, Inositol 1,4,5-triphosphate; DAG, Diacylglycerol; MAPK, Mitogen-activated protein kinases; MEK_{1/2}, Mitogen-activated protein kinase kinase 1/2; ERK_{1/2}, Extracellular signal-regulated kinase 1/2; PI3K, Phosphoinositide 3-kinase; Akt, Protein kinase B; mTOR, Mammalian target of rapamycin.

light:12-h dark photoperiod for at least 14 days prior to experimentation. During the process of tissue sampling and hepatocyte preparation, the fish was anesthetized in MS222 (0.05%; Sigma, St. Louis, MO) and spinosectomized according to the protocol approved by the Committee for Animal Use at the University of Hong Kong (Hong Kong). Human glucagon and pharmacological agents for signaling targets/kinases, including forskolin, MDL12330A, H89, GF109203X, TPA, PD98059, SCH772984, Ly294002, API-2, rapamycin, N-6-benzoyladenoxine-3',5'-cAMP (6Bnz-cAMP), 8-(4-chlorophenylthio)-3',5'-cAMP (8cpt-cAMP), and 2-aminoethoxy-diphenyl borate (2-APB) were acquired from Calbiochem (San Diego, CA), while chelerythrine, 1,2-dioctanoyl-*sn*-glycerol (DiC-8), actinomycin D and cycloheximide were obtained from Sigma and edelfosine and 8-(4-chlorophenylthio)-2'-O-methyladenosine-3',5'-cAMP (8cpt-2Me-cAMP) were purchased from Tocris (Ellisville, MO). For transfection studies, the expression vector for mouse HNF1 α and HNF1 α reporter pGL₃.HNF1 α .Luc carrying tandem repeats of HBE sites in its 5' promoter were procured from Yeasen Biotech (Shanghai, China).

Molecular Cloning and Characterization of Carp HNF1 α

Grass carp HNF1 α cDNA was cloned using 5'/3'RACE with primers designed according to the conserved regions of zebrafish HNF1 α with total RNA isolated from the carp liver as the template as described previously (26). Sequence alignment, 3D protein modeling and phylogenetic analysis were conducted using Clustal W (<http://www.ebi.ac.uk/clustalw>), SWISS-MODEL (<http://www.expasy.org/swissmod>) and MEGA 6.0 (<http://www.megasoftware.net/index.html>), respectively. To evaluate the gene copy number of HNF1 α , Southern blot was performed in DNA sample purified from the whole blood of grass carp according to the standard protocol (30). For tissue expression profiling of HNF1 α , RT-PCR was conducted in selected tissues and brain areas using primers specific for HNF1 α transcript with parallel PCR of β actin as the internal control. Using LC/MS/MS, protein expression of HNF1 α in the carp liver was also examined using a SCIEX TripleTOF-5600 system (AB SCIEX, Concord, ON, Canada) (31). To confirm the functionality of the newly cloned cDNA, the ORF of carp HNF1 α was cloned into pcDNA/Zeo to generate the expression vector pcDNA/HNF1 α . The vector was then used in promoter study in α T3 cells with co-transfection of the pGL₃.HNF1 α .Luc reporter and TK-Renilla (as internal control) to test the ability of carp HNF α expression in transactivating target promoter with $\times 4$ tandem repeats of HBE sites. In this experiment, parallel expression of mouse HNF1 α was used as a positive control.

IGF-I, CREB, and HNF1 α mRNA Measurement in Carp Hepatocytes

Primary culture of grass carp hepatocytes was prepared by collagenase digestion method (26) and maintained in DMEM/F12 medium at a seeding density of $\sim 0.8 \times 10^6$ cells/ml/well in PEI-precoated 24-well plates. After drug

treatment, total RNA was extracted with removal of genomic DNA and reversely transcribed as described previously (30). The samples prepared were then subjected to real-time PCR for IGF-I, CREB, and HNF1 α mRNA measurement using primers and PCR conditions as described in **Supplemental Table 1** using a RotorGene-Q System (Qiagen, Hilden, Germany). In our studies, serial dilutions of plasmids with the ORF of target genes were used as the standards for data calibration and parallel measurement of 18S RNA expression was used as the internal control. After the assays, the authenticity of the respective PCR products was routinely confirmed by melting curve analysis.

Western Blot for CREB and HNF1 α in Carp Hepatocytes

To study the effects of glucagon on CREB activation and CREB and HNF1 α protein expression in the carp liver, Western blot was performed in carp hepatocytes (26) after glucagon treatment for the duration as indicated. During the process, cell lysate prepared in RIPA buffer with inhibitor cocktails for proteases and phosphatases (Roche) was subjected to SDS-PAGE and transblotted onto Immobilon membrane (Millipore) followed by Western blot with antibodies for phosphorylated CREB (P-CREB, 1:500; Santa Cruz, Dallas, Texas), total CREB (T-CREB, 1:500; Calbiochem) and HNF1 α (1:2,500; Abcam, Eugene, OR), respectively. After incubation with HRP-conjugated secondary antibody (1: 5,000, Bio-Rad, Hercules, CA), Western blot signals were visualized using the SuperSignal WestPico Reagent (Pierce, Rockford, IL). Parallel blotting for β actin was also conducted to serve as the internal control using an Actin AB-1 Kit (Calbiochem). To test the effect of glucagon on protein:protein interaction of CREB and HNF1 α , cell lysate prepared from hepatocytes with glucagon treatment was subjected to co-immunoprecipitation (32) using a Crosslink Immunoprecipitation Kit (Thermo Scientific, Waltham, MA). Protein pull-down was conducted with HNF1 α antibody (1:100) followed by immunoblotting using the antibodies for phosphorylated and total CREB (1:500), respectively, and parallel pull-down with mouse IgG antibody (1:100) was used as a negative control.

RNA Pol-II ChIP and IGF-I Primary Transcript Measurement

To shed light on the effect of glucagon on IGF-I gene transcription in the carp liver, chromatin immunoprecipitation (ChIP) of IGF-I promoter was performed in carp hepatocytes using an EZ ChIP Assay Kit (Millipore, Billerica, MA) with the antibody for RNA polymerase II (Pol-II). After exposure to glucagon, hepatocytes were fixed in 1% formaldehyde for 10 min followed by quenching with 1 M glycine for 5 min at room temperature. Following cell harvest with a cell scraper (Thermo Scientific), hepatocytes ($\sim 4 \times 10^6$ cells) were pelleted by centrifugation, dissolved in 200 μ l SDS lysis buffer with protease inhibitor cocktail II (Roche) and sonicated using a M220 Focused-Ultrasonicator (Covaris, Woburn, MA) with peak incident power of 75 W and duty factor at 10% with 200 cycles per burst. A 5 μ l aliquot of the cell lysate containing sheared

chromatin with fragment size of 0.5–0.8 kb was saved as the input control (1:100 dilution) and the rest of the sample was subjected to immunoprecipitation using the antibody for RNA Pol-II (1:1,000, Millipore) according to the instructions of the assay kit with minor modifications (33). After decrosslinking of the chromatin pulled down by ChIP for 5 h with 65°C heating, the amount of IGF-I promoter pulled down with RNA Pol-II was monitored by real-time PCR using primers covering the proximal region of IGF-I promoter (**Supplemental Table 1**). In this experiment, parallel ChIP without Pol-II antibody (as a “no-antibody” control) or with the mouse IgG antibody (as the “antibody control”) was used as the negative control. The specificity of PCR signals detected was validated with genomic DNA as a positive control and “Mock ChIP” with Pol-II antibody but no chromatin as a negative control (as “Mock Ctrl”). To confirm that Pol-II association with IGF-I promoter could be correlated to IGF-I gene transcription, time course, and dose dependence studies were also performed in carp hepatocytes to examine the effects of glucagon on IGF-I primary transcript levels with real-time PCR using primers covering the junction between exon III and intron III of carp IGF-I gene (**Supplemental Table 1**). In our studies, serial dilutions of plasmids carrying the amplicon for IGF-I promoter/primary transcript were used as the standards for data calibration. Real-time PCR of IGF-I promoter in chromatin input was used as the loading control for Pol-II ChIP and parallel measurement of 18S RNA expression was used as the internal control for IGF-I primary transcript.

IGF-I Promoter Activity Expressed in α T3 Cells

To examine the role of promoter activation in glucagon-induced IGF-I gene transcription, a 1.07 kb 5' promoter of carp IGF-I gene was subcloned into the luciferase-expressing reporter pGL3.Basic (Promega) to generate the pIGF1(-1077).Luc construct for transfection studies in α T3 cells. Based on our validation in nine cell lines, α T3 cells were the only cell line with glucagon inducibility for carp IGF-I promoter and post-receptor signaling comparable with carp hepatocytes. To delineate the promoter region responsible for glucagon induction, 5' deletion analysis of the IGF-I promoter carried in pIGF1(-1077).Luc was conducted using a Mung Bean Nuclease 5' Deletion Kit for Kilo-Sequencing (TaKara Bio Inc., Kusatsu, Japan). For functional validation of the CRE and HBE sites identified in the proximal region of IGF-I promoter, sequence truncation, or loss-of-function mutation of these cis-acting elements was introduced in the IGF-I promoter within pIGF1.Luc construct using PCR-based deletion or Quick Change Site-Directed Mutagenesis Kit (Agilent, Santa Clara, CA). For promoter activity expression, α T3 cells seeded at $\sim 0.1 \times 10^6$ cells/ml/well in 24-well plate were transfected in Opti-MEM for 6 h with lipofectamine (Invitrogen) in the presence of 200 ng pIGF1.Luc construct (with or without 5' deletion of IGF-I promoter or CRE and HBE truncation/mutation), 50 ng glucagon receptor expression vector, 10 ng TK-Renilla (internal control), and 20 ng pEGFP-N1 (for tracking transfection efficiency) with pcDNA/Zeo as the carrier DNA to make up a total of

400 ng DNA per transfection. After a 15-h incubation for recovery, the cells were treated with glucagon for the dose and duration as indicated. To study the functional role of CREB and HNF1 α in glucagon-induced IGF-I promoter activation, parallel transfection with the expression vector for carp CREB or HNF1 α was also conducted prior to glucagon exposure. After drug treatment, α T3 cells were dissolved in passive lysis buffer (Promega) and the cell lysate prepared was subjected to luciferase activity measurement using a Dual-Glo[®] Luciferase Assay Kit (Promega, Madison, WI) as described previously (34).

Data Transformation and Statistical Analysis

For real-time PCR of IGF-I, CREB, and HNF1 α mRNA as well as IGF-I promoter and primary transcript, standard curves constructed with plasmids carrying the respective gene targets with dynamic range of $\geq 10^5$, amplification efficiency $\geq 98\%$, and correlation co-efficiency ≥ 0.95 were used for data calibration with the RotorGene Q.Rex software (Qiagen). Since the expression of 18S RNA (as internal control) did not show significant changes in our experiments, the raw data for respective gene targets were simply transformed as a percentage of the mean value in the control group (as “%Ctrl”). For the transfection studies using Luc reporters, luciferase activities expressed in α T3 cells were measured in terms of “arbitrary light unit.” To control for the variations in transfection efficiency between wells, the firefly luciferase activity conferred by the target gene promoter was normalized as a ratio of the Renilla luciferase activity caused by co-transfection of TK-Renilla (as internal control) in the same sample (as “LUC activity ratio”). Similar to the real-time PCR data, the normalized data for luciferase activity were then expressed as a percentage of the mean value in the control prior to statistical analysis. Data presented (mean \pm SEM) were pooled results from 4 to 6 separate experiments and analyzed with one-way ANOVA (for dose-dependence/interaction studies with pharmacological inhibitors) or two-way ANOVA (for time course) followed by Newman-Keuls *post-hoc* test. Differences were considered as significant at $p < 0.05$.

RESULTS

Structural Characterization, Tissue Distribution, and Functional Validation of Carp HNF1 α

As a first step to study the functional role of HNF1 α in IGF-I gene expression in grass carp, the structural identity of grass carp HNF1 α was established by 3'/5' RACE using total RNA from the liver as the template (**Supplemental Figure 1**). As revealed by the cDNA sequence obtained, the ORF of carp HNF1 α is 1,695 bp in size encoding a 564 a.a. protein (with MW of ~ 63 kDa). The deduced protein is composed of an N-terminal dimerization domain followed by the POU and homeobox domains in the central region and a transactivation domain in the C-terminal tail (**Figure 1A**). Sequence alignment at the protein level also confirms that the carp HNF1 α sequence is highly

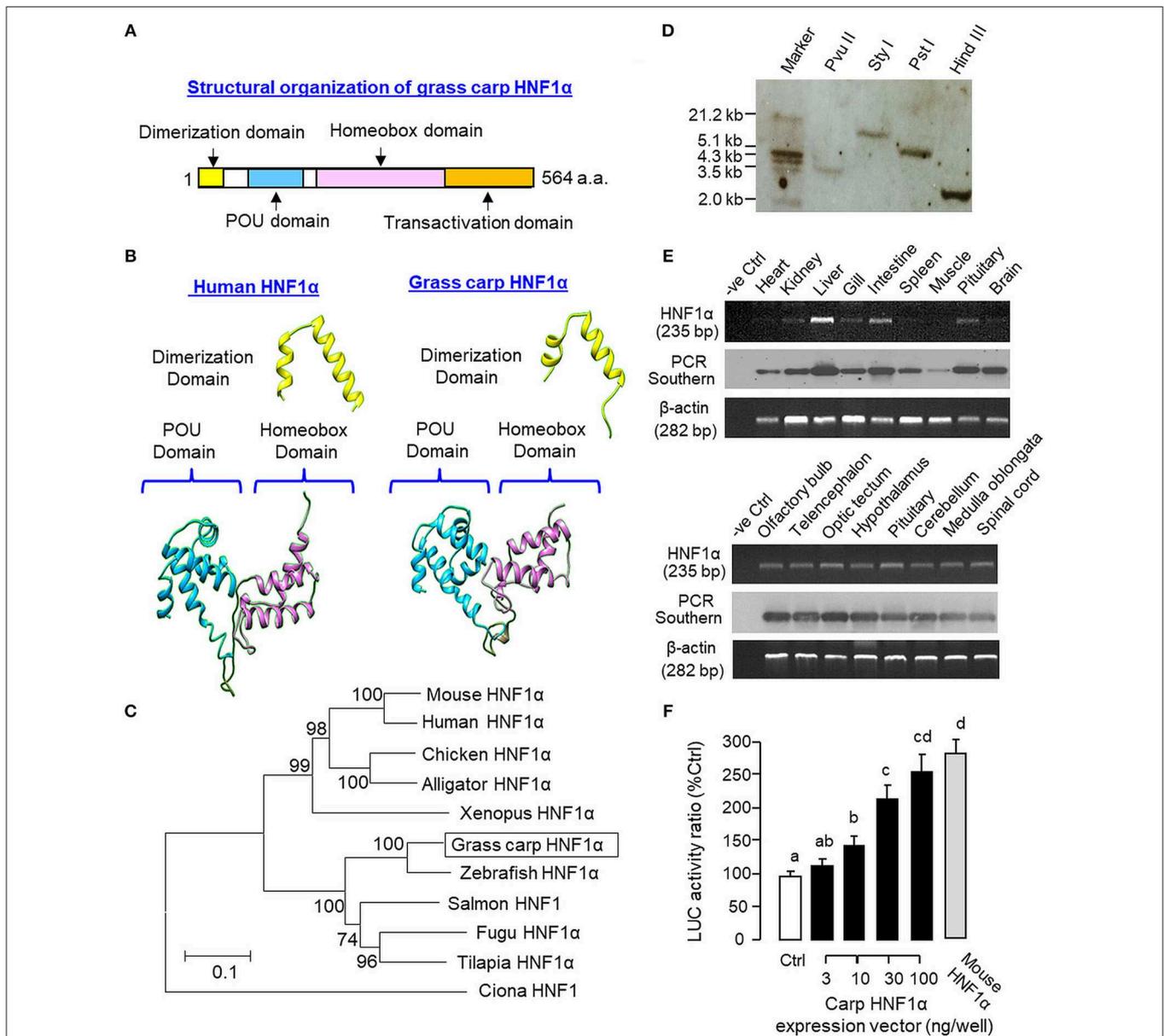


FIGURE 1 | Structural characterization, gene copy number, tissue distribution, and functional validation of grass carp HNF1α. **(A)** Structural organization of carp HNF1α a.a. sequence into four signature motifs, including the dimerization domain, POU domain, homeobox domain, and transactivation domain. **(B)** 3D Protein modeling of the dimerization domain, POU domain, and homeobox domain of carp HNF1α using the corresponding structures of their human counterparts as the template with SWISS-MODEL program. **(C)** Phylogenetic analysis of carp HNF1α cDNA sequence using the neighbor-joining method with MEGA 6.0 with the corresponding sequences identified in other species. The scale bar represents the evolutionary distance and the values for individual nodes of the guide tree are the percentage based on 1,000 bootstraps. **(D)** Copy number of HNF1α gene in the carp genome. Southern blot was conducted in genomic DNA after digestion with Pvu II, Sty I, Pst I, and Hind III, respectively, using a DIG-labeled cDNA probe for HNF1α. **(E)** Tissue expression profiling of HNF1α with RT-PCR. Total RNA was isolated from selected tissues and brain areas and subjected to RT-PCR using primers specific for carp HNF1α. The authenticity of PCR products was confirmed by PCR Southern and RT-PCR for β actin was used as the internal control. **(F)** Functional expression of carp HNF1α in αT3 cells. The ORF of grass carp HNF1α was subcloned into the eukaryotic expression vector pcDNA3.1 and transfected into αT3 cells with the Luc reporter pGL3.HNF1α.Luc with tandem repeats of HBE sites in its 5' promoter. In this study, parallel transfection with the expression vector for mouse HNF1α was used as a positive control. Data presented are expressed as mean ± SEM and groups denoted by different letters represent a significant difference at $p < 0.05$ (ANOVA followed by Newman-Keuls test).

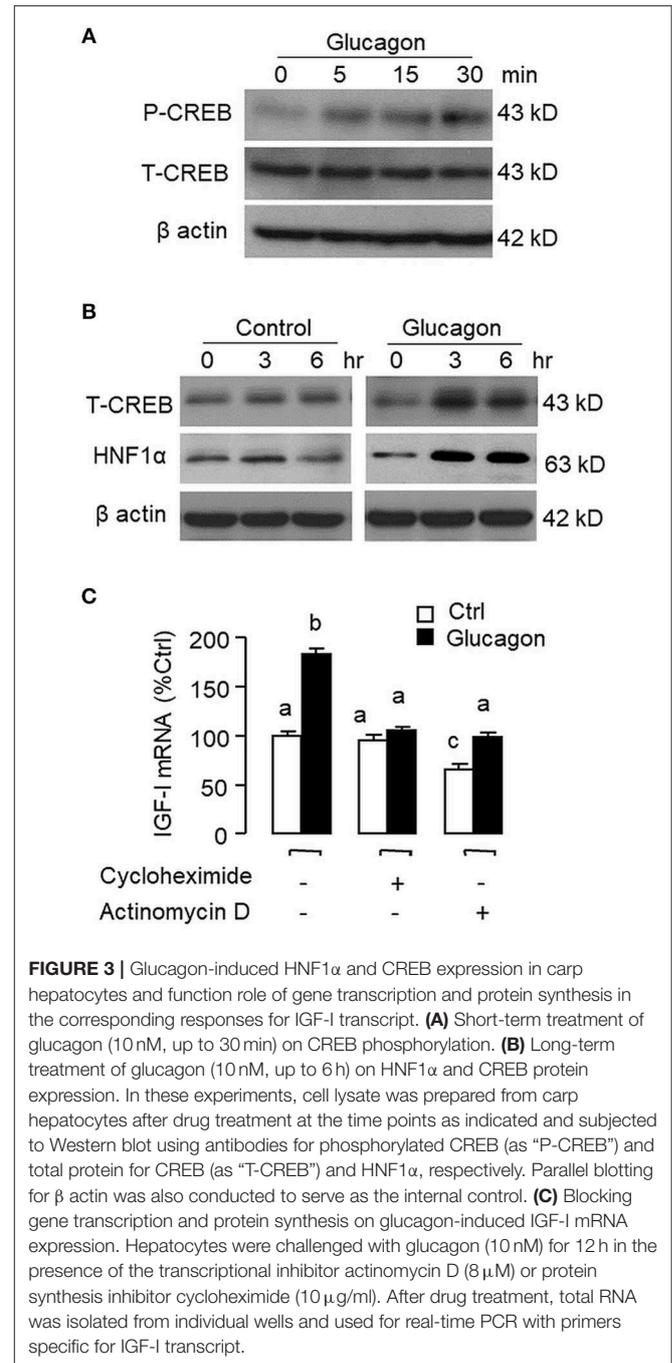
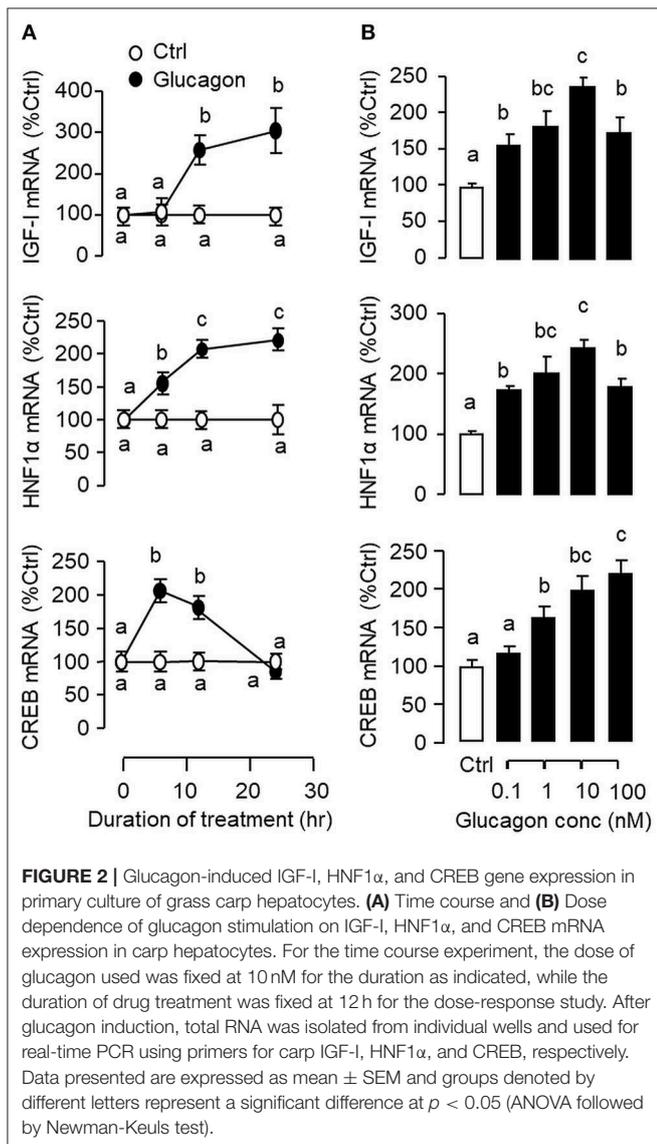
homologous to those reported in other vertebrates, especially in the POU domain (76.2–95.2%) and homeobox domain (73.2–97.9%; **Supplemental Figure 2**). Using 3D protein modeling, the spatial arrangement of the α helical structures within the

dimerization domain, POU domain, and homeobox domain of the carp HNF1α was found to be highly comparable, if not identical, with their counterparts in human HNF1α (**Figure 1B**). In parallel phylogenetic analysis using neighbor-joining method,

the nucleotide sequence of carp HNF1 α could also be clustered within the clade of fish HNF1 α with a very close evolution relationship with zebrafish HNF1 α (Figure 1C).

To deduce the copy number of HNF1 α gene in grass carp, Southern blot was performed in genomic DNA with digestion of Pvu II, Sty I, Pst I, and Hind III, respectively. As shown in Figure 1D, a single band was revealed in different samples after hybridization with the DIG-labeled probe for HNF1 α , implying that HNF1 α exists as a single-copy gene in the carp genome. For tissue expression of HNF1 α , RT-PCR was also performed in selected tissues and brain areas. In this case, PCR signals for HNF1 α were found to be ubiquitously expressed in tissues including the heart, kidney, liver, gill, intestine, spleen, muscle, and pituitary (Figure 1E, upper panels) as well as in brain areas including the olfactory bulb, telencephalon, optic tectum, hypothalamus, cerebellum, medulla oblongata, and spinal cord (Figure 1E, lower panels). The highest level of

HNF1 α signal was located in the liver while a lower extent of HNF1 α expression could also be noted in the intestine, pituitary, kidney, gill, and different brain areas. Using LC/MS/MS, peptide fragments originated from HNF1 α were consistently detected in protein samples prepared from the carp liver with trypsin digestion (protein coverage by peptides identified: 84.2% for the peptides with $\geq 75\%$ confidence and 57.3% for the peptides with 99% confidence; Supplemental Figure 3). To test if the newly cloned cDNA indeed encodes a HNF1 α protein with bioactivity, functional expression of carp HNF1 α was conducted in $\alpha T3$



cells transfected with a Luc reporter carrying tandem repeats of HBE sites in its 5' promoter. Similar to the study with mouse HNF1 α expression (as a positive control), transfection with the expression vector for carp HNF1 α was effective in inducing luciferase activity expression in a dose-dependent manner (Figure 1F).

Effects of Glucagon on HNF1 α , CREB, and IGF-I mRNA Expression in Carp Hepatocytes

To establish the temporal relationship of HNF1 α and CREB expression related to IGF-I regulation by glucagon in the carp liver, time-course study was conducted in primary culture of carp hepatocytes with glucagon treatment. As shown in Figure 2A, glucagon could elevate IGF-I mRNA levels with parallel rises of HNF1 α and CREB transcripts. Of note, the significant elevations in HNF1 α and CREB signals (occurred at 6 h) were detected well before the notable rise in IGF-I gene expression (occurred at 12 h). By fixing the duration of drug treatment at 12 h, increasing levels of glucagon were also found to induce IGF-I, HNF1 α , and CREB mRNA expression in a dose-dependent manner (Figure 2B). In parallel experiments, short-term stimulation with

glucagon (up to 30 min) could trigger rapid phosphorylation of CREB in carp hepatocytes (Figure 3A) while a longer duration of glucagon treatment (up to 6 h) was effective in increasing total protein of CREB and HNF1 α , respectively (Figure 3B). In the same cell model, glucagon-induced IGF-I mRNA expression was also sensitive to the blockade of gene transcription by actinomycin D or inhibiting protein synthesis using cycloheximide (Figure 3C), which raises the possibility that HNF1 α and CREB transcript expression/protein production may play a role in IGF-I regulation by glucagon.

Signal Transduction for IGF-I, HNF1 α , and CREB mRNA Expression Induced by Glucagon

Given that glucagon receptor is known to be functionally coupled with cAMP/PKA and PLC/IP₃/PKC pathways (35, 36), the potential involvement of the two post-receptor signaling cascades in IGF-I regulation by glucagon in the carp liver was examined. In carp hepatocytes, the stimulatory effects of glucagon on IGF-I, HNF1 α , and CREB mRNA expression were mimicked by treatment with the cAMP analog 8cpt-cAMP (Figure 4A) or by activating cAMP synthesis using

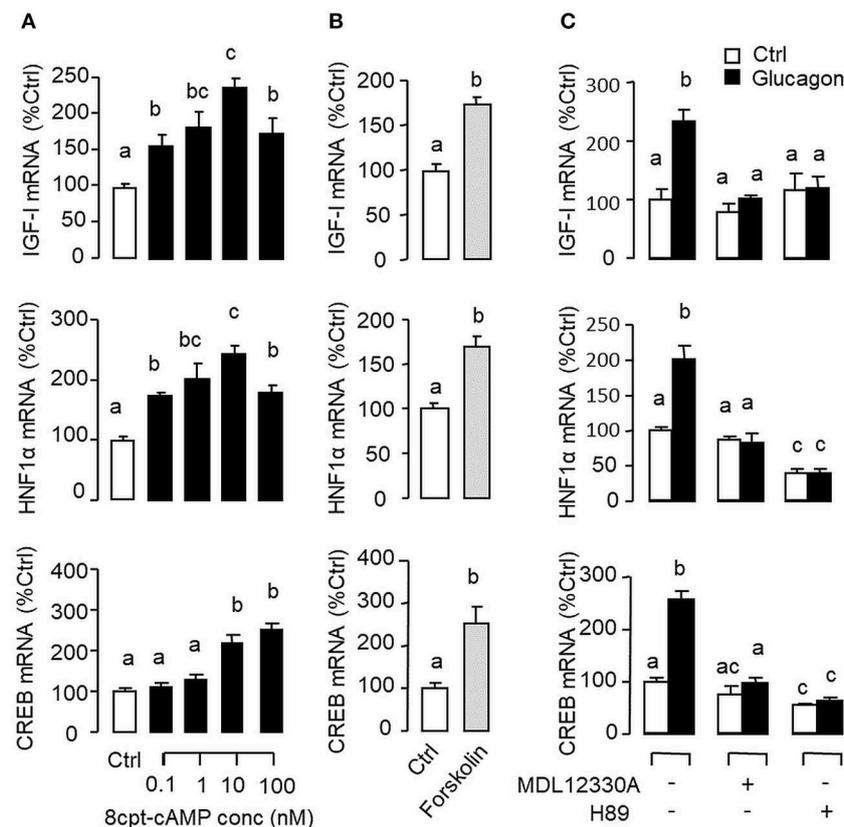
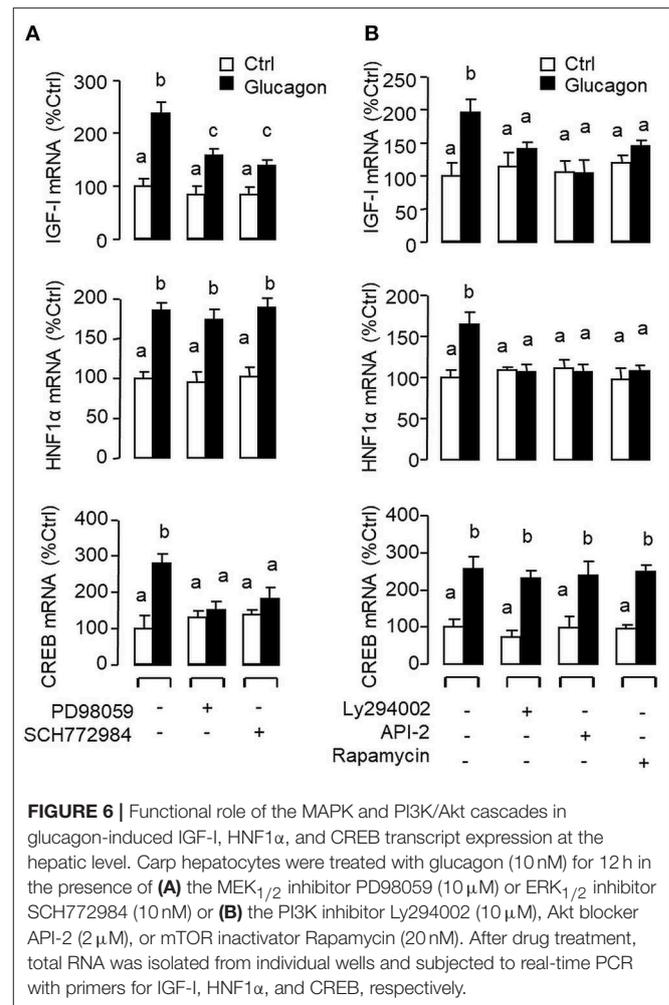
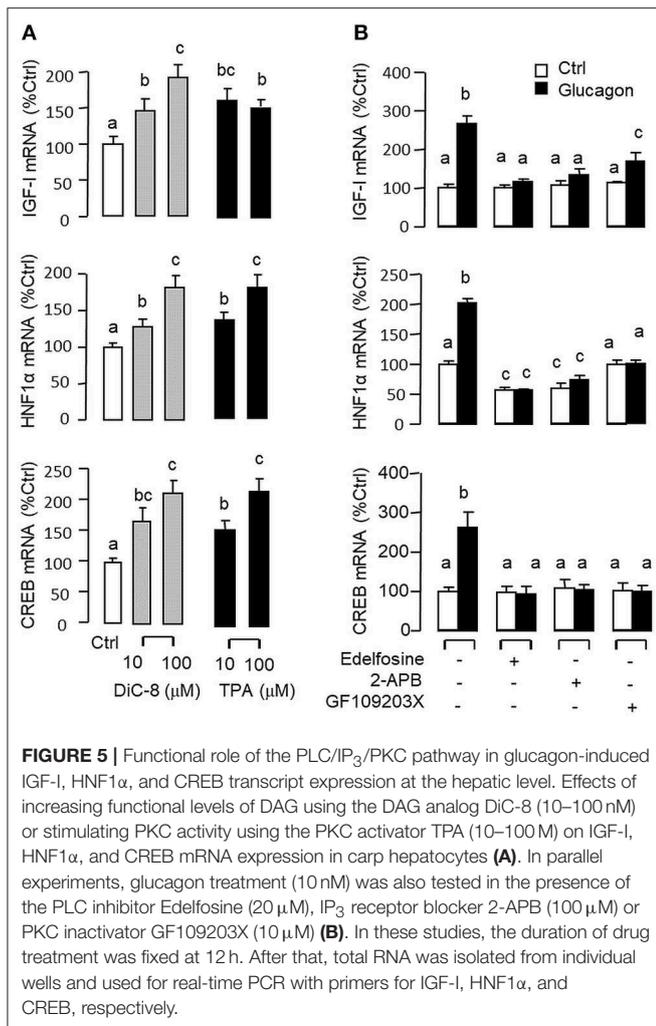


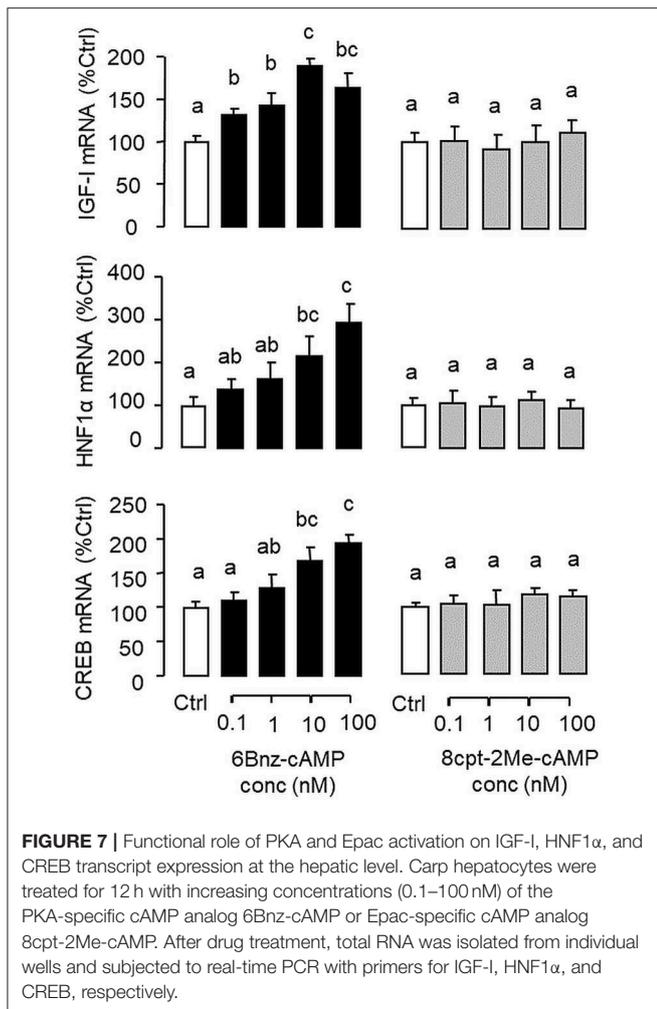
FIGURE 4 | Functional role of the AC/cAMP/PKA pathway in glucagon-induced IGF-I, HNF1 α , and CREB transcript expression at the hepatic level. Effects of (A) increasing functional levels of cAMP using the membrane-permeant cAMP analog 8cpt-cAMP (0.1–100 nM) or (B) stimulating cAMP synthesis with the adenylate cyclase (AC) activator forskolin (1 μ M) on IGF-I, HNF1 α , and CREB mRNA expression in carp hepatocytes. In parallel study, glucagon induction (10 nM) was also tested with co-treatment of the AC inhibitor MDL12330A (20 μ M) or PKA inactivator H89 (20 μ M) (C). In these experiments, the duration of drug treatment was fixed at 12 h. After that, total RNA was isolated from individual wells and used for real-time PCR with primers for IGF-I, HNF1 α , and CREB, respectively.



the adenylate cyclase (AC) activator forskolin (Figure 4B). Transcript expression of IGF-I, HNF1 α , and CREB induced by glucagon, in contrast, could be blocked by co-treatment of the AC inhibitor MDL12330A or PKA inhibitor H89 (Figure 4C). In parallel studies, IGF-I, HNF1 α , and CREB gene expression could also be up-regulated by treatment with the DAG analog DiC-8 or PKC activator TPA (Figure 5A) whereas the corresponding responses induced by glucagon were reduced/negated by the PLC inhibitor edelfosine, IP₃ receptor blocker 2-APB or PKC inactivator GF109203X (Figure 5B). In different cell models, glucagon actions mediated by MAPK (37, 38) and/or PI3K/Akt cascades (39) have also been documented. In our studies with carp hepatocytes, glucagon-induced IGF-I, and CREB but not HNF1 α transcript expression could be reduced/abolished by treatment with the MEK_{1/2} inhibitor PD98059 or ERK_{1/2} inhibitor SCH772984 (Figure 6A). However, parallel blockade with the PI3K inhibitor Ly294002, Akt blocker API-2, or mTOR inactivator rapamycin was effective in abating the actions of glucagon on IGF-I and HNF1 α mRNA levels but with no effects on CREB gene expression (Figure 6B).

Signaling Crosstalk in Glucagon-Induced IGF-I, HNF1 α , and CREB mRNA Expression

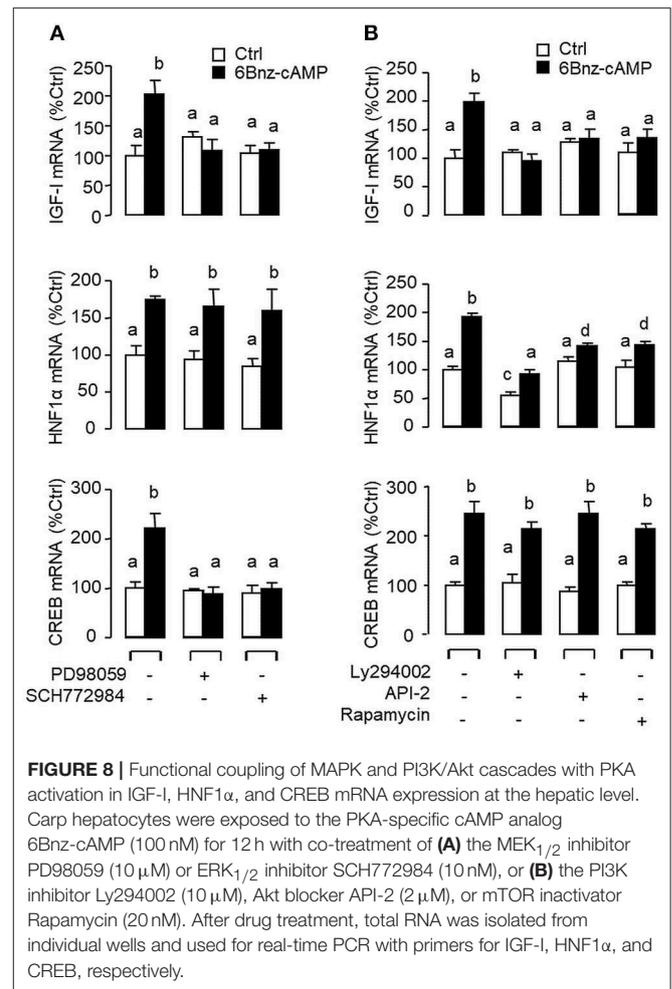
In mammals, functional crosstalk of cAMP with MAPK cascades through Epac activation of Rap1 is well-documented (40), e.g., for glucose homeostasis via insulin/glucagon signaling (41). To evaluate the functional role of Epac vs. PKA in cAMP dependence of glucagon actions, carp hepatocytes were exposed to increasing levels of the cAMP analogs 6Bnz-cAMP and 8cpt-2Me-cAMP, which are known to be specific for PKA and Epac activation, respectively (Figure 7). In this case, 6Bnz-cAMP but not 8cpt-2Me-cAMP was found to mimic the dose dependence of glucagon on IGF-I, HNF1 α , and CREB mRNA expression. To shed light on the possible interaction of cAMP/PKA and PLC/IP₃/PKC pathways with MAPK and PI3K/Akt cascades in mediating glucagon actions at the hepatic level, carp hepatocytes were challenged with the PKA-specific cAMP analog 6Bnz-cAMP (Figure 8) or PKC activator TPA (Figure 9) in the presence of the pharmacological inhibitors targeting MAPK and PI3K/Akt cascades. Similar to the preceding studies with glucagon, 6Bnz-cAMP consistently up-regulated IGF-I, HNF1 α , and CREB mRNA levels and the stimulatory effects on IGF-I and CREB



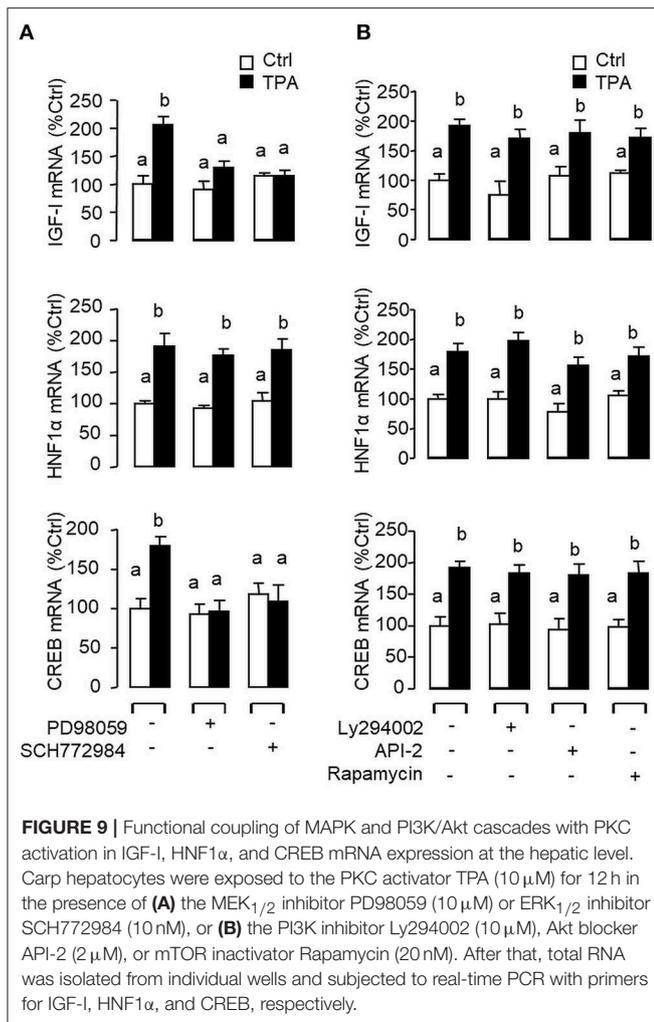
but not HNF1 α were totally blocked by co-treatment with the MEK_{1/2} inhibitor PD98059 or ERK_{1/2} inhibitor SCH772984 (**Figure 8A**). In parallel experiments, 8Bnz-cAMP induction of IGF-I and HNF1 α mRNA expression were also reduced/negated by the PI3K inhibitor Ly294002, Akt blocker API-2, or mTOR inactivator rapamycin. Similar treatment, however, did not affect the CREB signals induced by 8Bnz-cAMP (**Figure 8B**). For PKC crosstalk with MAPK and/or PI3K/Akt cascades, TPA-induced IGF-I and CREB, but not HNF1 α mRNA expression could be abated by inhibiting MEK_{1/2} with PD98059 or blocking ERK_{1/2} with SCH772984 (**Figure 9A**). Interestingly, the stimulatory effects on IGF-I, HNF1 α , and CREB expression induced by TPA were not altered by the PI3K inhibitor LY294002, Akt blocker API-2 or mTOR inactivator rapamycin (**Figure 9B**).

Glucagon-Induced Pol-II Recruitment and IGF-I Primary Transcript Expression

During the process of gene transcription, recruitment of RNA Pol-II to target gene promoter is a prerequisite for primary transcript production (42) and subsequent intron splicing can lead to formation of mature mRNA (43). To investigate the role of gene transcription in glucagon-induced IGF-I mRNA expression



at the hepatic level, Pol-II ChIP coupled to PCR detection of IGF-I promoter was performed in carp hepatocytes (**Figure 10A**). In chromatin sample pulled down by immunoprecipitation (IP) with the Pol-II antibody (as “Pol-II IP”), a single PCR product of 185 bp (covering –116 to +69 of IGF-I gene) was detected with primers flanking the proximal region of carp IGF-I promoter and the authenticity of the PCR product was confirmed with Southern blot using a DIG-labeled probe covering the same region. Although the 185 bp PCR product was also noted in parallel PCR with genomic DNA (as “+ve Ctrl”), chromatin input (as “Input Ctrl”) and “supernatant” of chromatin with “No Antibody (NA)” IP (as “NA Sup”), PCR signals were not observed in chromatin samples after IP with no antibody (as “NA IP”) or with mouse IgG (as “IgG IP”) as well as in mock IP without chromatin input (as “Mock IP”), indicating that the ChIP-PCR signal for IGF-I promoter is highly specific in our system. In parallel study with Pol-II ChIP coupled to real-time PCR for IGF-I promoter, prior treatment of hepatocytes with glucagon was found to enhance the PCR signal for IGF-I promoter pulled down with the Pol-II antibody (**Figure 10B**), implying that glucagon can promote Pol-II recruitment to IGF-I promoter at hepatic level. Consistent with the IGF-I mRNA



responses observed in preceding studies, glucagon treatment was also effective in up-regulating the levels of IGF-I primary transcript in carp hepatocytes in a time- (Figure 10C) and dose-dependent manner (Figure 10D).

Glucagon-Induced IGF-I Promoter Activity and Functional Role of HNF1 α and CREB

To evaluate the role of promoter activation in glucagon-induced IGF-I gene transcription, α T3 cells with glucagon receptor expression were used as the host cells to examine glucagon activation of IGF-I promoter of carp origin. After transfection with pIGF1(-1077).Luc carrying a \sim 1 kb 5' promoter of carp IGF-I gene, luciferase activity expressed in α T3 cells could be elevated in a time- (Figure 11A) and dose-related fashion with glucagon treatment (Figure 11B). In parallel studies, the luciferase activity induced by glucagon could be reduced/negated by (i) inhibiting the cAMP/PKA pathway using the AC blocker MDL12330A or PKA inhibitor H89 (Figure 12A), (ii) blocking the PLC/IP₃/PKA pathway with the PLC inhibitor edelfosine, IP₃ receptor blocker 2-APB or PKC inactivator GF109203X (Figure 12B), (iii) impeding MAPK signaling using

the MEK_{1/2} inhibitor PD98059 or ERK_{1/2} inhibitor SCH772984 (Figure 12C), and (iv) inactivating the PI3K/Akt cascade with the PI3K inhibitor LY294002, Akt blocker API-2 or mTOR inactivator rapamycin (Figure 12D). These results imply that the α T3 cells can faithfully mimic the post-receptor signaling for IGF-I regulation by glucagon in carp hepatocytes. To map the responsive sequence in the IGF-I promoter for glucagon action, 5' deletion analysis was conducted in the promoter of pIGF1(-1077).Luc to generate a series of deletion mutants with decreasing lengths of IGF-I promoter from -874 to -44 (Figure 13A). Transfection with these mutants revealed that both the basal and glucagon-induced luciferase activity were not affected by 5' deletion up to position -112. A further deletion from position -112 to -88 with a CRE site (TGACGTTA), however, was effective in attenuating both basal and glucagon-induced luciferase activity. Of note, the stimulatory effect of glucagon was totally ablated by further removal of the region from -88 to -44 containing a HBE site (CTTAATGAGTAAC). These results indicate that the proximal region downstream of position -112 with the CRE and HBE sites is essential for IGF-I promoter activation caused by glucagon.

To examine the functional role of HNF1 α and CREB in IGF-I promoter activation, over-expression of HNF1 α and CREB were performed in α T3 cells transfected with pIGF1(-112).Luc carrying a 112 bp IGF-I promoter with the CRE and HBE sites in the proximal region. In this case, transfection with the HNF1 α expression vector (Figure 13B) or CREB expression vector (Figure 13C) was effective in stimulating luciferase activity expression in a dose-dependent manner. Over-expression of HNF1 α or CREB not only could increase basal but also enhance glucagon-induced luciferase activity and these stimulatory effects were found to be additive with co-expression of the two transcription factors (Figure 13D). In carp hepatocytes, Western blot for CREB in the protein pull-down (IP) using HNF1 α antibody revealed that the immunoblotting (IB) signals for phosphorylated and total protein of CREB could be enhanced by glucagon treatment (Figure 13E). Since no CREB signals could be noted in the protein pull-down by IgG antibody, our results suggest that the association of HNF1 α with activated CREB is specific and can be induced by glucagon at the hepatic level. To shed light on the functionality of the HBE and CRE sites located in the proximal region of IGF-I promoter, site-directed mutagenesis and PCR-based truncation of these cis-acting elements were also conducted in pIGF1(-112).Luc. Similar to our preceding study, both basal and glucagon-induced luciferase activity were up-regulated in α T3 cells co-transfected with pIGF1(-112).Luc and HNF1 α expression vector and these stimulatory actions were reduced/obliterated with mutation (mHBE, CATATGGAGTAAC) or truncation of the HBE site (Δ HBE) within the IGF-I promoter (Figure 14A). In parallel study, similar enhancement in basal and glucagon-induced luciferase activity were also noted with co-transfection of pIGF1(-112).Luc and CREB expression vector. Again, these stimulatory effects could be blocked by mutation (mCRE, TACGGTTA) or truncation of the CRE site (Δ CRE) within the proximal region of IGF-I promoter (Figure 14B).

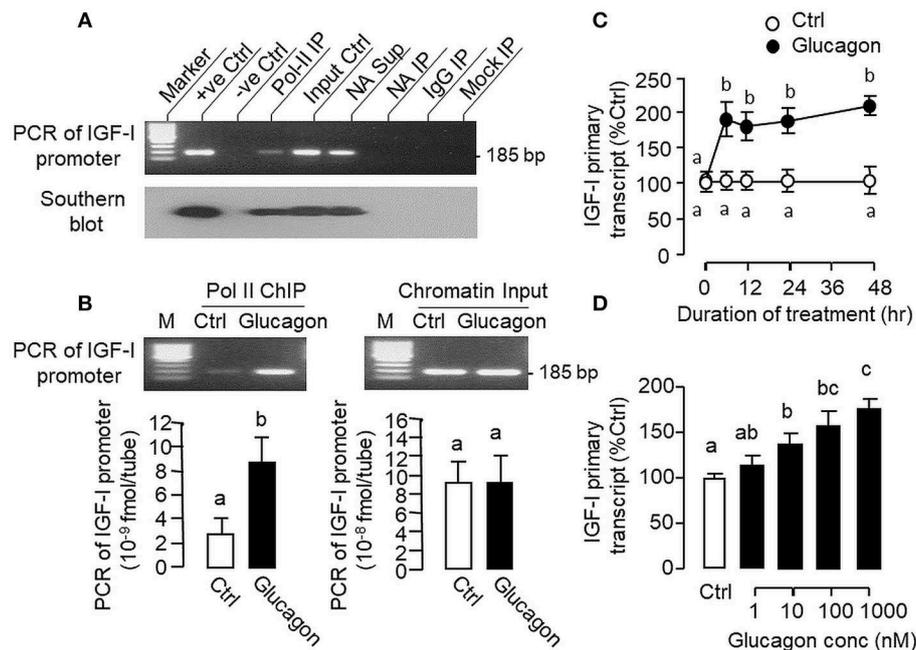


FIGURE 10 | Glucagon-induced Pol-II recruitment to IGF-I promoter and production of IGF-I primary transcript in carp hepatocytes. **(A)** Pol-II binding with IGF-I promoter revealed by PCR coupled with chromatin immunoprecipitation (ChIP). Chromatin samples with fragment size of 0.5–0.8 kb after sonication were prepared from carp hepatocytes and used for ChIP with the antibody for RNA Pol-II (as “Pol-II IP”). After decrosslinking, PCR was performed using primers covering the proximal region of the carp IGF-I promoter. In this experiment, genomic DNA (as “+ve Ctrl”), chromatin input (as “Input Ctrl”) and “supernatant” of chromatin sample after IP with no antibody (as “NA Supp”) were used as the positive control. PCR without adding the template (as “-ve Ctrl”) or with chromatin samples after IP with “No Antibody” (as “NA IP”)/parallel ChIP with antibody for mouse IgG (as “IgG IP”) or without chromatin input (as “Mock IP”) were used as the negative control. The authenticity of PCR products detected was also confirmed with Southern blot using a DIG-labeled probe covering the same region of the IGF-I promoter. **(B)** Glucagon-induced Pol-II recruitment to IGF-I promoter in carp hepatocytes. Hepatocytes were challenged for 1 h with glucagon (10 nM) and subjected to chromatin preparation followed by ChIP with Pol-II antibody. After that, real-time PCR with primers covering the proximal region of IGF-I promoter was performed for quantitation of IGF-I promoter pulled down with RNA Pol-II. Real-time PCR for IGF-I promoter was also conducted in the chromatin input prior to Pol-II ChIP to serve as the loading control. **(C)** Time course and **(D)** Dose dependence of glucagon treatment on IGF-I primary transcript expression in carp hepatocytes. For the time course experiment, the dose of glucagon used was fixed at 100 nM for the duration as indicated, while the duration of drug treatment was fixed at 12 h for the dose-response study. After that, total RNA was isolated, digested with DNase I to remove genomic DNA contamination, and subjected to real-time PCR using primers covering the junction of exon III and intron III of carp IGF-I gene.

DISCUSSION

Among the transcription factors involved in IGF-I gene expression, the role of HNF1 α appears to be well-conserved and its binding site(s), namely HBE, can be identified within the IGF-I promoter from fish to mammals (22). In previous studies, over-expression of HNF1 α was shown to elevate basal and/or confer GH inducibility for IGF-I gene transcription while mutation of HBE site(s) can suppress GH-induced IGF-I promoter activation, e.g., in human (44) and fish models including the salmon (23) and common carp (25). However, endocrine regulation of HNF1 α expression as a means to modify/control IGF-I gene transcription has not been examined and remains an unexplored area for IGF-I research. In our study with grass carp, a CRE site was located right next to the HBE site in the proximal promoter of IGF-I gene and the close proximity of the two cis-acting elements raises the possibility that HNF1 α may act with CREB to regulate IGF-I gene transcription. As a first step to investigate HNF1 α regulation related to glucagon modulation of IGF-I expression in carp model, grass carp HNF1 α was cloned and confirmed to be

a single-copy gene. Phylogenetic analysis revealed that the newly cloned cDNA could be grouped within the family of fish HNF1 α and the signature motifs for HNF1 α , e.g., the dimerization domain for HNF1 dimer formation (45), POU and homeobox domains for DNA binding (46, 47) and transactivation domain for promoter activation (48), were also identified in the protein sequence of carp HNF1 α . By *in silico* modeling, the 3D structures of dimerization domain, POU domain, and homeobox domain of carp HNF1 α were found to be highly comparable with their human counterparts. Functional expression in α T3 cells also confirmed that HNF1 α of carp origin could transactivate target promoter with HBE sites. Our findings, taken together, indicate that the newly cloned cDNA indeed encodes the bona fide HNF1 α with bioactivity in grass carp.

HNF1 α is a liver-enriched transcription factor involved in glucose and lipid metabolism (49) and its mutation can lead to MODY3 subtype of diabetes (50). In mammals, “extra-hepatic” expression of HNF1 α has also been reported, e.g., in the stomach, pancreas, intestine, and kidney of the mice (51). In agreement with its expression in the liver, pancreas, and kidney, HNF1 α

knockout in mouse model not only can induce metabolic defects at the hepatic level but also alter insulin secretion and reduce renal glucose reabsorption (52). In fish models, HNF1 α has been cloned in salmon (53) and zebrafish (54), and its tissue expression is highly comparable to that in mammals (53, 54). In zebrafish, genome-wide promoter analysis reveals the presence of binding sites for HNF1, HNF3, HNF4, and HNF6 in a large number of liver-specific/enriched genes, suggesting that HNF1 α by working with other HNFs may form a “transcriptional network” regulating hepatic functions (55). In tilapia, HNF1 α expression can also be detected in the gonad (56) and it raises the possibility that HNF1 α may play a role in steroid production/gametogenesis in fish models (57). In our study with grass carp, PCR signals for HNF1 α were found to be ubiquitously expressed with the highest level detected in the liver, to a lower extent in the intestine, pituitary, kidney and gills, and with low levels in the heart, spleen, muscle, and various brain areas. The high level of HNF1 α expression in the liver is in agreement with its role as a liver-enriched transcription factor (49), while the HNF1 α signals detected in the gut and kidney are also comparable to the previous studies in zebrafish (54). However, our findings of HNF1 α expression in the gills and pituitary, together with the signals within the brain, have not been reported previously and may suggest a possible role of HNF1 α in osmoregulation at the branchial level and neurotransmission/neuroendocrine functions in the brain-pituitary axis. In parallel study with LC/MS/MS, protein fragments originated from HNF1 α were also identified in protein samples prepared from the carp liver. These results not only provide evidence for hepatic expression of HNF1 α at the protein level but also support the idea that the transcript signals for HNF1 α detected by RT-PCR can be properly translated into target protein at the tissue level in grass carp.

Pancreatic hormones, including insulin and glucagon, are known to play a key role in regulating hepatic functions mainly via the portal vascular link between the liver and pancreas (58). Apart from their metabolic effects, insulin and glucagon can also modify hepatic expression of IGF-I, the down-stream effector of GH, but their effects are highly variable among different studies. In rat hepatocytes, insulin could induce IGF-I expression by increasing its transcript stability without affecting its gene transcription (59). In other reports with the same model, insulin was found to have no effect on basal (60) but enhance GH-induced IGF-I mRNA expression (61), probably by up-regulation of GH receptor expression (62). Similar inconsistency has also been documented for the studies with glucagon. In this case, glucagon was shown to have stimulatory (9), inhibitory (10) or no effects on IGF-I expression/secretion (63) and the cause for the discrepancy is still unclear. In fish models, similar information for IGF-I regulation is restricted to a limited number of studies. For examples, in tilapia, insulin treatment could inhibit basal and did not alter GH-induced IGF-I gene expression at the hepatic level (64). In salmon hepatocytes, interestingly, glucagon had no effect on basal but suppressed GH stimulation on IGF-I mRNA level (15). In our study with carp hepatocytes, we have the novel findings that insulin not only could increase basal but also potentiate GH-induced IGF-I gene expression through protein:protein interaction between GH receptor and insulin receptor with concurrent enhancement of STAT5, ERK1/2, and

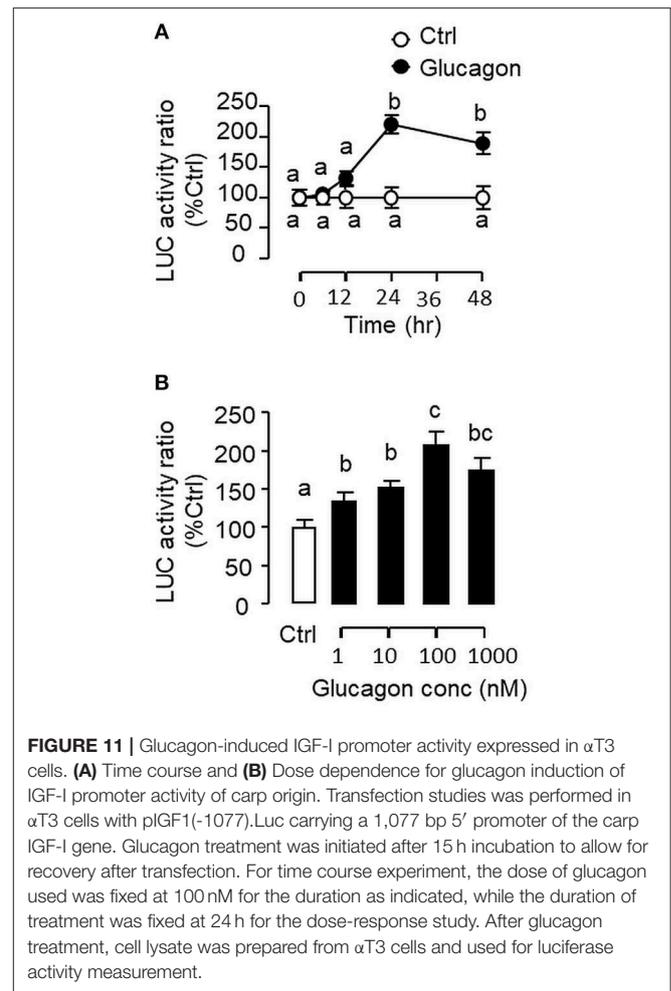


FIGURE 11 | Glucagon-induced IGF-I promoter activity expressed in α T3 cells. **(A)** Time course and **(B)** Dose dependence for glucagon induction of IGF-I promoter activity of carp origin. Transfection studies was performed in α T3 cells with pIGF1(-1077).Luc carrying a 1,077 bp 5' promoter of the carp IGF-I gene. Glucagon treatment was initiated after 15 h incubation to allow for recovery after transfection. For time course experiment, the dose of glucagon used was fixed at 100 nM for the duration as indicated, while the duration of treatment was fixed at 24 h for the dose-response study. After glucagon treatment, cell lysate was prepared from α T3 cells and used for luciferase activity measurement.

Akt signaling (32). The same model was also used in our current study to examine the mechanisms for IGF-I regulation by glucagon in the carp liver. In this case, glucagon treatment was found to elevate IGF-I mRNA levels with parallel rises in HNF1 α and CREB transcript expression in carp hepatocytes. During the process, rapid phosphorylation of CREB and elevations in protein expression of HNF1 α and CREB were also noted. Given that (i) HBE and CRE sites are present in the proximal promoter of carp IGF-I gene, (ii) the HNF1 α and CREB responses (both mRNA and protein) could occur prior to the rise in IGF-I signals after glucagon induction, and (iii) glucagon-induced IGF-I mRNA expression could be blocked by inhibition of gene transcription and protein translation, it raises the possibility that HNF1 α and CREB gene expression followed by their protein translation and subsequent activation (for CREB) may contribute to glucagon-induced IGF-I gene transcription in the carp liver.

In mammals, glucagon receptor is known to be functionally coupled with the cAMP/PKA and PLC/IP₃/PKC pathways (35, 65). Glucagon activation of MAPK (66) and PI3K/Akt cascades (67) has also been reported, which can be attributed to the signaling crosstalk by Epac, a guanine nucleotide exchange factor activated by cAMP (66, 68). In carp hepatocytes, increasing cAMP synthesis by AC activation (using forskolin) or treatment with cAMP analog (e.g., 8cpt-cAMP) could mimic glucagon

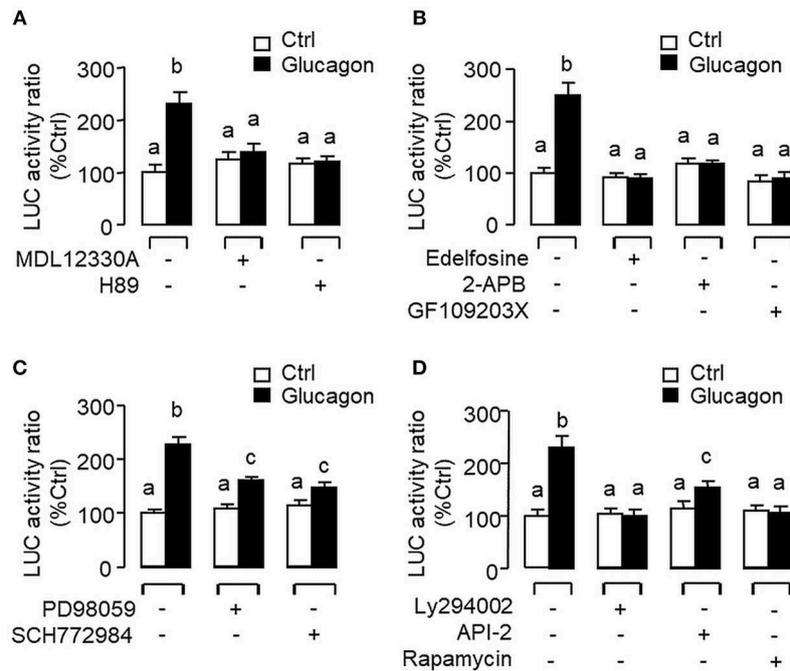


FIGURE 12 | Post-receptor signaling for glucagon-induced IGF-I promoter activity expressed in α T3 cells. After transfection with pIGF1(-1077).Luc, α T3 cells were treated with glucagon (100 nM) for 24 h in the presence of (A) the AC inhibitor MDL12330A (20 μ M) or PKA blocker H89 (20 μ M), (B) the PLC inhibitor Edelfosine (20 μ M), IP₃ receptor blocker 2-APB (100 μ M) or PKC inactivator GF109203X (10 μ M), (C) the MEK_{1/2} inhibitor PD98059 (10 μ M) or ERK_{1/2} inhibitor SCH772984 (10 nM), or (D) the PI3K inhibitor Ly294002 (10 μ M), Akt blocker API-2 (2 μ M), or mTOR inactivator Rapamycin (20 nM). After drug treatment, cell lysate was prepared and used for luciferase activity measurement.

stimulation of IGF-I, HNF1 α , and CREB mRNA whereas the corresponding responses triggered by glucagon were totally negated by inactivating AC (by MDL12330A) or PKA (by H89). In parallel experiments, IGF-I, HNF1 α , and CREB mRNA levels were also elevated by increasing functional levels of DAG (by DiC8) or direct activation of PKC (using TPA) but their corresponding stimulation induced by glucagon were found to be highly sensitive to PLC blockade (by edelfosine), IP₃ receptor inactivation (by 2-APB) or inhibiting PKC activity (by GF109203X). These findings, as a whole, support the idea that glucagon-induced IGF-I, HNF1 α , and CREB expression in the carp liver were mediated by the AC/cAMP/PKA and PLC/IP₃/PKC pathways. Regarding the role of MAPK and PI3K/Akt cascades, their involvement in hepatic expression of IGF-I induced by GH has been reported in fish models, e.g., in rainbow trout (69) and grass carp (26). In our study with carp hepatocytes, glucagon-induced IGF-I and CREB but not HNF1 α mRNA expression could be reduced/totally abolished by blocking MEK_{1/2} (by PD98059) or ERK_{1/2} (by SCH772984). However, inhibiting PI3K (by Ly294002), Akt (by API-2), and mTOR (by rapamycin) were found to ablate IGF-I and HNF1 α transcript expression induced by glucagon but the corresponding rise in CREB signals was not affected. These findings imply that (i) the MEK_{1/2}/ERK_{1/2} and PI3K/Akt/mTOR cascades were involved in IGF-I induction by glucagon in addition to the cAMP/PKA and PLC/IP₃/PKC pathways, (ii) hepatic expression of HNF1 α induced by glucagon was mediated by the PI3K/Akt/mTOR but

not MEK_{1/2}/ERK_{1/2} pathway, and (iii) the parallel response for CREB, in contrast, was mediated by MEK_{1/2}/ERK_{1/2} pathway and the PI3K/Akt/mTOR cascade was not involved. Apparently, glucagon induction of IGF-I, HNF1 α and CREB expression in the carp liver was mediated by overlapping and yet distinct post-receptor signaling mechanisms.

Since Epac is known to mediate cAMP crosstalk with the MAPK and PI3K/Akt pathways (40) and glucagon actions mediated by Epac, e.g., for ghrelin regulation (66) and glucose homeostasis (41), have also been reported, it is tempting to speculate that Epac may be involved in glucagon induction of IGF-I, HNF1 α , and CREB expression in carp liver. However, the idea is not supported by our findings based on cAMP analogs with differential selectivity for PKA and Epac. In carp hepatocytes, unlike the PKA-specific 6Bnz-cAMP which could mimic the stimulatory actions of glucagon, the Epac-specific 8cpt-2Me-cAMP was found to have no effects on transcript expression of IGF-I, HNF1 α , and CREB. Similar to the results of glucagon treatment, the stimulatory effects of 6Bnz-cAMP on IGF-I and CREB but not HNF1 α mRNA expression were sensitive to the blockade of MEK_{1/2} (by PD98059) and ERK_{1/2} (using SCH772984). Meanwhile, inhibiting PI3K (by Ly294002), Akt (by APB-2), and mTOR (by rapamycin) were also effective in blocking transcript expression of IGF-I and HNF1 α induced by 6Bnz-cAMP but with no effect on the corresponding responses for CREB. These findings suggest that (i) the MEK_{1/2}/ERK_{1/2} and PI3K/Akt/mTOR cascades were acting downstream of

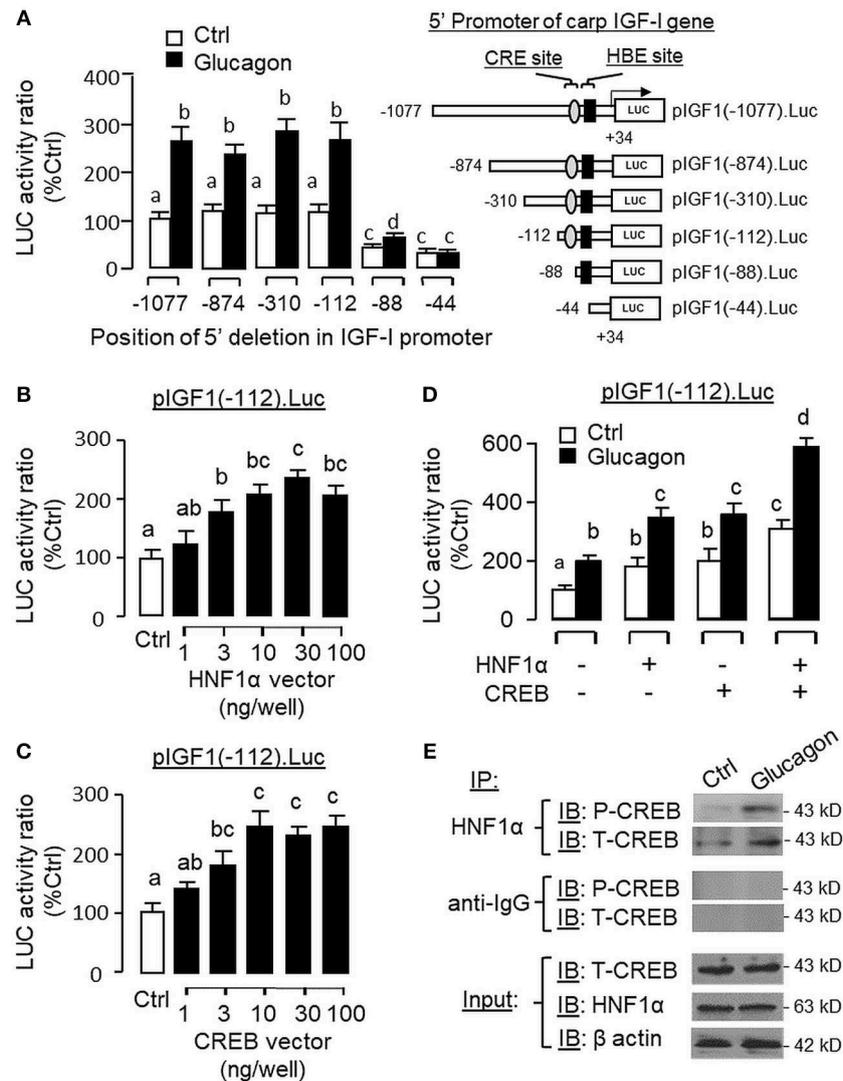


FIGURE 13 | Function role of HNF1 α and CREB in glucagon-induced IGF-I promoter activity in α T3 cells. **(A)** Mapping of glucagon responsive sequence to the proximal promoter of IGF-I gene with CRE and HBE sites. 5' Deletion was conducted in the 1,077 bp promoter of pIGF1(-1077).Luc to generate a series of 5' deleted Luc reporters with decreasing lengths of IGF-I promoter from position -874 to -44. These 5' deletion constructs were then used for transfection study in α T3 cells followed by 24 h treatment with glucagon (100 nM). Over-expression of **(B)** HNF1 α and **(C)** CREB on IGF-I promoter activation. Co-transfection was performed in α T3 cells with pIGF1(-112).Luc (with CRE and HBE sites in proximal promoter) together with increasing levels of the expression vector for carp HNF1 α or CREB as indicated. **(D)** Functional interaction of HNF1 α and CREB on glucagon-induced IGF-I promoter activity expressed in α T3 cells. In parallel study with pIGF1(-112).Luc, similar transfection was conducted with the HNF1 α vector alone (30 ng/ml), CREB vector alone (30 ng/ml) or co-transfection with the two vectors (30 ng/well each). After that, the cells were treated with glucagon (100 nM) for 24 h. In our experiments with IGF-I promoter, cell lysate was prepared from α T3 cells after the drug treatment/transfection with expression vectors and used for luciferase activity measurement. **(E)** Protein:protein interaction of HNF1 α and CREB induced by glucagon in carp hepatocytes. Immunoprecipitation (IP) was performed in cell lysate prepared from carp hepatocytes after 1-h treatment with glucagon (10 nM) using the antibody for HNF1 α . After that, the protein pull-down was subjected to immunoblotting (IB) with antibodies for phosphorylated and total protein of CREB (as "P-CREB" and "T-CREB," respectively). In this experiment, parallel IP with the antibody for mouse IgG (as "anti-IgG") was used as the negative control while the corresponding IB for HNF1 α , T-CREB, and β actin were conducted in the cell lysate prior to the pull-down with HNF1 α antibody to serve as the input control.

PKA activation for IGF-I expression, (ii) functional coupling of PKA with the PI3K/Akt/mTOR pathway was involved in HNF1 α expression, and (iii) the corresponding responses for CREB expression were mediated by PKA coupling with the MEK_{1/2}/ERK_{1/2} cascades and the PI3K/Akt/mTOR pathway was not involved. Since PKC activation of MAPK and PI3K/Akt

cascades has also been documented, e.g., in curcumin prevention of diabetic cardiomyopathy (70) and actin remodeling in chemotaxing amoeboid cells (71), the possibility of PKC crosstalk with MAPK and PI3K/Akt cascades in the hepatic actions of glucagon cannot be excluded. In carp hepatocytes, PKC activation (by TPA) consistently induced transcript expression of

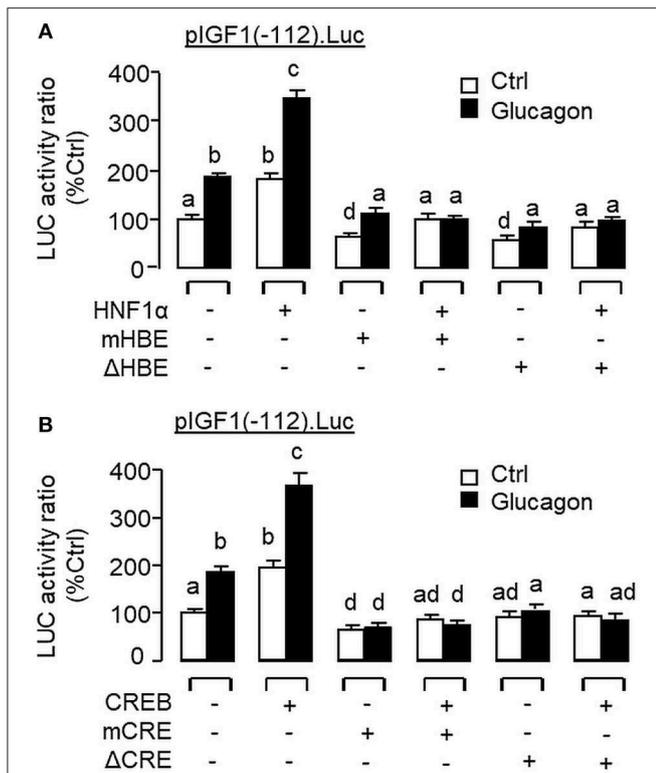
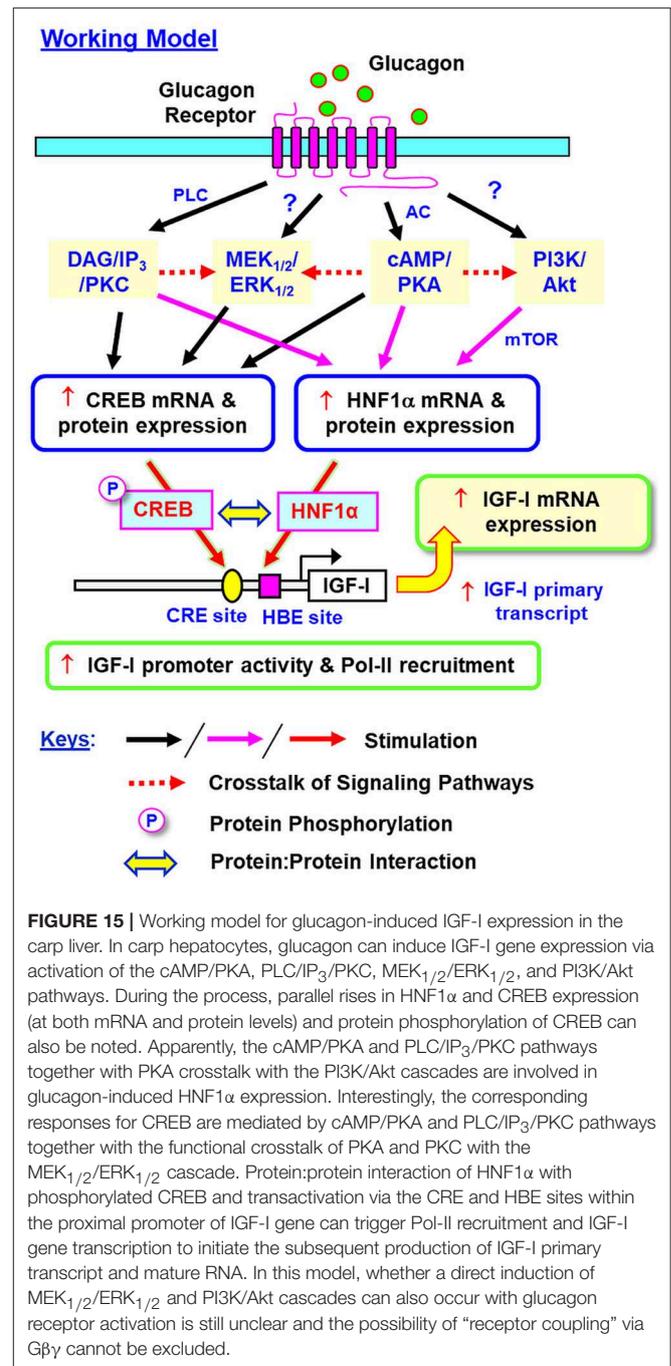


FIGURE 14 | Functional role of HBE and CRE sites within the proximal promoter of IGF-I gene in glucagon-induced IGF-I promoter activity in α T3 cells. Site-directed mutation and PCR-based truncation were conducted in (A) the HBE site (as “mHBE” and “ Δ HBE,” respectively) and (B) CRE site (as “mCRE” and “ Δ CRE,” respectively) within the IGF-I promoter of pIGF1(-112).Luc. After that, the constructs with HBE and CRE mutation/truncation were used for co-transfection studies in α T3 cells together with the expression vector for HNF1 α and CREB, respectively, followed by a 24-h treatment with glucagon (100 nM). In these experiments, parallel transfection with the wild type pIGF1(-112).Luc was used as the control. After drug treatment, cell lysate was prepared and used for luciferase activity measurement.

IGF-I, HNF1 α , and CREB and the stimulatory actions on IGF-I and CREB but not HNF1 α mRNA could be blocked by MEK_{1/2} (using PD98059) or ERK_{1/2} inactivation (by SCH772984). Parallel inhibition of PI3K (by Ly294002), Akt (by API-2), or mTOR (by rapamycin), however, were found to have no effects on the stimulatory responses induced by PKC activation. Our results indicate that, unlike PKA coupling with both MARK and PI3K/Akt cascades, the MEK_{1/2}/ERK_{1/2} pathway was acting downstream of PKC and involved in IGF-I and CREB but not HNF1 α gene expression. Although our studies have provided evidence for differential crosstalk of PKA and PKC with the MARK and PI3K/Akt cascades, we do not exclude the possibility that direct stimulation of MARK and PI3K/Akt signaling can also be achieved by glucagon receptor activation, e.g., by signal transduction via G β γ protein (72).

In previous reports with mammalian models (e.g., rat), the studies on IGF-I regulation by glucagon have been focused on IGF-I secretion/transcript expression (9, 10) and no information



is available for glucagon regulation of IGF-I gene transcription. In carp hepatocytes, glucagon treatment was found to enhance the recruitment of Pol-II to IGF-I promoter with parallel elevation in IGF-I primary transcript. Since Pol-II recruitment at promoter level (42) followed by primary transcript production (43) are the crucial steps for gene transcription, our findings may imply that the responses for IGF-I mRNA were caused by glucagon induction of IGF-I gene transcription. This idea is also supported by the results of transfection studies in α T3 cells with Luc

reporters carrying the carp IGF-I promoter. In this case, IGF-I promoter activity could be elevated by glucagon treatment, and similar to carp hepatocytes, the trans-activating effect of glucagon on IGF-I promoter was mediated by cAMP/PKA, PLC/IP₃/PKC, MAPK, and PI3K/Akt cascades as revealed by pharmacological blockade of different components of respective signaling pathways. Using 5' deletion, the region of IGF-I promoter responsive to glucagon induction was mapped to the proximal promoter downstream of position -112 containing a CRE site sitting right next to a HBE site. Although the presence of CRE site in IGF-I promoter has not been reported previously, the HBE site located in IGF-I promoter of fish species, e.g., in salmon (24) and common carp (25), is well-conserved and known to be involved in GH-induced IGF-I promoter activity. In our study with carp hepatocytes, interestingly, we have the novel finding that glucagon treatment could induce rapid association of HNF1 α with phosphorylated CREB. For parallel transfection studies in α T3 cells, over-expression of HNF1 α or CREB increased both basal and glucagon-induced IGF-I promoter activity and these stimulatory actions could be further enhanced by co-expression of the two transcription factors. Of note, the enhancing effects on glucagon induction caused by HNF1 α or CREB over-expression could be blocked by site-directed mutagenesis/truncation of the respective cis-acting elements within the IGF-I promoter. Our findings, as a whole, suggest that HNF1 α and CREB expression (accompanied with CREB activation) induced by glucagon followed by their protein:protein interaction and transactivation via the CRE and HBE sites within the proximal region of IGF-I promoter can lead to Pol-II recruitment and IGF-I gene transcription in the carp liver.

In summary, grass carp HNF1 α was cloned and confirmed to be a single-copy gene expressed in the liver with the ability to transactivate target promoter with HBE sites. Based on our studies in grass carp hepatocytes and α T3 cells, a working model has been proposed for the signal transduction and transcriptional mechanisms mediating glucagon-induced IGF-I gene expression in the carp liver (**Figure 15**). In grass carp, glucagon can trigger IGF-I mRNA expression at the hepatic level via activation of the cAMP/PKA, PLC/IP₃/PKC, MAPK, and PI3K/Akt cascades with parallel rises of HNF1 α and CREB expression and CREB phosphorylation. The cAMP/PKA and PLC/IP₃/PKC pathways activated by glucagon together with PKA crosstalk with PI3K/Akt/mTOR cascades are involved in the stimulatory effect on HNF1 α expression. The corresponding actions on CREB expression, however, are mediated by glucagon activation of cAMP/PKA and PLC/IP₃/PKC cascades with PKA and PKC crosstalk with the MEK_{1/2}/ERK_{1/2} pathway. During glucagon stimulation, protein:protein interaction between HNF1 α and phosphorylated CREB and transactivation of IGF-I promoter via the CRE and HBE sites within the proximal promoter region also occur, which then trigger RNA Pol-II recruitment and initiation of IGF-I gene transcription for subsequent production of IGF-I primary transcript and mature mRNA. Our studies for the first time provide information on (i) the post-receptor signaling mechanisms for glucagon-induced IGF-I gene expression at the

hepatic level, and (ii) the novel responses of HNF1 α and CREB expression induced by glucagon and their functional role in glucagon-induced IGF-I promoter activation. In mammals, Pol-II binding to IGF-I promoter (e.g., rat) with p300-dependent histone hyperacetylation (20) and HNF6 association with Foxa2 (73) or C/EBP α (74) to enhance promoter activity via p300/CBP recruitment have been reported (e.g., in HepG2 cells). In different cell models, the histone acetylases p300/CBP are known to bind with CREB and act as the co-activators for its transactivation activity via complex formation with CRT2 (27). Whether p300/CBP recruitment can occur during HNF1 α /CREB interaction induced by glucagon and contribute to IGF-I gene transcription by chromatin acetylation is unclear, and for sure, can be as an interesting direction for our on-going research.

DATA AVAILABILITY

The datasets generated for this study can be found in the GenBank.

ETHICS STATEMENT

This study was carried out in accordance with the recommended guidelines for the care and use of animals for research and teaching at the University of Hong Kong (Hong Kong). The protocol used in our study (CULATR4608-18) was approved by the Committee on the Use of Live Animal for Teaching and Research, University of Hong Kong.

AUTHOR CONTRIBUTIONS

AW was the PI and grant holder. AW and JB were responsible for project planning and data analysis. JB and XJ were involved in molecular cloning and functional studies for IGF-I, HNF1 α , and CREB expression. MH was responsible for LC/MS/MS detection of HNF1 α protein expression in the liver. Manuscript preparation and editing were done by AW, BC, and JB.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fendo.2019.00605/full#supplementary-material>

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