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ORIGINAL PAPER

Activation of GH signaling and GH-independent stimulation of growth in zebrafish by introduction of a constitutively activated GHR construct

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Abstract *Growth hormone* (*GH*) gene transfer can markedly increase growth in transgenic fish. In the present study we have developed a transcriptional assay to evaluate GH-signal activation (GHSA) in zebrafish embryos. By analyzing the transcription of *c-fos* and *igf1,* and the promoter activity of *spi2.1*, in zebrafish embryos injected with different constructs, we found that overexpression of either *GH* or growth hormone receptor (*GHR*) resulted in GHSA, while a synergetic overexpression of *GH* and *GHR* gave greater activation. Conversely, overexpression of a C-terminal truncated dominant-negative *GHR* (ΔC-*GHR*) efficiently blocked GHSA epistatic to *GH* overexpression, demonstrating the requirement for a full GHR homodimer in signaling. In view of the importance of signal-competent GHR dimerization by extracellular GH, we introduced into zebrafish embryos a constitutively activated *GHR* (CA-*GHR*) construct, which protein products constitutively dimerize the GHR productively by Jun-zippers to activate downstream signaling in vitro. Importantly, overexpression of CA-*GHR* led to markedly higher level of GHSA than the synergetic overexpression of *GH* and *GHR*. CA-*GHR* transgenic zebrafish were then studied in a growth trial. The transgenic zebrafish showed higher growth rate than the control fish, which was not achievable by *GH* transgenesis in these zebrafish. Our study demonstrates GH-independent growth by CA-*GHR* in vivo which bypasses normal IGF-1 feedback control of GH secretion. This provides a novel means of producing growth enhanced transgenic animals based on molecular protein design.

Keywords GH · GHR · Constitutively active GHR · Protein design · Zebrafish

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Introduction

The potential economic benefits of transgenic technology to aquaculture are enormous, following the introduction of novel desirable traits to farmed fishes (Chen et al. 1996). With the expansion of the global population and overfishing, advanced aquaculture techniques are needed to meet the increasing demand

for fish protein. Transgenic technology offers the opportunity to improve both the quantity and quality of conventional fish strains currently exploited in aquaculture (Fu et al. 2005). It is also an important methodology for studying the function of genes and genomes in model animals (Kikuta and Kawakami 2009). Successful production of transgenic fish was first demonstrated in goldfish (Zhu et al. 1985) and 3 years later in zebrafish (Stuart et al. 1988). More than 30 fish species, including many of the major aquaculture species like carp, tilapia, catfish and salmonids, have been genetically engineered with most efforts targeted to enhancing growth and feed conversion efficiency through the transfer of *growth hormone* (*GH*) gene constructs (Zhu and Sun 2000; Wu et al. 2003; Devlin et al. 2006). *GH* transgenesis has shown to result in different effects of growth enhancement in host fish depending on different genetic backgrounds (Devlin et al. 2001, 2009). For instance, one report showed that *GH* transgenesis is effective to growth only in hemizygous but not in homozygous individuals of those transgenic zebrafish (Studzinski et al. 2009) and we have never obtained fast-growing transgenic zebrafish in our laboratory by *GH* transgenesis.

It is well known that GH is the major regulator of postnatal growth and metabolism (Lichanska and Waters 2008) *via* GH receptor (GHR) signaling pathways (Rowland et al. 2005). The GHR is a type I cytokine receptor consisting of extracellular, transmembrane and intracellular domains. GH activates the GHR by realigning two identical receptor subunits in a constitutive dimer through binding with their extracellular domains, leading to the activation of JAK2 kinase associated with the intracellular domain of GHR (Herrington and Carter-Su 2001; Waters et al. 2006). STATs 5a, and 5b bind to particular phospho-tyrosines in the cytoplasmic domain of the GHR following their phosphorylation by JAK2, are themselves tyrosine phosphorylated by JAK2, dimerize, and translocate to the nucleus to activate *igf1* transcription in particular. JAK2 also directly activates STAT1 and 3 by tyrosine phosphorylation, and again, these STATs translocate to the nucleus, bind to particular STAT responsive elements and together with other trans-factors, activate the transcription of target genes such *c-fos* (Cesena et al. 2007; Ihle and Gilliland 2007). Intriguingly, in our previous study, GH-independent activation of GHR can be achieved in cell culture by fusing the transmembrane and intracellular domains of GHR to the leucine zippers to achieve an active dimer orientation. This stabilizes the receptor dimer in a conformation that holds the box 1 sequences in proximity, facilitating signaling (Behncken et al. 2000). Here we have utilized the leucine zipper constructs to demonstrate for the first time enhanced growth by a molecularly designed constitutively activated *GHR* (CA-*GHR*) in zebrafish, which we have found is normally resistant to *GH* transgene-mediated growth.

In previous studies, the expression profiles of *GH* and *GHR* have been analyzed either during development or in different tissues of zebrafish (Zhu et al. 2007; Di Prinzio et al. 2010). However, a comparative expression analysis of *GH*, *GHR* and the signaling targets, *c-fos* and *igf1*, in zebrafish, has not been described. In our present study, we utilized zebrafish to study the comparative expression of GH signaling factors during embryonic development and in different tissues of adults. By setting up an in vivo model to evaluate the GH-signal activation (GHSA) levels in zebrafish embryos, furthermore, we were able to perform functional analysis of GH signaling during zebrafish early development by overexpression of *GH* or *GHR*.

Materials and methods

Fish maintenance and embryo injection

Fish were cultured in the fish culture facility, Institute of Hydrobiology, according to the *Zebrafish Book* (Westerfield 2000). Embryos were obtained from artificial fertilization and microinjected with indicated reagents with a pressure microinjector as described (Liu et al. 2005). mRNA was injected in the yolk below the first developing cell and DNA was injected inside the cytoplasm at 1-cell stage.

Constructs design and mRNA synthesis

The CA-*GHR* construct was described previously (Behncken et al. 2000). As shown in Fig. 1a, the transgene codes for a porcine GHR signal peptide (amino acids 1-27), the mouse *c-jun* leucine zipper (amino acids 277–315) fused upstream of the porcine

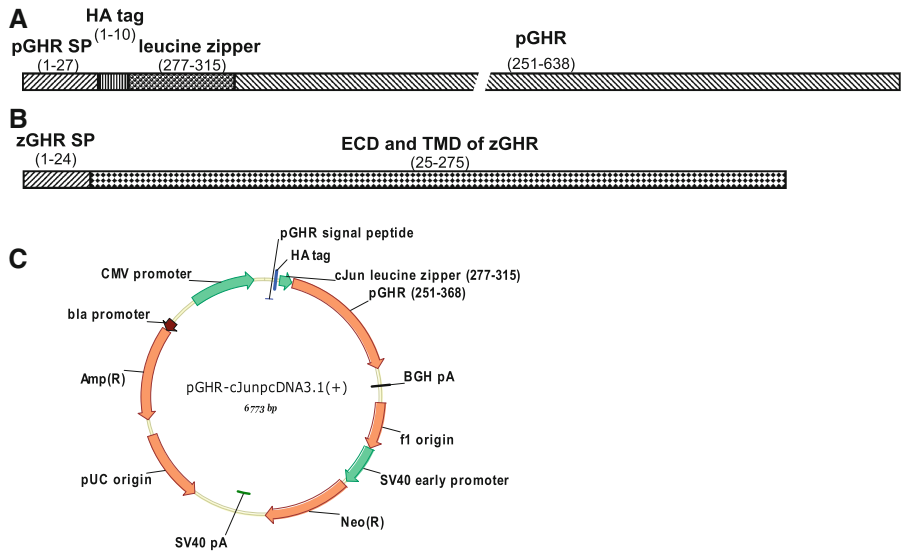

Fig. 1 Schematic description of leucine zipper transgene chimera *Jun-GHR* (**a**) and C-terminal truncated zebrafish *GHR* (**b**) and the plasmid pGHR-cJun-pcDNA3.1(+) (**c**). Numbers in parentheses refer to the amino acid sequence from which the respective cDNA segments were taken. *SP* signal peptide, *pGHR* porcine GHR, *zGHR* zebrafish GHR, *ECD* extracellular domain, *TMD* transmembrane domain, *BGH pA* bovine GH polyA

GHR transmembrane and cytoplasmic domains (amino acids 251–638), cloned into the pcDNA3.1(+) vector. The transgene expression cassette is driven by a CMV promoter and utilizes a bovine GH polyA (bGH pA) (Fig. 1c) and the construct pGHR-cJun-pcDNA3.1 is hereafter termed as *Jun-GHR*. cDNA of 3 dpf (day-post-fertilization) zebrafish was used to amplify the full length cDNA and the sequence coding the extracellular and transmembrane domains (dominant negative C-terminal truncated protein, ΔC-*GHR*, Fig. 1b) of *GHRa* via high-fidelity PCR by using Kod-plus enzyme (Toyobo, Japan) and appropriate primers (Table 1). The primers were designed according to zebrafish *GHRa* cDNA sequence available in GenBank (NM_001083578). Common carp *GH* (c*GH*) cDNA was amplified from cDNA pool of common carp pituitary with primers cGH_P1 and cGH_P2 (Table 1). For overexpression of c*GH* and z*GHR*, the PCR products were cloned into pCS2+ expression vector after double digestion of the PCR products. For mRNA synthesis, ΔC-*GHR*-pCS2+ construct was linearized with *Kpn*I and capped mRNA was synthesized using the Message Machine kit (Ambion Inc., USA) as previously described (Chen et al. 2009). All the DNA constructs have the same constitutive CMV promoter.

RT-PCR (reverse-transcription PCR) analysis of GHR signaling related genes

Total RNA of 50 zebrafish embryos, pooled tissues from 3 wildtype individuals or tissues from one transgenic individual was extracted using TRIzol® reagent (Invitrogen, USA) according to the manufacturer's instructions. All RNA samples were treated with RNase-Free DNase (Promega, USA) according to manufacturer's protocol. cDNA was synthesized by reverse transcription (RT) from 1 μg of total RNA using the ReverTra Ace enzyme (TOYOBO, Japan), dNTPs and Oligo(dT)20 RT primer (TOYOBO, Japan). The RT reaction was performed at 42°C for 60 min followed by 95°C for 5 min. PCR was performed to study the expression of different genes, such as *GH, GHR, c-fos* and *igf1* in different samples. The primer sequences are shown in Table 1. Each pair of primer sequences locate in different exons of the respective gene, to avoid the amplification of genomic DNA potentially contaminated in the samples. PCR was carried out in a 25 μl reaction volume containing 2.5 μl of 10× PCR buffer, 0.2 μM of each primer, 0.2 mM of each dNTP, 0.75 mM of $MgCl_2$, 0.5 unit of Taq DNA polymerase (Fermentas, Canada) and 50 ng of cDNA solution. The PCR

Table 1 Primers used in the study

Primer name	Sequence (5′–3′)	Gene (purpose)	Expected size (bp)
GH_P1	GTTGGTGGTGGTTAGTTTGCT	*GH* (RT-PCR)	331
GH_P2	CAGGCTGTTTGAGATAGTGGAG		
GHRa_P1	ACCGATAAAGAGTATGAAGTGCG	*GHR* (RT-PCR)	541
GHRa_P2	TGTGAAGAAGGGAAGCCAAG		
c-fos_P1	GTGGGAGCAGGAACTGAGGG	*c-fos* (RT-PCR)	618
c-fos_P2	GGTTCTTGTTTACTGGAGGGATA		
igf1_P1	GTCTAGCGGTCATTTCTTCCA	*igf1* (RT-PCR)	320
igf1_P2	CAGGCGCACAATACATCTCG		
BA_P1	ATCTGGCATCACACCTTCTACAAC	*β-actin* (RT-PCR)	254
BA_P2	TAACCCTCATAGATGGGCACGGT		
Jun-GHR_P1	TGCTCAGGGAACAGGTGG	Transgene detection	715
Jun-GHR_P2	TGGGCAGTTTGATGAGTTGA		
GHR_P3	GCAGGATCCATGGCCCACTCGCTCTCTCTC	*GHR* (fulL length cloning)	1,731
GHR_P4	AGGCTCGAGTTATCTGGGTTGCGCAGATAAG		
GHR_P5	AGGCTCGAGTCACCTCTGCTGCTGGGAGATGAC	*GHR* (C-terminal truncated cloning)	846
cGH_P1	TTCGAATTCTGAGCGAAATGGCTAGAGTA	carp *GH* (cDNA cloning)	924
cGH_P2	AGTTCTAGATAAATTGCTTAGACACCACTGT		
c-fos_P3	TCGCTGATTCGCAGACACGC	*c-fos* (real-time PCR)	199
c-fos_P4	ATCGCTCTACATCCATCTCACAGTCC		
igf1_P3	TTCAGCAAACCGACAGGATA	*igf1* (real-time PCR)	124
igf1_P4	TCTTCACAGGCGCACAATAC		
BA_P3	TCACCACCACAGCCGAAAG	*β-actin* (real-time PCR)	98
BA_P4	AGAGGCAGCGGTTCCCAT		

reaction consists of a denaturation at 94°C for 5 min, 35 cycles of 30 s at 94°C, 30 s at 56°C and 45 s at 72°C, with a final elongation step of 10 min at 72°C. *β-actin* primer pairs (Table 1) were used to normalize the cDNA concentration of all samples. The products were run on 1% agarose gels stained with ethidium bromide (0.5 μg/ml), and amplified bands were visualized by ultraviolet transillumination and semi-quantified by Glyko Bandscan software (Novato, CA).

Real-time quantitative PCR analysis of *c-fos* and *igf1*

Fertilized zebrafish zygotes were microinjected with the DNA constructs *Jun-GHR*, zebrafish *GHRa* (z*GHRa*), carp *GH* (c*GH*), c*GH* and z*GHRa* at a concentration of 50 ng/μl for each construct. To illustrate the importance of functional GHR, *cGH* expression construct of 50 ng/μl and ΔC-*GHR* mRNA of 800 ng/μl were co-injected into zebrafish embryos. Each embryo was injected with 1 nl of DNA or RNA sample and each sample was injected with 300 embryos. cDNA from the manipulated zebrafish embryos at 2-cell stage, 1 and 3 dpf and from different tissues of adult transgenic zebrafish was synthesized as described above and analyzed with real-time quantitative PCR for *c-fos* and *igf1* expression level. Real-time PCR was performed on an ABI PRISM® 7000 Sequence Detector (Applied Biosystems, Inc. USA) according to the manufacturer's instructions. Reactions were performed in a 20 μl volume with 150 ng of cDNA, 0.2 μM primers and 10 μl of SYBR® Green Realtime PCR Master Mix (TOYOBO, Japan). The primer pairs were shown in Table 1. All reactions were run using the following conditions: 1 min denaturation at 95°C followed by 40 cycles of 95°C denaturation for 15 s, 55°C annealing for 15 s, and 72°C extension for 45 s. Detection of the fluorescent product was carried out at the end of the 72°C extension period. The data (Ct value), obtained from the software (7000 system SDS

software), was transferred to Windows Excel (Microsoft co.) sheet and fold-change was calculated using $2^{-\Delta\Delta Ct}$ method, with *β-actin* as the calibrator. To validate the stability of *β-actin* for real-time PCR normalization, several pairs of primers specific for *PSB7*, *eTIF-2B*, *eEF-1A* and *GAPDH* were designed as described in our previous study and used for GeNorm analysis (Vandesompele et al. 2002; Pei et al. 2007). Three samples were run for each analysis and all real-time PCR reactions were run in triplicates. The significance of the mean differences between various experimental groups was determined by one way ANOVA followed by Duncan's multiple range test analyses. A P value <0.05 was considered statistically significant.

In vivo luciferase assay of *spi2.1* promoter activity

Each zebrafish zygote was co-injected with *spi2.1-luc* (Jiao et al. 2006) of 50 pg and a constitutively expressed *TK-Renilla* luciferase construct (Promega) of 0.5 pg and the manipulated embryos were subsequently injected with indicated DNA or RNA samples. Embryos were allowed to develop until 3 dpf, then sets of 20 embryos were lysed in passive lysis buffer (Dual-Luciferase Reporter Assay System, Promega) and the luciferase activity were measured with a Berthold luminometer and relative luciferase activity was calculated as described (Liu et al. 2009). Each sample was analyzed in triplicate, and mean value and standard deviation were calculated. The significance of the mean differences between various experimental groups was determined by one way ANOVA followed by Duncan's multiple range test analyses. A P value < 0.05 was considered statistically significant.

Generation, screening and growth trial of transgenic zebrafish

Fertilized zebrafish zygotes were microinjected with *Jun-GHR* construct of 50 ng/μl. Transgenic embryos and fish were raised according to standard procedure (Westerfield 2000). Transgene positive P0 fish were screened out by PCR assay of the total DNA of caudal fin. DNA samples were extracted from caudal fin samples or embryo samples by means of treatment with DNA extraction buffer and phenol/chloroform purification, followed by ethanol precipitation as described (Sun et al. 2005). PCR reactions were performed in a reaction mix of 25 μl, containing 50 ng of total DNA, 0.5 unit of Taq DNA polymerase (Fermentas, Canada), 1× Taq buffer, 0.5 μl of 10 mM dNTPs (Generay Biotech, China), 1 μl of each 10 μM primer, and a suitable amount of sterile deionized water. The PCR protocol used for transgene detection was as follows: 5 min denaturation at 95°C, 30 cycles of 30 s at 95°C, 30 s at 55°C and 45 s at 72°C and a final elongation step for 7 min at 72°C. *Jun-GHR*_P1 and *Jun-GHR*_ P2 (Table 1) were used as PCR primers. All PCR products were separated by 1% agarose gel electrophoresis and visualized by ultraviolet transillumination.

Each matured P0 transgenic zebrafish was mated with wildtype zebrafish and a pool of 10 F1 embryos from each cross was checked by PCR detection of the transgene. Each batch of embryos showing transgene positive was considered as an individual F1 transgenic batch. At 1 month-post-fertilization (mpf), tail fin DNA was extracted to check the presence of transgene in each F1 fish. 100 transgenic individuals from one batch of F1 embryos and 100 non-transgenics were raised in aquaria at the same condition. The body length and weight were measured from 20 individuals of transgenic and non-transgenic zebrafish after random selection from 1 to 4 mpf. Student's t-test was applied for statistical comparison and the differences were considered significant at $P < 0.01$. All values in the text and figures refer to mean ± standard deviation (SD).

Results

Spatio-temporal expression of GH signaling components

RNA from various tissues was extracted and RT-PCR was used to analyze the transcription of *GH*, *GHR*, *c-fos* (Cesena et al. 2007) and *igf1* (Wood et al. 2005), which represent the ligand, receptor and targets in the signaling, in different tissues as well as in a series of developmental stages of wildtype zebrafish (Fig. 2a, b).

In adult fish (Fig. 2a), *GH* was expressed in brain, liver, ovary and muscle with the highest expression level in the brain that includes pituitary gland by

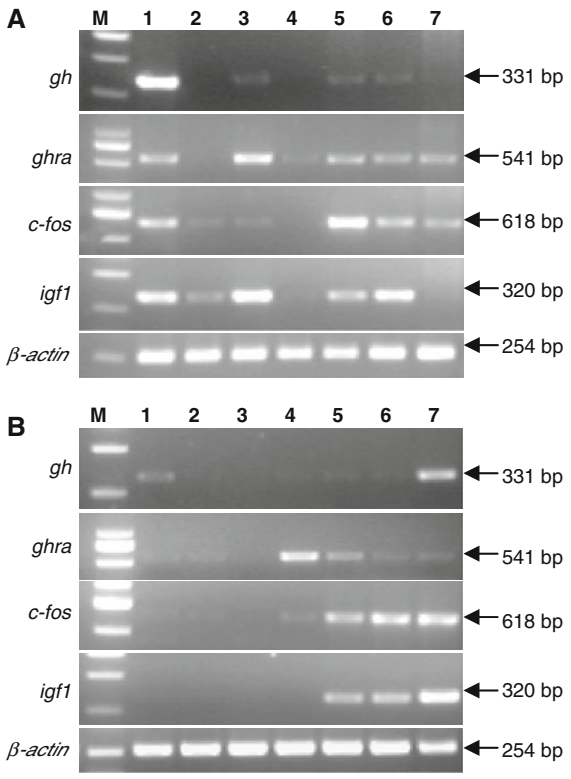

Fig. 2 The transcription analysis of *GH*, *GHR*, *c-fos* and *igf1* in wildtype zebrafish. **a** RT-PCR analysis in different tissues of adult zebrafish. *1* brain, *2* heart, *3* liver, *4* kidney, *5* ovary, *6* muscle, *7* fin, and *M* DNA Ladder (DL 2000). **b** RT-PCR analysis in different developmental stages of zebrafish embryo. *1* 2-cell, *2* high, *3* 80%-epiboly, *4* early somite, *5* 1 dpf, *6* 2 dpf, *7* 3 dpf, and *M* DNA ladder (DL 2000). *β-actin* was used as the internal control

analysis with Glyko Bandscan software. *GHR* transcript was found in all tissues examined except for heart. The highest levels of *GHR* expression were observed in liver, followed by brain and ovary. In the case of *c-fos*, the highest expression was observed in ovary followed by brain, and in all other tissues except the kidney. *igf1* transcripts were present in all tissues except kidney with the highest expression level in liver.

During different stages of embryonic development (Fig. 2b), *GH* transcript was detected in 2-cell stage embryos but not at later stages, reappearing from 1 dpf, and increasing with development. This indicates that *GH* is maternally expressed and its zygotic expression starts from 1 dpf. *GHR* is also maternally expressed although the expression level might be not as high as that of *GH*. The embryonic expression of *GHR* starts from the early somite stage, and in contrast to *GH*, the expression of *GHR* decreased from the early somite stage to 3 dpf. Although *c-fos* transcript was in 2-cell embryos, from the early somite stage expression of *c-fos* increased gradually with the time, mimicking the *GH* expression profile. *igf1* was minimally expressed maternally, with expression from 1 dpf, and increasing thereafter.

Activation of GHR signaling by combined overexpression of GH and GHR in zebrafish embryos

In order to quantify GH signaling in transgenic fish, we measured expression of the GH target genes *c-fos* and *igf1*. Since *c-fos* reached a rather high expression level at 1 dpf and *igf1* reached its highest expression level at 3 dpf, we used 1 dpf and 3 dpf embryos to check the expression levels of *c-fos* and *igf1*, respectively, by normalizing values to 2-cell embryo levels, since these should not be altered by the transgene. By GeNorm analysis, we found that *β-actin* is the most stable gene among all the candidate reference genes (data not shown) and it could be used to quantify the relative expression levels of *c-fos* and *igf1*. First, we injected c*GH* expression construct into zebrafish embryos and found that *c-fos* expression was 1.7-fold and *igf1* expression was 3.1-fold in the *GH*-injected embryos in comparison to wildtype embryos (Fig. 3a, b), suggesting significant GHSA in the injected embryos. Second, we overexpressed zebrafish *GHR* and found 1.7-fold of *c-fos* expression and 3.5-fold of *igf1* expression, demonstrating that overexpression of *GHR* could also activate GH signaling in the presence of endogenous GH. Third, we used a synergetic expression of *GH* and *GHR*, and this overexpression showed 2.0-fold of *c-fos* expression and 4.5-fold of *igf1* expression, higher than these constructs separately. In contrast, overexpression of ΔC-*GHR* efficiently attenuated the GHSA activity of *GH* overexpression, as shown by a lower expression level of *igf1* (1.5-fold) than the overexpression of *GH* alone (3.1-fold). These results clearly indicate the importance of GHR level in GH signaling, and by implication, fish growth, since Igf1 is one of the major effectors of growth performance (Eppler et al. 2010).

On the other hand, we conducted luciferase assay by utilizing *spi2.1-luc* construct (Jiao et al. 2006), to

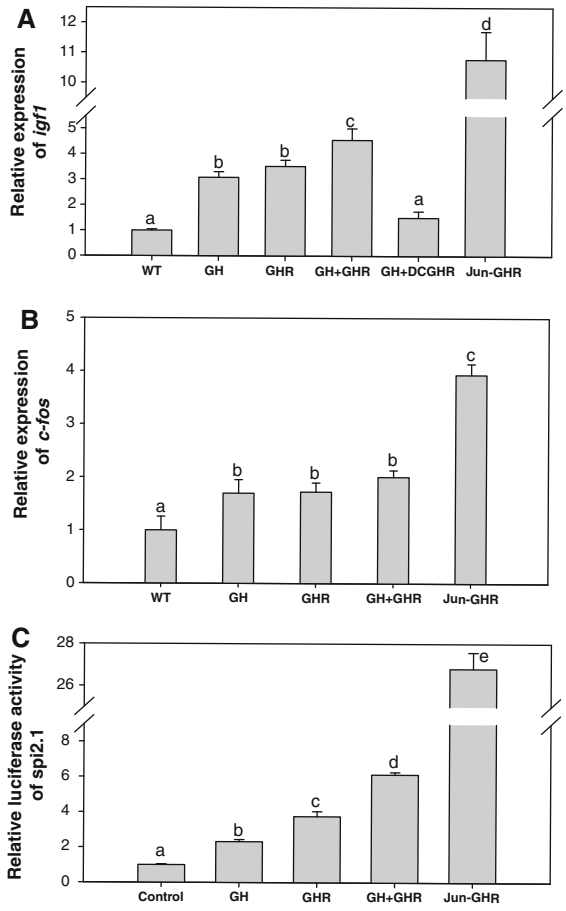

Fig. 3 Analysis of GH-signal activation levels in zebrafish embryos receiving different constructs. **a** Relative expression of *igf1* in zebrafish embryos at 3 dpf by real-time PCR analysis. **b** Relative expression of *c-fos* in zebrafish embryos at 1 dpf by real-time PCR analysis. **c** Relative promoter activities of *spi2.1* in zebrafish embryos at 3 dpf by luciferase assay. Control refers to wildtype embryos that were only injected with *spi2.1-luc* and *TK-Renilla* constructs. In **a**, **b** and **c**, each *bar* presents the mean value and the corresponding standard deviation from triplicate analysis. A statistically significant difference ($P < 0.05$) is indicated by a *different letter* above the bar, as determined by one-way ANOVA followed by Duncan's test

evaluate the overall GHSA level in zebrafish embryos. As shown in Fig. 3c, c*GH* overexpression induced 2.3-fold GHSA in comparison with wildtype embryos, z*GHR* overexpression induced 3.7-fold GHSA and the synergetic overexpression of GH and GHR could even result in a 6.1-fold GHSA. Thus the in vivo analysis of GHR signaling activity is validated by the luciferase assay of *spi2.1* promoter activity. Both expression analysis of *c-fos* and *igf1* and promoter activity analysis of *spi2.1* showed that higher GHR signaling activation could be achieved by synergetic expression of GH and GHR.

Elevated activation of GHR signaling by Jun-GHR overexpression in zebrafish embryos

To test whether our designed *Jun-GHR* constructs could activate GH signaling, we injected *Jun-GHR* construct into zebrafish embryos and checked the expression of *c-fos* in 1 dpf and *igf1* in 3 dpf embryos (Fig. 3a, b). The relative expression levels of *c-fos* and *igf1* were 3.9-fold and 10.8-fold, respectively, in *Jun-GHR* transgenic embryos, relative to the expression level of wildtype embryos. By contrast, the synergetic overexpression of *GH* and *GHR*, which showed the highest relative expression levels of *c-fos* and *igf1* in our earlier injection combinations, were only 2.0-fold and 4.5-fold of wildtype embryos. We also checked the promoter activity of *spi2.1* in *Jun-GHR* injected embryos. The relative luciferase activity of the *Jun-GHR* injected embryos was 26.8-fold, significantly higher than that of the cGH + zGHR injected embryos, which was only 6.1-fold. Herein the overexpression of *Jun-GHR* gave a higher efficiency of activation of signaling in zebrafish embryos when compared with the synergetic overexpression of *GH* and *GHR*. This result has been revealed by the stimulation of two GH target genes, *c-fos* and *igf1*, and the activation of GH signaling responsive *spi2.1* promoter. Thus it is expected that we would obtain transgenic zebrafish with accelerated growth performance using *Jun-GHR* construct as the transgene.

Activation of GH targets and accelerated growth of transgenic fish

As shown by RT-PCR analysis during development of transgenic embryos, the transcription of *Jun-GHR* starts from high stage, reached the highest level in 3 dpf embryos (Fig. 4a). By PCR screening of transgenic P0 generation we obtained 16 *Jun-GHR* transgene positive founders. We further sampled one P0 *Jun-GHR* transgenic fish, and *Jun-GHR* transcript expression was found in almost all the tissues tested, although expression in the brain was weak (Fig. 4b). More interestingly, by both bandscan analysis of the agarose gel bands (Fig. 4b) and real-time PCR assay

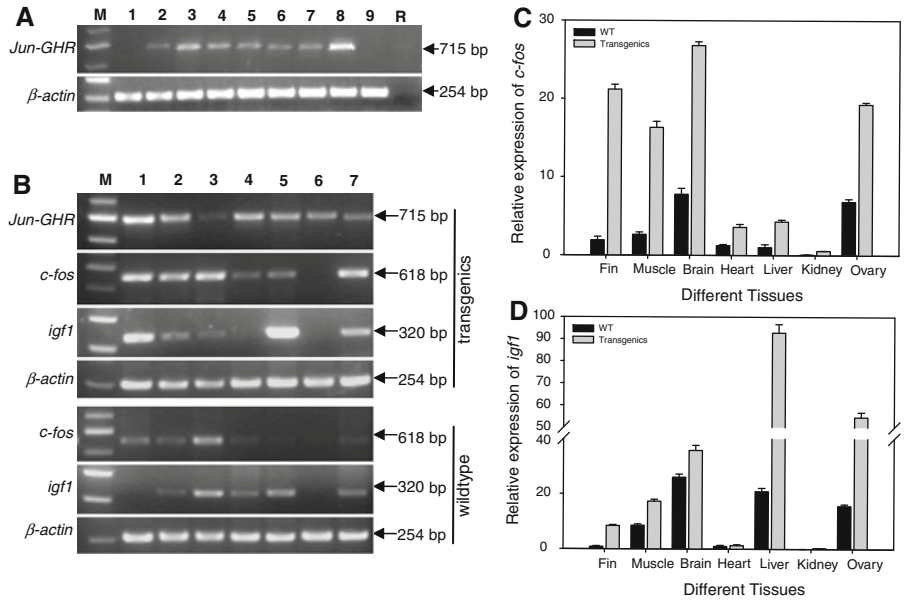

Fig. 4 The transcription of *Jun-GHR*, *c-fos* and *igf1* in *Jun-GHR* transgenic zebrafish. **a** *Jun-GHR* transcription in transgenic embryos at different developmental stages. *1* 2-cell, *2* high, *3* shield, *4* 80%-epiboly, *5* early somite, *6* 1 dpf, *7* 2 dpf, *8* 3 dpf, *9* wildtype embryos, *R* template of total RNA without reverse transcription. **b** The transcription of *Jun-GHR* transgene, *c-fos* and *igf1* in different tissues of transgenic zebrafish. *1* fin, *2* muscle, *3* brain, *4* heart, *5* liver, *6* kidney, *7* ovary, and *M* DNA Ladder (DL 2000). *β-actin* amplified from the same samples was used as the internal control. **c** The relative expression levels of *c-fos* in different tissues of wildtype and in transgenic zebrafish. **d** The relative expression levels of *igf1* in different tissues of wildtype and in transgenic zebrafish. In **c** and **d**, each *bar* presents the mean value and the corresponding standard deviation from triplicate analysis

(Fig. 4c, d), we detected higher expression of GH target genes, *c-fos* and *igf1* in different tissues of transgenic zebrafish. For instance, higher expression of *c-fos* was observed in all the tissues except kidney (Fig. 4b, c). Ectopic expression of *igf1* was even found in the fin tissue of transgenic fish, since there was nearly no expression of *igf1* in the fin tissue of wildtype fish (Fig. 4b, d). These results strongly indicate that *Jun-GHR* transgene is faithfully transcribed in transgenic fish, and as a result strongly activates GH/GHR target genes in various tissues.

All the transgene positive founders were crossed with wildtype zebrafish to produce the F1 generation. Among them, 2 crosses gave transgene positive F1 embryos. At 1 mpf, we screened out 100 transgene positive zebrafish from 1 cross and raised them in the aquaria, with 100 non-transgenic zebrafish in the same condition as control. From 2 mpf, the average body weight and body length (mean ± SD) of *Jun-GHR* transgenic F1 fish showed to be significantly higher ($P < 0.01$) than those of the non-transgenics (Fig. 5a, b). At 4 mpf, the transgenic fish weighed 0.56 ± 0.08 g at average, 86.1% higher than the average body weight of controls (0.30 ± 0.08 g) ($P < 0.01$), and the average body length of the transgenics was 3.61 ± 0.18 cm, 25.2% higher than that of the controls (2.88 ± 0.29 cm) ($P < 0.01$). As shown in Fig. 5c, the transgenic fish showed wider lateral bodies with wider and thicker dorsal bodies than the non-transgenic control fish as what we found in *GH*-transgenic common carp (Wang et al. 2001). These demonstrate that the expression of *Jun-GHR* transgene resulted in growth acceleration of transgenic zebrafish.

Discussion

We have previously demonstrated that GH and GHR are present from a very early embryonic stage in mice (Pantaleon et al. 1997), and summarized potential contributions of GH to prenatal development in mammals (Waters and Kaye 2002). In this study, the expression of *GH, GHR*, *c-fos* and *igf1* genes

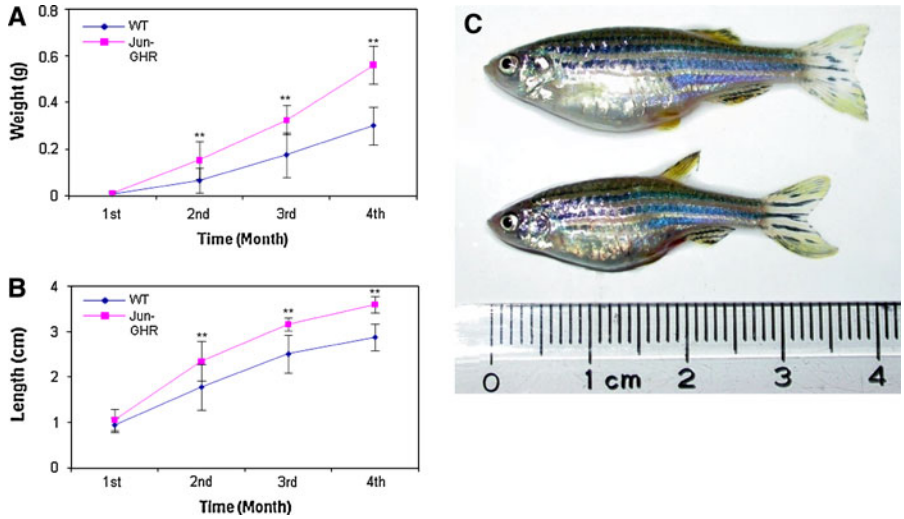

Fig. 5 Growth trial of F1 transgenic and non-transgenic zebrafish. **a** The increase of body weight along development. **b** The increase of body length along development. **c** The typical phenotype of *Jun-GHR* transgenic (*upper*) and non-transgenic (*lower*) zebrafish. Weight and length of transgenic and non-transgenic zebrafish were statistically compared by student's *t*-test (** $P < 0.01$)

were extensively and comparatively analyzed during 7 stages of embryonic development and in 7 different tissues of adult zebrafish. Transcription of *GH, GHR* and *c-fos* was observed at the 2-cell stage, indicating maternal expression of these genes, and detection of their expression in the ovary further suggests that they start to be accumulated in the oocytes during maturation. For most of them, the zygotic expression was detected from the end of gastrulation, i.e., early somite stage, far prior to the formation of functional pituitary gland. Expression of *GH* increased with expression of *igf1* at 3 dpf. The maternal expression and early expression of *GH* and *igf1* in fishes have also been reported by several investigators (Li et al. 2006, 2007). These observations suggest that GH/GHR pathway also plays a role in early development of fish, and in our present study, overexpression of GHR or GH alone led to increased GHSA in 1 and 3 dpf embryos, strongly indicating the existence of functional GH and GHR expression in zebrafish embryos from 1 dpf. Moreover, blocking GHSA in zebrafish during early development by overexpression a dominant-negative *GHR*, ΔC-*GHR*, resulted in embryos with early developmental defects (Ishtiaq Ahmed et al., unpublished data). In previous studies, it has been found that GH can stimulate actin rearrangement (Goh et al. 1997), microtubule polymerization (Goh et al. 1998), and the assembly of multiprotein complex involved in cell adhesion and cell movement (Zhu et al. 1998a, b). Given that the fish embryos undergo dynamic cell movement in early development, e.g., gastrulation movements (Chen et al. 2009), it is possible that the GH/GHR pathway may participate in the regulation of cell movement during early development. It was also reported that GH/Stat5b can directly regulate the transcription of a Wnt signaling element (*frizzled4*) and suppressors of cytokine signaling (Vidal et al. 2007), thus it is also possible that GH/GHR pathway may crosstalk with the other pathways to regulate the early development of fish.

In most of previous studies, the transcriptional activity of GH signaling in fish has been measured in cultured cells or tissues (Björnsson et al. 2002; Jiao et al. 2006). Here we have utilized zebrafish embryos as a host system to analyze the GH signaling target gene *igf1* and the promoter activity of *spi2.1*, after injection of different combination of transgene constructs, such as *GH*, *GHR*, *GH* + *GHR*, and *GH* + ΔC-*GHR* into zygotes. This has allowed us to compare the signaling activity of GH/GHR pathway upon treatment with different GH pathway-related components in vivo. As compared with overexpression of *GH* or *GHR* alone, the combined overexpression of *GH* and *GHR* increased GHSA, and overexpression of ΔC-*GHR* which lacks the

intracellular signal transducer domain efficiently attenuated the transcriptional activity. These results validate the efficacy of this in vivo system for studying GH/GHR signaling.

Because GH-induced activation of the GHR is the critical step in GH signaling, we utilized a constitutively activated form of *GHR*, *Jun-GHR* to ascertain the effect of continuous activation of GHR in the absence of the IGF-1 feedback on GH secretion. Although shown to be highly active in cell cultures (Behncken et al. 2000), this construct has not been studied in vivo. By using zebrafish as a host animal, we found that *Jun-GHR* transgenics could not only strongly activate the GH/GHR signaling but also stimulate the growth performance of the transgenic fish. Notably, we have never previously been able to stimulate growth of these zebrafish with *GH* transgenes alone. In terms of GH target gene activation, *Jun-GHR* increased the transcriptional activity of the GH/GHR pathway very significantly more than the overexpression of *GH*, *GHR*, and even *GH* + *GHR*. This demonstrates that CA-*GHR* transgenesis should be much more powerful than the common *GH* transgenesis in the aspect of signaling activation and growth stimulation. Intriguingly, *igf1* was even ectopically activated in the fin tissue of transgenic zebrafish, which tissue did not display detectable *igf1* transcription in wildtype zebrafish. Surprisingly, although *Jun-GHR* was transcribed in the kidney of transgenic fish, we could not detect visible transcription of *igf1* or *c-fos* in the kidney, which suggests that the zebrafish kidney may lack some intracellular transducer for the GH/GHR pathway. The broad distribution of *Jun-GHR* transcripts in various tissues and strong activation of GH/GHR target genes have presumably contributed to the allometric growth stimulation of transgenic zebrafish, which will be tremendously useful for increasing yields of transgenic farmed fish. Nevertheless, in future studies we should use "all-fish" CA-*GHR* constructs for production of transgenic commercial fishes, as CMV promoter was used in our present study.

In previous studies of transgenic animals, researchers usually utilized chimeric constructs. Although the promoters and the coding sequences are from different genes (Laible 2009), the final products are usually the wildtype forms of certain proteins. Here we propose that researchers may produce transgenic animals by introducing the conception of molecular designed protein, e.g., the *Jun-GHR* product does not exist in the nature but show higher efficiency than the natural proteins. As the comprehensive understanding of many pathways has been extensively advanced, it is certain that other transgenes could be usefully and efficiently modified based on protein design.

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References

Behncken SN, Billestrup N, Brown R et al (2000) Growth hormone (GH)-independent dimerization of GH receptor by a leucine zipper results in constitutive activation. J Biol Chem 275(22):17000–17007

Björnsson B, Johansson V, Benedet S et al (2002) Growth hormone endocrinology of salmonids: regulatory mechanisms and mode of action. Fish Physiol Biochem 27(3): 227–242

Cesena TI, Cui TX, Piwien-Pilipuk G et al (2007) Multiple mechanisms of growth hormone-regulated gene transcription. Mol Genet Metab 90(2):126–133

Chen TT, Vrolijk NH, Lu JK et al (1996) Transgenic fish and its application in basic and applied research. Biotechnol Ann Rev 2:205–236

Chen CH, Sun YH, Pei DS et al (2009) Comparative expression of zebrafish lats1 and lats2 and their implication in gastrulation movements. Dev Dyn 238(11):2850–2859

Devlin RH, Biagi CA, Yesaki TY et al (2001) Growth of domesticated transgenic fish. Nature 409(6822):781–782

Devlin RH, Sundstrom LF, Muir WM (2006) Interface of biotechnology and ecology for environmental risk assessments of transgenic fish. Trends Biotechnol 24(2): 89–97

Devlin RH, Sakhrani D, Tymchuk WE et al (2009) Domestication and growth hormone transgenesis cause similar changes in gene expression in coho salmon (Oncorhynchus kisutch). Proc Natl Acad Sci USA 106(9):3047–3052

Di Prinzio CM, Botta PE, Barriga EH et al (2010) Growth hormone receptors in zebrafish (Danio rerio): adult and embryonic expression patterns. Gene Expr Patterns 10(4–5):214–225

Eppler E, Berishvili G, Mazel P et al (2010) Distinct organ-specific up- and down-regulation of IGF-I and IGF-II mRNA in various organs of a GH-overexpressing transgenic Nile tilapia. Transgenic Res 19(2):231–240

Fu C, Hu W, Wang Y et al (2005) Developments in transgenic fish in the People's Republic of China. Rev Sci Tech 24(1):299–307

Goh EL, Pircher TJ, Wood TJ et al (1997) Growth hormone-induced reorganization of the actin cytoskeleton is not required for STAT5 (signal transducer and activator of transcription-5)-mediated transcription. Endocrinology 138(8):3207–3215

Goh EL, Pircher TJ, Lobie PE (1998) Growth hormone promotion of tubulin polymerization stabilizes the microtubule network and protects against colchicine-induced apoptosis. Endocrinology 139(10):4364–4372

Herrington J, Carter-Su C (2001) Signaling pathways activated by the growth hormone receptor. Trends Endocrinol Metab 12(6):252–257

Ihle JN, Gilliland DG (2007) Jak2: normal function and role in hematopoietic disorders. Curr Opin Genet Dev 17(1):8–14

Jiao B, Huang X, Chan CB et al (2006) The co-existence of two growth hormone receptors in teleost fish and their differential signal transduction, tissue distribution and hormonal regulation of expression in seabream. J Mol Endocrinol 36(1):23–40

Kikuta H, Kawakami K (2009) Transient and stable transgenesis using Tol2 transposon vectors. In: Graham JL, Andrew CO, Kawakami K (eds) Zebrafish, methods in Molecular Biology, vol 546. Humana Press, New York, pp 69–84

Laible G (2009) Enhancing livestock through genetic engineering—recent advances and future prospects. Comparative immunology. Microbiol Infect Dis 32(2):123–137

Li M, Greenaway J, Raine J et al (2006) Growth hormone and insulin-like growth factor gene expression prior to the development of the pituitary gland in rainbow trout (Oncorhynchus mykiss) embryos reared at two temperatures. Comp Biochem Physiol A Mol Integr Physiol 143(4): 514–522

Li M, Raine JC, Leatherland JF (2007) Expression profiles of growth-related genes during the very early development of rainbow trout embryos reared at two incubation temperatures. Gen Comp Endocrinol 153(1–3):302–310

Lichanska AM, Waters MJ (2008) How growth hormone controls growth, obesity and sexual dimorphism. Trends Genet 24(1):41–47

Liu WY, Wang Y, Sun YH et al (2005) Efficient RNA interference in zebrafish embryos using siRNA synthesized with SP6 RNA polymerase. Dev Growth Differ 47(5): 323–331

Liu Y, Bourgeois C, Pang S et al (2009) The germ cell nuclear proteins hnRNP G-T and RBMY activate a testis-specific exon. Plos Genetics 5(11):e1000707

Pantaleon M, Whiteside EJ, Harvey MB et al (1997) Functional growth hormone (GH) receptors and GH are expressed by preimplantation mouse embryos: a role for GH in early embryogenesis? Proc Natl Acad Sci USA 94(10): 5125–5130

Pei DS, Sun YH, Chen SP et al (2007) Zebrafish GAPDH can be used as a reference gene for expression analysis in cross-subfamily cloned embryos. Anal Biochem 363(2): 291–293

Rowland JE, Lichanska AM, Kerr LM et al (2005) In vivo analysis of growth hormone receptor signaling domains and their associated transcripts. Mol Cell Biol 25(1):66–77

Stuart GW, McMurray JV, Westerfield M (1988) Replication, integration and stable germ-line transmission of foreign sequences injected into early zebrafish embryos. Development 103(2):403–412

Studzinski AL, Almeida DV, Lanes CF et al (2009) SOCS1 and SOCS3 are the main negative modulators of the somatotrophic axis in liver of homozygous GH-transgenic zebrafish (Danio rerio). Gen Comp Endocrinol 161:67–72

Sun YH, Chen SP, Wang YP et al (2005) Cytoplasmic impact on cross-genus cloned fish derived from transgenic common carp (Cyprinus carpio) nuclei and goldfish (*Carassius auratus*) enucleated eggs. Biol Reprod 72(3):510–515

Vandesompele J, De Preter K, Pattyn F et al (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3(7):RESEARCH0034

Vidal OM, Merino R, Rico-Bautista E et al (2007) In vivo transcript profiling and phylogenetic analysis identifies suppressor of cytokine signaling 2 as a direct signal transducer and activator of transcription 5b target in liver. Mol Endocrinol 21(1):293–311

Wang Y, Hu W, Wu G et al (2001) Genetic analysis of all fish growth hormone gene transferred carp (*Cyprinus carpio* L.) and its F1 generation. Chinese Sci Bull 46(14):1174–1177

Waters MJ, Kaye PL (2002) The role of growth hormone in fetal development. Growth Horm IGF Res 12(3):137–146

Waters MJ, Hoang HN, Fairlie DP et al (2006) New insights into growth hormone action. J Mol Endocrinol 36(1):1–7

Westerfield M (2000) The zebrafish book. A guide for the laboratory use of zebrafish (Danio rerio). Eugene, University of Oregon Press

Wood AW, Duan C, Bern HA (2005) Insulin-like growth factor signaling in fish. Int Rev Cytol 243:215–285

Wu G, Sun Y, Zhu Z (2003) Growth hormone gene transfer in common carp. Aquat Living Resour 16:416–420

Zhu ZY, Sun YH (2000) Embryonic and genetic manipulation in fish. Cell Res 10(1):17–27

Zhu Z, Li G, He L et al (1985) Novel gene transfer into the fertilized eggs of gold fish (*Carassius auratus* L. 1758). J Appl Ichthyol 1(1):31–34

Zhu T, Goh EL, LeRoith D et al (1998a) Growth hormone stimulates the formation of a multiprotein signaling complex involving p130(Cas) and CrkII. Resultant activation of c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK). J Biol Chem 273(50):33864–33875

Zhu T, Goh EL, Lobie PE (1998b) Growth hormone stimulates the tyrosine phosphorylation and association of p125 focal adhesion kinase (FAK) with JAK2. Fak is not required for stat-mediated transcription. J Biol Chem 273(17): 10682–10689

Zhu Y, Song D, Tran NT et al (2007) The effects of the members of growth hormone family knockdown in zebrafish development. Gen Comp Endocrinol 150(3):395–404