A study of somatolactin actions by ectopic expression in transgenic zebrafish larvae

Guohui Wan and King Ming Chan

Department of Biochemistry, Faculty of Science, Chinese University of Hong Kong, Sha Tin, N.T., Hong Kong SAR, People’s Republic of China

(A correspondence should be addressed to K M Chan; Email: kingchan@cuhk.edu.hk)

Abstract

Somatolactin (SL) is a fish-specific hormone that belongs to the prolactin (PRL) and GH family. Recently, two forms of SL, SLα and SLβ, have been found in some species, and may have different actions and functions. To investigate the role of SL in fish growth and metabolism, we generated transgenic fish founders with ectopic expression of SLα and SLβ to study the physiological functions and actions of these SLs among several marker genes. We fused the cDNAs encoding the precursor SLs in frame to a zebrafish β-actin gene promoter to generate transgenic zebrafish lines that were coinjected with a green fluorescent protein (GFP) driven by the same promoter. The transgenic zebrafish were selected based on GFP expression and confirmed by genomic PCR, Southern blot analysis, and transgene expression. Investigations into the expression of marker genes in larvae on different pathways using real-time PCR have provided a general understanding of the actions of SLs. This study found that the overexpression of SLα and SLβ in vivo significantly enhanced the transcription of IGFs, insulin, leptin, sterol regulatory element binding protein 1, and fatty acid synthase, as well as the expression level of vitellogenin and proopiromelanocortin, while causing reduced levels of catalase and glutathione S-transferase in the larvae of transgenic zebrafish.

Journal of Molecular Endocrinology (2010) 45, 301–315

Introduction

Somatolactin (SL) is a recently discovered glycoprotein hormone in fish of GH/prolactin (PRL) superfamily, with significant structural homology in all fish taxa studied to date (Ono et al. 1990, Rand-Weaver et al. 1991, 1992, Chen et al. 1994). According to sequence comparisons, it is generally believed that SL and PRL evolved from a common ancestral gene related to GH with two successive rounds of gene duplication before the divergence between vertebrates and invertebrates (Chen et al. 1994, Fukamachi & Meyer 2007). However, the SL gene is not found in tetrapods and might have been lost during the evolution of early land vertebrates (Fukamachi & Meyer 2007). Fish SL is mainly expressed in the pars intermedia of the pituitary gland and distinct from proopiromelanocortin (POMC)-producing cells (Kaneko 1996); hence, it should have physiological actions different from those of GH and PRL. In addition, despite the conserved helical bundles, the number of disulfide bonds (6 or 7) is different from that found in GH and PRL (4), and the low sequence identity found between SL and GH (50–60%) also suggests that they have dissimilar functions in fish (Chen et al. 1994, Fukamachi & Meyer 2007).

Two isoforms of SL, SLα and SLβ, have been identified in zebrafish (Zhu et al. 2004) and grass carp (Jiang et al. 2008), and phylogenetic analyses suggest that they are paralogs that result from an ancient duplication of SL genes in ray-finned fish probably related to reproduction (Planas et al. 1992, Chen et al. 1994, Yang & Chen 2003). The tertiary structures of these two SLs may be different because there is an extra third cysteine in SLα, and they share only 60% sequence identity within the same species (Jiang et al. 2008). Therefore, it would be of interest to know whether the actions and functions of these two SLs differ. The main physiological function of the SL gene remains a matter of debate, but it is likely that SL is involved in multiple and even overlapping functions with other members of the GH/PRL family. Recent studies suggest that SLs are involved in steroidogenesis and reproductive maturation (Planas et al. 1992, Rand-Weaver et al. 1992, Olivereau & Rand-Weaver 1994, Johnson et al. 1997), acid–base balance (Kakizawa et al. 1996), background adaptation (Kakizawa et al. 1995, Zhu & Thomas 1995), immune function (Calduch-Giner et al. 1998), energy mobilization and stress (Rand-Weaver et al. 1993), lipid metabolism and pigmentation (Zhu & Thomas 1997, Fukamachi et al. 2004, 2005, 2009), and the regulation of chromatophores (Zhu & Thomas 1995, 1996, 1997). The recent discovery of an SL-deficient mutant in medaka (Oryzias latipes) (color interfere, ci) indicated that SL might function in the proliferation and morphogenesis of epidermal chromatophores, body color regulation, or cortisol secretion in vivo (Fukamachi et al. 2005, 2009).
The gene silencing of SLs in zebrafish (Danio rerio) by antisense morpholino oligonucleotides during embryonic development, however, only showed their different effects on the delay in swim bladder inflation (Zhu et al. 2007).

The presence of two distinct GH receptors (GHRs), GHR clade 1 and GHR clade 2, has been detected in several teleost species (Fukamachi et al. 2005, Jiao et al. 2006, Pierce et al. 2007, Benedet et al. 2008), and phylogenetic analyses of salmon GHRs suggest that GHR clade 1 is an SL receptor (SLR), whereas GHR clade 2 is the actual GHR (Benedet et al. 2008). A recent study showed that SL could act as a novel regulator of insulin-like growth factor (IGF) gene expression in fish (Wan et al. 2009), which suggests that SL may exert its effects indirectly by way of the GH/IGF axis. Whether the actions of SL are on the GHR or SLR remains to be investigated; however, SL and GH may have overlapping functions. Among mammals are a large number of paralogous genes (e.g. there are 26 PRL-related genes in rats and mice), and the non-classical actions of these ligands are mediated through neither the PRL nor the GHR (Ain et al. 2004, Green 2004). Therefore, it is believed that the functional study of SLs by knockdown assay in embryos has been impaired by the compensational effects of other genes because of redundant functions in the GH/PRL family. Nevertheless, the overexpression of SLs in vivo can reveal a gain of function that may be unrecognized by targeted mutational analysis because of functional redundancy.

To study the actions of two closely related SL genes by ectopic expression in transgenic zebrafish, we cloned cDNAs encoding the precursor SLs in frame to a zebrafish β-actin (βA) gene promoter that would overexpress SL genes ubiquitously to generate transgenic zebrafish lines coinjected with a green fluorescent protein (GFP) driven by the same promoter. Larvae of these transgenic fish founders were used for the analysis of marker gene expression in growth, development, metabolism, reproduction, pigmentation, and the antioxidant defense system to reveal the main functions of SLs.

Materials and methods

Zebrafish

Zebrafish were obtained from a local pet shop. This is a local strain of zebrafish derived from India that is used for many experiments. The protocols for rearing and keeping zebrafish were adopted from Westerfield (1995). A license (no. AL928650) for cloning into pGL3-Basic vector (Promega) according to the zebrafish genomic sequence (NCBI accession no. AL928650). PCR was performed on the TaKaRa PCR Thermal Cycler Dice with the following conditions: one cycle at 94 °C for 2 min, 30 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 3 min, followed by one cycle of 72 °C for 7 min. The gel-extracted PCR product was cloned into the pGEM-T Easy Vector System (Promega) and sent to a commercial DNA sequence service, Tech-Dragon, for nucleotide sequence determination.

The cDNA obtained by reverse transcriptase (Promega) was used as a template for zebrafish SLα, SLβ, and GH gene amplification using the following specific primer sets:

| SLα (5‘-ATGATGTGCTAGGCTGAGTT-3‘) | SLβ (5‘-ATGATGTGCTAGGCTGAGTT-3‘) |
| SLα (5‘-ATGATGTGCTAGGCTGAGTT-3‘) | SLβ (5‘-ATGATGTGCTAGGCTGAGTT-3‘) |
| GH (5‘-ATGATGTGCTAGGCTGAGTT-3‘) | GH (5‘-ATGATGTGCTAGGCTGAGTT-3‘) |

The primers were designed according to the zebrafish database of the NCBI (accession no. NM_001037706 for SLz, NM_001037674 for SLβ, and BC116501 for GH). GFP was amplified from a commercial vector, phrGFP II-1 (Stratagene, La Jolla, CA, USA).

DNA constructs

Genomic DNA samples were purified from whole zebrafish by grinding in liquid nitrogen and further digestion with proteinase K and phenol–chloroform extraction (Westerfield 1995). The zebrafish βA gene promoter (Liu et al. 1990, 1991) was amplified using the Expand High Fidelity PCR System (Roche). Purified zebrafish genomic DNA (200 ng) was mixed with 5 μl of 10× Expand High Fidelity buffer, 15 mM of MgCl₂, 1·5 μl of 10 μM βA forward primer (5‘-GGGTTACCGAGTAATACGACGCTT-3‘), 1·5 μl of 10 μM βA reverse primer (5‘-GTCGACTGATCCATGATCATT-3‘), 1 μl of 10 mM of dNTP mix, and 0·75 μl of Expand High Fidelity Enzyme Mix (Applied Biosystems, Foster City, CA, USA). Finally, distilled water was added to increase the reaction volume to 50 μl. The DNA primers were designed with enzyme sites (KpnI and Smal) for cloning into pGL3-Basic vector (Promega) to study the promoter activity that via free access
Generation of transgenic zebrafish

Adult zebrafish were reared in a closed water circulation system at 28 °C under a controlled photoperiod of 14 h light:10 h darkness cycle (Westerfield 1995). An egg collection device was placed into a tank of fish at the beginning of light cycle. Eggs were collected ~20 min after being laid (one-to-two-cell stage) and coinjected with the linearized vectors dissolved in 0.25% phenol red in 0.1 M Tris–HCl (zβAzSLα and zβAhrGFP; zβAzSLβ and zβAhrGFP; zβAzSLβ and zβAhrGFP; zβAzSLβ and zβAhrGFP) using the PV820 Pneumatic PicoPump (World Precision Instruments; Chong & Vielkind 1989, Hamada et al. 1998). Approximately, 500 pl of DNA solution represented a final number of 10^6 copies of each transgene per injected embryo (Zhu et al. 1985). The microinjection needles were made from Narishige GD-1 glass capillaries using the MODEL P-97 microelectrode puller (Sutter Instrument Co., Novato, CA, USA; www.endocrinology-journals.org

Figure 1 Schematic diagrams of hormone overexpression vectors. (a) A total of 2.2 kb region of zebrafish β-actin gene promoter before translation start codon (ATG) was isolated for the hormone overexpression constructs, containing some conserved elements, e.g. SRF element in intron 1, which can enhance the activity of β-actin gene promoter. (b–d) The zβAzSLα, zβAzSLβ, and zβAzGH constructs isolated from the plasmids after digestion with KpnI and used for microinjection, showing some restriction sites for transgene detection. Primers P1 and P2, P3 and P4, and P5 and P6 were used in the genomic DNA PCR reactions to identify transgenic fish for zβAzSLα, zβAzSLβ, and zβAzGH respectively. Probes in zβAzSLα and zβAzSLβ were used in Southern blot analysis for further confirmation of transgenic fish, while D indicates intron in the genomic DNA of wild-type fish. (e) The zβAhrGFP construct isolated from the plasmid after digestion with KpnI and used as a coinjection strategy for positive GFP signal detection.
Chong & Vielkind 1989). The injected embryos were incubated at 28°C (Chong & Vielkind 1989). GFP expression was analyzed 24 h after fertilization using a Leica DM1L fluorescence microscope with a Leica filter set (excitation = 485 nm; emission = 520 nm), and pictures were taken using the Leica DFC420 digital camera system (shown in Fig. 2). Mosaicism in the first transgenic generation (G₀) was classified according to the GFP expression patterns as follows: weak, a few cells expressing GFP; moderate, <50% of the body expressing GFP; or strong, more than 50% of the body expressing GFP (Gibbs & Schmale 2000). For the generation of stable transgenic lines, the founders were raised to sexual maturity. Transgenic screening was performed by crosses with wild-type (WT) zebrafish. At least 100 embryos from each founder were examined for GFP fluorescence.

Investigation of transgenes

Genomic DNA was isolated from G₀ and G₁ transgenic fish larvae as mentioned above to confirm the positive transgenes by PCR and Southern blotting. The following primers were used for PCR analysis of the transgenes:

\[ z\beta AzSL \alpha P1 \ (5'-GAATCGGCGGACTTGAG-3') \];
\[ z\beta AzSL \alpha P2 \ (5'-TCCAGAGGACGCACC-3') \];
\[ z\beta AzSL \beta P3 \ (5'-ATCATGTCATCGCGCCTAT-3') \];
\[ z\beta AzSL \beta P4 \ (5'-GTCGAGCACTCGTCAA-3') \];
\[ z\beta AzGH P5 \ (5'-GAATCGGCGGACTTGAG-3') \]; and
\[ z\beta AzGH P6 \ (5'-AAGACGAGGCCCATCTTG-3') \].

PCR was carried out in a 25 μl volume containing 25 pM of each primer, 1 mM of each dNTP, and 1 unit of Expand High Fidelity Enzyme (Applied Biosystems) for 35 cycles. Each cycle included 1 min at 94°C, 30 s at 60°C, and 1 min at 72°C.

The probes for Southern blot analysis were amplified from the hormone overexpression constructs using the following primers:

\[ z\beta AzSL \alpha F: 5'-CCCATCCCAACATCCAAGA-3' \];
\[ z\beta AzSL \alpha R: 5'-ATACAGCAGGCCGCTCCATC-3' \];
\[ z\beta AzSL \beta F: 5'-CTCGAGGCTTATTGCAGGAGGTATG-3' \]; and
\[ z\beta AzSL \beta R: 5'-CCAAGGCTCCTGACC-3' \].

Double-stranded oligonucleotide probes, \( z\beta AzSL \alpha -102 \text{ bp} \) and \( z\beta AzSL \beta -308 \text{ bp} \) (Δ in the probes indicated intron in the genomic DNA of the WT fish, which is shown in Fig. 1), were end labeled with digoxigenin (DIG)-11-ddUTP using Terminal transferase (Roche). Purified genomic DNA (5 μg) was digested with EcoRI or Smal and XhoI overnight, resolved by electrophoresis in a 0.8% agarose gel, and blotted onto a nylon membrane (Immobilon-N; Millipore, Billerica, MA, USA) using an electroblotting apparatus (TE70 ECL Semi-Dry Transfer Unit, Amersham) according to the manufacturer’s instructions. The DNA was fixed onto the membrane by cross-linking it with a transilluminator, and prehybridized in a solution containing 5× SSC.

Figure 2 Investigation of GFP expression in transgenic zebrafish. GFP expression was driven by β-actin gene promoter to evaluate mosaicism in transgenic zebrafish with hormone (SLα, SLβ, and GH) overexpression in the coinjection strategy. Excitation = 485 nm; Emission = 520 nm.
(3 M NaCl and 0·3 M trisodium citrate, pH 8·0), 1% SDS, 5× blocking reaction (Roche), 50% (v/v) formamide, and denatured salmon sperm DNA at 68°C for 4 h. The prehybridization solution was then removed, and a fresh hybridization solution (5× SSC, 1% SDS, 5× blocking reaction, and 50% (v/v) formamide) containing the denatured DIG-labeled DNA probe (about 25 ng/ml) was added and incubated at 68°C overnight. The hybridized membrane was washed first in 2× SSC, 1% SDS at 25°C with constant agitation for 5 min, and 0·5× SSC, 0·1% SDS at 68°C under constant agitation for 15 min. After the immunological detection of the DIG-11-ddUTP, positive signals were detected using FUJI X-ray film for autoradiography.

**Rearing of larvae and growth analysis**

Transgenic and non-transgenic fish were reared until 6 months of age in a closed circulation water system composed of 15-l tanks. Triplicate samples were collected from each genetic group (SLα-transgenic G₀, SLβ-transgenic G₀, and GH-transgenic G₀), with twenty fish each. Water quality was monitored once a day, and temperature, pH, nitrogenous compounds, and photoperiod were maintained according to zebrafish requirements (Chong & Vielkind 1989, Westerfield 1995, Gibbs & Schmale 2000). The fish were fed a high-quality commercial fish diet (ZM Systems, Winchester, UK): all fry were fed with infusoria grade ZM-000 (30–50 μm, 52% protein) at week 1, ZM-100 (80–200 μm, 55% protein) at week 2, ZM-200 (150–300 μm, 60% protein) at weeks 3–5, and Mediaquafish Guppy Food (300–500 μm, 48% protein, Japan) together with freshly hatched brine shrimp (Brine Shrimp Direct, Ogden, UT, USA) from week 6 on. In all situations, the food was completely consumed within 10 min. After the first month, the fish were anesthetized (Tricaine, 0·1 mg/ml) and weighed every 2 weeks to maintain the percentage of food by biomass in each tank. At the end of the experiment, the mean weight was compared among the three test groups. In addition, the total length of individual fish was measured, and the condition factor (K) was calculated using the formula $K = (W/L^3) \times 10^3$, where $W$ is the mass in milligrams and $L$ is the total length in millimeters.

**Quantification of mRNA levels using real-time PCR**

Using ABI Primer Express software (Applied Biosystems), specific primers (Table 1) for zebrafish βA (AF057040), SLα (AY376857), SLβ (AJ867249), GH (AJ937858), PRL (NM_181437), IGF1 (NM_131825), IGF2a (NM_131433), IGF2b (NM_001001815), IGF3 (EU272146), leptin (LEP, NM_001128576), sterol regulatory element binding protein 1 (SREBP1, DQ836065), SREBP2 (ENSDARG00000063438), fatty acid synthase (FAS, XM_682295), acetyl-coA carboxylase 2 (ACC2, XM_678989), vitellogenin (VTG, NM_001044897), POMC (NM_181438), phosphoenolpyruvate carboxykinase (PEPCK, NM_214751), insulin (INS, NM_131056), catalase (CAT, AF170069), and glutathione S-transferase (GST, AB194127) were designed to span two exons at the intron–exon junction to prevent the primers from binding to the genomic DNA sequences amplifying the contaminated DNA in the samples, and hence minimize genomic contamination in reverse transcription-PCR (Table 1).

### Table 1 Nucleotide sequences of gene-specific primers for real-time PCR in zebrafish

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward primer (5’–3’)</th>
<th>Reverse primer (5’–3’)</th>
<th>Amplicon size (bp)</th>
</tr>
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<tr>
<td>βA</td>
<td>CGAGCAGGAGATGGGAACC</td>
<td>CAACGAAACGCTATTGC</td>
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<tr>
<td>SLα</td>
<td>CAACAGGCTGGAAACACC</td>
<td>GGCTCATTCCACAGCTGAA</td>
<td>129</td>
</tr>
<tr>
<td>SLβ</td>
<td>GTGGTGGTCTGTGCTGTTAAA</td>
<td>CTTCTCCGCTGAGATTTGC</td>
<td>102</td>
</tr>
<tr>
<td>GH</td>
<td>GCATCAGCCGTGCTCACTCA</td>
<td>CCCCAGGTAGTGAATACTC</td>
<td>101</td>
</tr>
<tr>
<td>PRL</td>
<td>ATCTCAGCACCCTACCTCA</td>
<td>GCTGATCCTCCGGCATT</td>
<td>147</td>
</tr>
<tr>
<td>IGF1</td>
<td>TGTAGAGAAGACCGCCGAGG</td>
<td>TTTGTCAGTGTATGGAATCA</td>
<td>211</td>
</tr>
<tr>
<td>IGF2a</td>
<td>CTCGTGCGGGAGAGACTA</td>
<td>CCTGTGACACTGGGAGA</td>
<td>229</td>
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<tr>
<td>IGF2b</td>
<td>TCTGCGATGGTGGAGG</td>
<td>CGCAAGATGCAGGAGAT</td>
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<td>IGF3</td>
<td>AGAAGTGGGCCTGCTAG</td>
<td>TGAGAGGTGGTGCTG</td>
<td>194</td>
</tr>
<tr>
<td>PRL</td>
<td>TGCCAGAAATCCTACCAAGAG</td>
<td>TTTGTCAGTGTATGGAATCA</td>
<td>211</td>
</tr>
<tr>
<td>SREBP1</td>
<td>GGCACGGAAAGACCGCAAGG</td>
<td>TCCACCGTGCTGATTG</td>
<td>108</td>
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<tr>
<td>SREBP2</td>
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<tr>
<td>VTG</td>
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<td>GATAGAACCTTGCTGAG</td>
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<td>POMC</td>
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<td>PEPCK</td>
<td>GACTGGGCCCGAGGAGG</td>
<td>AAAGGCGGAGGGAGA</td>
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<td>INS</td>
<td>TGAGTCTGCTTTCTCCTCCT</td>
<td>CACTACGTACATGGCTC</td>
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</tr>
<tr>
<td>CAT</td>
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<td>AAAGGCGGAAACGCGAGG</td>
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<tr>
<td>GST</td>
<td>CGTACAGACATCTGCGAGA</td>
<td>AGATCTCTCAACTCCGCTGTT</td>
<td>92</td>
</tr>
</tbody>
</table>
Transgenic larvae and non-transgenic larvae (N=6) at week 5 were sampled to grind in liquid nitrogen. TRIzol reagent (Invitrogen) was used for total RNA extraction. Then, DNase treatment was performed with RNase-free DNase (Takara, Kyoto, Japan). Reverse Transcriptase (Promega) was used for cDNA synthesis. To perform real-time PCR, a reaction mixture was set up in a 0.2 ml clear thin-walled, optical-grade PCR tube that contained 12.5 μl Brilliant SYBR Green QPCR master mix (Applied Biosystems), 0.5 μl of forward primer (10 μM), 0.5 μl of reverse primer (10 μM), 2 μl cDNA template, and 9.5 μl nuclease-free water for a final volume of 25 μl.

Real-time quantitative PCR was carried out on the ABI 7700 detection system (Applied Biosystems). The PCR cycles were 1 cycle at 95°C for 10 min, 40 cycles at 95°C for 30 s, 58°C for 30 s, and 72°C for 1 min. All PCR assays were performed in triplicate. The fold inductions of candidate genes were determined by dividing the relative mRNA levels of transgenic samples by those of the control samples, all normalized with the level of βA. All of the data were analyzed using one-way ANOVA and Tukey’s multiple comparison tests with a 95% confidence level on GraphPad Prism 5.

**Results**

**Production of SL-transgenic fish**

From a local strain of zebrafish, a 2.2 kb region of zebrafish βA gene promoter was isolated using genomic PCR. Potential transcription factor binding sites that may be involved in regulating the βA gene promoter were found (Fig. 1a). SLβ-transgenic and SLβ-transgenic zebrafish were produced by the coinjection of the transgenes βAzSL and βAzAhrGFP, and βAzSLβ and βAzAhrGFP respectively in an equimolar ratio (1:1), using one-cell embryos (Fig. 1).

At the time of assessment by fluorescence microscopy (Fig. 2), which was 1 week after fertilization, the survival rate of the untreated fish embryos was observed and recorded as 786 out of 1120 (70.2%), whereas the survival rate of the microinjected embryos was 352 out of 1100 (32%) for the SLβ-transgenic ones and 343 out of 1300 (26.4%) for the SLβ-transgenic ones respectively. Among the 352 SLβ-transgenic embryos, 118 (33.5%) were classified as GFP negative (no expression), 111 (31.5%) as weakly GFP positive, 89 (25.3%) as moderately GFP positive, and 34 (9.7%) as strongly GFP positive. Among the 343 SLβ-transgenic embryos, 127 (37%) were classified as GFP negative (no expression), 135 (39.4%) as weak GFP positive, 62 (18.1%) as moderately GFP positive, and 19 (5.5%) as strongly GFP positive. GH-transgenic zebrafish were used as positive controls by coinjecting the transgenes βAzGH and βAhrGFP. The strongly GFP-positive samples were collected and used for further analysis.

**Identification of transgenic zebrafish by genomic DNA PCR and Southern blot**

Genomic DNA was extracted from the samples of 5-week-old transgenic fish with strong GFP expression to determine the presence of the βAzSLβ, βAzSLβ, and βAzGH transgenes. In the PCR analysis, a 299 bp fragment was generated from SLβ-transgenic G0 zebrafish #14 and #28 using primers P1 and P2, and a 298 bp fragment was generated from SLβ-transgenic G0 zebrafish #5 and #11 using primers P3 and P4 (Fig. 3a). A 673 bp fragment was detected in the positive control.
samples (GH-transgenic G0 zebrafish #25 and #26) using primers P5 and P6 (Fig. 3b). Southern blot analysis revealed that three bands were present separately in SLα-transgenic G0 zebrafish #14 and #28 (Fig. 4a), one band in SLβ-transgenic G0 zebrafish #5 and two bands in SLα-transgenic G0 zebrafish #11 in the digestion of EcoRI (Fig. 4b). It is suggested that the transgene zβAZSLα was integrated into three different sites in the genome of SLα-transgenic G0 zebrafish #14 and #28, and the transgene zβAZSLβ was integrated into only one site in SLβ-transgenic G0 zebrafish #5 and two sites in SLβ-transgenic G0 zebrafish #11. Simultaneously, in the digestion of Smal and XhoI, which cut through the whole length of SLα and SLβ cDNA in the transgenes, a 700 bp band was found as expected and detected in both the SL-transgenic G0 and G1 samples (Fig. 4c and d). All of these data suggest that the transgenes zβAZSLα, zβAZSLβ, and zβAZGH were integrated into the host genome, consistent with hrGFP expression.

Growth analysis of SL-transgenic G0 founders

The SL-transgenic G0 founders did not grow faster or bigger than the non-transgenic fish; however, a special stripe phenotype was found in the SLα-transgenic G0 line (Fig. 5). At the end of the growth experiment, the SLα-transgenic G0 fish reached a final average body weight of 421.1 ± 78.3 mg; their SLα-transgenic G0 siblings reached a final average body weight of 443.4 ± 82.6 mg; and their GH-transgenic G0 siblings reached a final average body weight of 542.9 ± 103.7 mg. The transgenic fish all show higher body mass in comparison with their non-transgenic siblings (328.6 ± 68.2 mg; Fig. 6a). The SL subtypes had a similar growth enhancement effect, which was less than that of GH. The condition factors calculated for all transgenic and non-transgenic zebrafish used in this experiment are shown in Fig. 6b. However, the K values of the transgenic and the non-transgenic fish show no significant difference.

Assessment of the physiological effects of SL among SL-transgenic G0 founders

To find out whether the overexpression of hormones would affect the expression of their family members, the endogenous mRNA levels of SLα, SLβ, and GH genes in 5-week-old transgenic zebrafish were investigated (Fig. 7). The results show a 24.3-fold induction of SLα in the SLα-transgenic fish without obvious up- or down-regulation of other members in the GH family.
A similar result is found for the SLβ-transgenic fish, with a 14·5-fold induction of SLβ; however, only a 9·81-fold induction of GH is found in the GH-transgenic fish. The results indicate the successful overexpression of hormones in vivo, as anticipated.

Three types of IGF have been identified in zebrafish so far: IGF1, IGF2, and IGF3, with two subtypes of IGF2: IGF2α and IGF2β. Figure 8 shows the gene expression levels (fold induction over non-transgenic control) of IGFs in transgenic fish. The level of IGF1 was up-regulated by 5·46- and 6·77-fold in the SLα-transgenic and the SLβ-transgenic fish respectively, whereas there was a sharp induction of 88·34-fold in the SL-transgenic fish, with a 2·13-fold increase in the SLα-transgenic fish. A study of somatolactin actions in transgenic zebrafish, SREBP1 and SREBP2, and only ACC2 were selected to understand the effect of SL on glucose synthesis and lipid metabolism. The two types of SREBP were chosen to understand the role of SLs in pigmentation, and a moderate increase of 2·53- and 2·26-fold was found in the SLα-transgenic and SLβ-transgenic fish respectively, but only 1·45-fold in the GH-transgenic fish (Fig. 12).

CAT and GST mRNA levels were used to investigate the effect of redundant SLs on the antioxidant defence system. CAT gene expression was found to decrease slightly in both the SLα-transgenic (0·75-fold) and the SLβ-transgenic (0·82-fold) fish, but it was found to increase a little in the positive GH-transgenic fish (1·27-fold; Fig. 13). A moderate reduction in GST gene expression was observed in the SLα-transgenic (0·55-fold) and the SLβ-transgenic (0·46-fold) fish, whereas almost no change was observed in the GH-transgenic fish (Fig. 14).

**Discussion**

It is generally assumed that the direct microinjection of gene constructs into the pronucleus or nucleus of a fertilized egg before the onset of cleavage is the most effective way to incorporate new genetic material into 2·81-fold, whereas ACC2 expression decreased 0·66- and 0·76-fold in the SLα-transgenic and SLβ-transgenic lines respectively. GH-transgenic fish demonstrated a 4·82-fold increase in FAS mRNA level and a 3·41-fold increase in ACC2 mRNA level by comparison (Fig. 10).

VTG was selected to analyze the possible role of SLs in reproduction. It was found that VTG mRNA was up-regulated in the SLα-transgenic and SLβ-transgenic fish with a 5·94- and 6·02-fold increase respectively over the control, and VTG mRNA was up-regulated in the GH-transgenic fish with a 2·04-fold increase (Fig. 11). POMC was chosen to understand the role of SLs in pigmentation, and a moderate increase of 2·53- and 2·26-fold was found in the SLα-transgenic and SLβ-transgenic fish respectively, but only 1·45-fold in the GH-transgenic fish (Fig. 12).

**Figure 6** Body weight (a) and calculation of condition factor K (b) in 6-month-old zebrafish (*Danio rerio*) of transgenic zebrafish. WT, wild-type, non-transgenic fish; SLα, SLα-transgenic G0 fish; SLβ, SLβ-transgenic G0 fish; GH, GH-transgenic G0 fish. Different letters indicate significant differences (*P*<0·05) among the fish groups; *N* = 10.

**Figure 7** Gene expression of endogenous SLα, SLβ, and GH in the SLα-transgenic, SLβ-transgenic, and GH-transgenic lines respectively. The hormones were successfully overexpressed without inducing or suppressing other members’ levels *in vivo*. Each value represents the mean ± s.e.m. of the three replicates. Different letters indicate significant differences (*P*<0·05) from each other (one-way ANOVA).

**Figure 8** shows the gene expression levels (fold induction over non-transgenic control) of IGFs in transgenic fish. The level of IGF1 was up-regulated by 5·46- and 6·77-fold in the SLα-transgenic and the SLβ-transgenic fish respectively, whereas there was a sharp induction of 88·34-fold in the SL-transgenic fish, with a 2·13-fold increase in the SLα-transgenic fish.
the genome of injected embryos (Zhu et al. 1985, Ozato et al. 1986, Culp et al. 1991, Chen et al. 1993, Morales et al. 2001); however, the exact time of gene integration after microinjection is not guaranteed (Hamada et al. 1998, Figueiredo et al. 2007). Recently, the use of reporter genes that allow the evaluation of the degree of in vivo mosaicism in transgenic fish has facilitated the identification of probable germline founders (Rahman et al. 1997, 1998, Hamada et al. 1998, Ju et al. 1999, Liu et al. 2003), and the coinjection of a reporter transgene along with the gene construct of interest represents a considerable reduction in the effort needed to establish transgenic germlines (Rahman et al. 1997, 1998). The analysis of the GFP expression patterns permitted the selection of possible germline founders in the G0 generation. One week after microinjection, 66.5% (SLα) and 63% (SLβ) of the fish embryos expressed GFP, indicating highly efficient transgenic fish production. However, part of this observed expression may be attributed to transitory expression due to the transcription of unintegrated transgenes (Chong & Vielkind 1989, Thermes et al. 2002). However, as the DNA injected contained no plasmid DNA, the chance of DNA replication following embryogenesis was reduced. We found that 9-7% (SLα) and 5-5% (SLβ) of the fish that we analyzed showed strong GFP reporter gene expression, similar to 5% of the fish with strong GFP expression reported by Gibbs & Schmale (2000) for G0-transgenic zebrafish and >5% of the fish with strong GFP expression reported by Gibbs & Schmale (2000) and Thermes et al. (2002) for G0-transgenic medaka. Our conditions were similar to those of Gibbs & Schmale (2000) and Thermes et al. (2002), who also used linearized transgenes in which GFP gene expression was under the control of ubiquitous promoters (α- or βA).

In addition to genomic PCR confirmation, Southern blot analysis was used to reconfirm the GFP-positive transgenic fish. The probes designed in SL gene cDNA contained several introns in WT genomic DNA to avoid a false positive signal. After digestion with EcoRI, three different bands were detected in SLα-transgenic G0 sample #116 and #117, whereas only one band and two bands appeared in SLβ-transgenic G0 sample #103 and #104 respectively. We used SmaI and XhoI to digest the whole genomic DNA to see whether the transgenes of total SL cDNA would be released, and the results showed only one band, as expected, but with different copies in the genomic DNA.

Measurement of the mRNA expression of SLs using real-time PCR revealed that the zβAsSLα and zβAsSLβ
transgenes produced high levels of ubiquitous active hormone mRNAs, with an increase of 24.3-fold for SLα and 14.5-fold for SLβ, compared with the non-transgenic controls, but without any increase in the GH mRNA level. A primary function of GH is to promote somatic growth in fish; however, this function is accomplished indirectly through the GH/IGF1/IGF2 axis. This axis has been shown to play important roles in fish growth and development. After GH binds to the GHR in the target tissue and activates it, a tyrosine kinase called janus kinase (JAK) is activated to transduce the GH signal in the target tissue to release IGF1 or IGF2 (Carter-Su & Smit 1998). To find out whether SLs affect fish growth and development as GHS are mediated by IGFs, the expression level of IGFs in SL-transgenic fish was investigated. In mammals, IGF1 plays an important role in both embryonic and postnatal growth (Baker et al. 1993), primarily through its stimulatory effects on cell proliferation and inhibition of cell death (apoptosis). Mice carrying null mutations in the IGF1 gene are born small and grow very poorly postnatally (Baker et al. 1993, Powell-Braxton et al. 1993, Liu et al. 1998). IGF2 is thought to be a primary growth factor required for early development, whereas IGF1 expression is required for achieving maximal growth (O’Dell & Day 1998). In our in vivo study, a significant increase in IGF1 (5.46- and 6.77-fold), IGF2a (4.38- and 4.35-fold), and IGF2b (2.83- and 3.94-fold) in the SLα-transgenic and the SLβ-transgenic zebrafish larvae respectively brought about a moderate enhancement of zebrafish growth, with a respective 1.28- and 1.35-fold increase in body weight gain, by comparison with a 1.65-fold increase in the GH-transgenic zebrafish.

Our data agree with those of the transfer and overexpression of GH transgenes from other groups, including salmonid (2–6-fold; Du et al. 1992), common carp (1.2–1.8-fold; Chen et al. 1993), zebrafish (2–6-fold; Figueiredo et al. 2007), and tilapia (3.5–4-fold; Rahman et al. 1998). SLα and SLβ could act on growth development to some extent as GH does—through IGF signaling. Recently, IGFs have been found to be expressed in teleosts in a wide range of tissues throughout life, which suggests that the autocrine/paracrine role of this hormone may be of particular physiological importance in bony fish (Schlueter et al. 2007, Sang et al. 2008, Wang et al. 2008). For example, gonad-specific IGF3 was found to be present only in the fish genome (Sang et al. 2008), which suggests its potential function in gonad development and

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**Figure 11** Vitellogenin (VTG) gene expression in the non-transgenic and different transgenic lines. Overexpressed SLs induced a higher level of VTG than did GH in the transgenic lines. Each value represents the mean ± S.D. of the three replicates. Different letters indicate significant differences (P < 0.05) from each other (one-way ANOVA).

**Figure 12** Proopiomelanocortin (POMC) gene expression in the non-transgenic and different transgenic lines. Both SLs and GH slightly regulated the expression of POMC in the transgenic lines. Each value represents the mean ± S.D. of the three replicates. Different letters indicate significant differences (P < 0.05) from each other (one-way ANOVA).

**Figure 13** Catalase (CAT) gene expression in the non-transgenic and different transgenic lines. Neither the SLs nor GH had any effect on the level of CAT. Each value represents the mean ± S.D. of the three replicates. Different letters indicate significant differences (P < 0.05) from each other (one-way ANOVA).
reproduction in teleosts. IGFs have also been found to be required for primordial germ cell migration and survival (Mommsen & Plisetskaya 1991, Papasani et al. 2006). However, SLs did not appear to enhance IGF3 gene expression in this study.

Glucose is stored as glycogen in the liver and muscle (Wang et al. 1994, Elo et al. 2007). PEPCK is an indicator of blood glucose levels for the examination of glucose metabolism, which catalyzes a rate-limiting step in gluconeogenesis, and is transcriptionally regulated by glucagon and insulin (Brown & Goldstein 1997). Our study showed that the overexpression of SLz could induce more mRNAs of insulin (2.96-fold), and thus PEPCK (1.32-fold), than SLβ could (insulin: 1.92-fold; PEPCK: 1.14-fold). SREBP can up-regulate the synthesis of enzymes involved in sterol biosynthesis. For instance, SREBP1 regulates the genes needed to make fatty acids, while SREBP2 regulates the genes involved in cholesterol metabolism (Company et al. 1999, Browney et al. 2006). The overexpression of both SLz and SLβ increased SREBP1 expression but inhibited SREBP2 expression in zebrafish. The abundant SLz resulted in more fatty acids through activation of a higher level of FAS expression than did SLβ (Fig. 10). A transient increase in plasma SL levels was found in short-term fasted fish (Vega-Rubin de Celis et al. 2003), which suggests that SL may play a role as an anti-obesity hormone helping to expedite growth and reproductive processes following the replenishment of fat stores, and/or mediate the adaptation to fasting until the lipolytic action of GH and/or other endocrine factors is fully accomplished. This is consistent with the inhibition of ACC expression in SL-transgenic fish, in which the fatty mechanism is regulated by the inhibition of the β-oxidation of the fatty acid in mitochondria, as the presence of fatty acids inhibits its activities (Sahu 2003). A similar result was also found in European sea bass (Johnson et al. 1997). Leptin plays a key role in regulating energy intake and energy expenditure, including appetite and metabolism (Mayer et al. 1998). A moderate induction (2.58- and 2.14-fold) of LEP in SLz-transgenic and SLβ-transgenic fish helps to execute anti-obesity effects.

However, studies of seasonal changes in SL mRNA showed the gradual stimulation of SL synthesis and release during sexual maturation, and spawning females tend to have more SL cells than equivalent males, indicating that SL may play a role in the control of some steps of reproduction (Planas et al. 1992, Rand-Weaver et al. 1992, Oliverau & Rand-Weaver 1994). Plasma SL concentrations were found to remain relatively constant throughout gonad development but were found to drop during or following ovulation in sole and halibut (Schneider 1996). SL was found to stimulate gonad steroidogenesis produced by testicular fragments and ovarian follicles in vitro in coho salmon; however, this steroidogenic activity was considerably less than that of GH (Planas et al. 1992). SL synthesis and release were found to be inhibited by gonadectomy (Specker & Sullivan 1994). VTG is an egg yolk precursor protein crucial for reproductive success, such as oocyte growth, oocyte maturation, early embryogenesis, and later, larval development (Nguyen et al. 2006). Our study showed that the ectopic expression of SLz and SLβ in vivo significantly induced the mRNA expression of VTG, indicating that SLs play a role in vitellogenesis. The stimulation and secretion of VTG might pass through the granulose and thecal cell layers, bind to specific receptors on the oocyte surface, and are sequestered via receptor-mediated endocytosis (Schallreuter et al. 2008).

SL has also been found to be related to pigmentation, melanosome aggregation, chromatophore proliferation, and morphogenesis. A medaka (O. latipes) ci mutant in which the proliferation and morphogenesis of chromatophore pigment cells are deficient was recently found to have SL gene mutation (Fukamachi et al. 2006). An increased number and more dendritically shaped leucophores were observed, and fewer xanthophores were visible in the mutant, so the body of the WT was darker than that of the ci mutant (Fukamachi et al. 2006). The effect of SL on melanosome aggregation was studied in red drum (Zhu & Thomas 1997) and zebrafish (Castrucci et al. 1997) in a dose-dependent manner. A ci-if double mutant (ci and lacking leucophores) was used to investigate the role of SL in the regulation of body color, and the results showed that the if gene played an indispensable role in leucophore development of epistatic to SL signaling (Fukamachi et al. 2006). In the present study, the POMC gene was used to study the effect of SL on pigmentation. A twofold induction of POMC was found in both the

Figure 14 Glutathione S-transferase (GST) gene expression in the non-transgenic and different transgenic lines. SLs reduced the expression of GST, whereas GH had no effect on it. Each value represents the mean ± s.d. of the three replicates. Different letters indicate significant differences (P<0.05) from each other (one-way ANOVA).
SLα-transgenic and the SLβ-transgenic zebrafish larvae, indicating the role of SL in body color and background adaptation. POMC (expressing melanocyte-stimulating hormone) can stimulate the production and release of melanin (melanogenesis) by melanocytes in the skin (Brown-Borg & Rakoczy 2005, Brown-Borg et al. 2005). An interesting disrupted stripe phenotype was found only in the SLα-transgenic fish (Fig. 5). The molecular mechanism of SLα in stripe morphogenesis in fish remains to be investigated.

GH is a key player in the physiological mechanisms of altered stress resistance, such as the alteration in the activity of multiple enzymatic components of methionine and glutathione metabolism in Ames dwarf mice (Brown-Borg et al. 2009). Studies show that Ames dwarf mice live significantly longer than their WT siblings and exhibit elevated antioxidative defences and reduced oxidative damage, perhaps leading to their extended longevity (Chelikani et al. 2004, Leggatt et al. 2007).

Research into GH-transgenic salmon shows the up-regulation of the components of the glutathione antioxidant system to combat potentially higher reactive oxygen species production, based on the accelerated growth and increased metabolic rates. We, however, found slight reductions in the expression of the catalase (CAT) gene and GST gene in both the SLα-transgenic and the SLβ-transgenic fish. Catalase is a common central enzyme involved in reactive oxygen species (ROS) scavenging through the degradation of hydrogen peroxide into oxygen and water (Chelikani et al. 2004). GSTs involved in xenobiotic metabolism are cytosolic proteins that catalyze the conjugation of glutathione with a substrate bearing an electrophilic atom. Free reactive electrophilic intermediates of xenobiotics can produce damage to important cellular constituents. The decreases in these two key enzymes in the antioxidant defence system in the SL-transgenic fish suggest a reduction in the capability to deal with an oxidative stress situation (Leggatt et al. 2003). An increased growth rate in transgenic zebrafish was found to be associated with an increased metabolic rate (Rosa et al. 2008). It is expected that the antioxidant system in GH-transgenic fish might be up-regulated to cope with increased ROS production based on the high metabolic demand due to accelerated growth rate in these transgenic fish. The glutathione antioxidant system was found to be enhanced in GH-transgenic coho salmon (Leggatt et al. 2003); however, a resistance effect to excess GH caused a decrease in the catalytic subunit expression of glutamate-cysteine ligase, an enzyme responsible for glutathione synthesis, in GH-transgenic zebrafish (Rosa et al. 2008). The reduction in the efficiency of the antioxidant defence system due to the overexpression of SLs may result in the greater susceptibility of SL-overexpressing fish to oxidative stress, and such altered stress capacity may affect their health and life span.

In summary, we have successfully generated transgenic zebrafish with the ectopic expression of SLα and SLβ of zebrafish in vivo using the coinjection strategy. The data obtained here indicate that the overexpression of SLs can induce IGFs, but that the level of induction is well below that of GH; however, overexpressing SLs could affect growth, development, glucose synthesis, lipid metabolism, reproduction, pigmentation, and the antioxidant defence system. Whether these observed effects were mediated via GHRs or SLR remain to be investigated. To find out the full pictures of the actions of SLs on growth and metabolisms, adult transgenic fish should be employed for further investigations.

Declaration of interest

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

Funding

This research did not receive any specific grant from any funding agency in the public, commercial or not-for-profit sector, all equipments used are already available in the Department of Biochemistry, and consumables were paid by research allowance from the Department of Biochemistry, Chinese University.

Acknowledgements

The authors thank the graduate division of Biochemistry (Science) for the provision of a post-graduate studentship to GW and the Department of Biochemistry for its support of this research with a high degree of academic freedom.

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Received in final form 4 August 2010
Accepted 26 August 2010
Made available online as an Accepted Preprint 26 August 2010