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

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Abstract

Somatolactin (SL) is a fish-specific hormone that belongs to the prolactin (PRL) and GH family. Recently, two forms of SL, SL α and SL β , 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 SL α and SL β 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 SL α and SL β *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 proopiomelanocortin, while causing reduced levels of catalase and glutathione S-transferase in the larvae of transgenic zebrafish.

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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 proopiomelanocortin (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, SL α and SL β , 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 SL α , 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, Oliverau & 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,

2006, 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 defence 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 (09-142 in DH/HA&P/8/2/1 Pt.6) to conduct experiments was obtained from the Department of Health, Government of the Hong Kong Special Administrative Region, China.

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 μ l of 10 $\times$ Expand High Fidelity buffer, 15 mM of $MgCl_2$, 1.5 μ l of 10 μ M α z βA forward primer (5'-GGGGTACCAGTAATGCAGCAGCGTGAAG-3'), 1.5 μ l of 10 μ M α z βA reverse primer (5'-TCCCCCGGGGGCTGTAGGGA-AAAAAGCG-3'), 1 μ l of 10 mM of dNTP mix, and 0.75 μ l of Expand High Fidelity Enzyme Mix (Applied Biosystems, Foster City, CA, USA). Finally, distilled water was added to increase the reaction volume to 50 μ l. The DNA primers were designed with enzyme sites (KpnI and SmaI) for cloning into pGL3-Basic vector (Promega) according to the zebrafish genomic sequence in the GenBank database of the National Center for Biotechnology Information (NCBI) (accession no. AL928650). PCR was performed on the TaKaRa PCR Thermal Cycler Dice with the following conditions: one cycle at 94 $^{\circ}C$ for 2 min, 30 cycles at 94 $^{\circ}C$ for 30 s, 60 $^{\circ}C$ for 30 s, and 72 $^{\circ}C$ for 3 min, followed by one cycle of 72 $^{\circ}C$ 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 $SL\alpha$, $SL\beta$, and GH gene amplification using the following specific primer sets:

α z $SL\alpha$ F (5'-ATGAACACAGTTAAAGTTCTGCAGG-3') and α z $SL\alpha$ R (5'-CTATTATTGGGGCAGGCAGCTGAGTT-3');
 α z $SL\beta$ F (5'-ATGATGAAGAAAGCTACAGC-3') and α z $SL\beta$ R (5'-CTATTAGAAGAGGGAGCAGTTTTC-3');
 α zGHF (5'-ATGGCTAGAGCATTGGTGCT-3') and α zGHR (5'-CTATTACAGGTACAGTTGGAATCCA-3').

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

The βA gene promoter was cloned into the pGL3-Basic vector (Promega) to study the promoter activity that drives the firefly luciferase reporter gene. The cDNAs of zebrafish $SL\alpha$, $SL\beta$, GH, and humanized renilla GFP (hrGFP) were respectively replaced with luciferase reporter gene, and the hormone overexpression constructs were linearized, containing only the promoter element, hormone cDNA, and polyA signal elements, with plasmid and its antibiotic resistance gene removed (Fig. 1).

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 z β AzSL α , z β AzSL β , and z β AzGH 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 z β AzSL α , z β AzSL β , and z β AzGH respectively. Probes in z β AzSL α and z β AzSL β were used in Southern blot analysis for further confirmation of transgenic fish, while Δ indicates intron in the genomic DNA of wild-type fish. (e) The z β AhrGFP construct isolated from the plasmid after digestion with KpnI and used as a coinjection strategy for positive GFP signal detection.

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 the 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 (z β AzSL α and z β AhrGFP;

z β AzSL β and z β AhrGFP; z β AzGH and z β AhrGFP) using the PV820 Pneumatic PicoPump (World Precision Instruments; Chong & Vielkind 1989, Hamada *et al.* 1998). Approximately, 500 μ l 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;

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 DMIL 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 (G_0) 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 G_0 and G_1 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 β AzSL α P1 (5'-GAATCGGCGGCATTGAG-3');
z β AzSL α P2 (5'-TCCAGAGGAACCGCACC-3');
z β AzSL β P3 (5'-CATCAGTCATCACCGCCTAT-3');
z β AzSL β P4 (5'-GTGAGCAACTCGTCAA-3');

z β AzGH P5 (5'-GAATCGGCGGCATTGAG-3'); and
z β AzGH P6 (5'-AAGACGAGCCCATCTTG-3').

PCR was carried out in a 25 μ l 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 β AzSL α F: 5'-CCCATCCCCAACATCCAAGA-3';
z β AzSL α R: 5'-ATACAGCAGCGGCTCCATC-3';
z β AzSL β F: 5'-GCTCGACTTATTCGGAGGTATG-3'; and
z β AzSL β R: 5'-CCAAGCCTCCTTCACCC-3'.

Double-stranded oligonucleotide probes, z β AzSL α -102 bp and z β AzSL β -308 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 μ g) was digested with EcoRI or SmaI 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 $\times$ 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 (SL α , SL β , 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 (SL α -transgenic G₀, SL β -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 μ m, 52% protein) at week 1, ZM-100 (80–200 μ m, 55% protein) at week 2, ZM-200 (150–300 μ m, 60% protein) at weeks 3–5, and Mediaaquafish Guppy Food (300–500 μ m, 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 $K = (W \cdot L^{-3}) \times 10^3$, 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 β A (AF057040), SL α (AY376857), SL β (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 (ENSARG00000063438), 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

Genes	Forward primer (5'–3')	Reverse primer (5'–3')	Amplicon size (bp)
β A	CGAGCAGGAGATGGGAACC	CAACGGAACGCTCATTGC	102
SL α	CAACCAGGCTGGAAACACC	GGCTCCATCCACGACTGAA	129
SL β	GTGGTGTTCTGGTCTGCTTAA	CTTCTCCGCTGAGATTGTGC	102
GH	GCATCAGCGTGCTCATCAA	CCCTACGGTCAGGTAGAAATCC	101
PRL	ATCTCAGCACCTCACTCACCAA	GCTCATCTCCGGCACTTT	147
IGF1	TGTAGAGGACAGCGGGAGG	TTGGTGAGTGTATGGAAATCA	211
IGF2a	CTCTGTGGCGGAGAACTA	CCTGTGACACTGGGAAGA	229
IGF2b	TCTGTGGGATTGTGGAGG	CGCAAGATGGCAGGGAT	243
IGF3	AGAAAGTGCGGCGTGAT	TGAGGAGGTTTGGGTGC	194
LEP	TCCAGGACATTATCCAGAG	TCCAGGTAACCCAGAAGT	159
SREBP1	GGCGGAGACAGCAAGG	GCCAGCGGGTTAAAGGA	108
SREBP2	CGAGACGACGGTTAGGC	TCCAGCGTGCGTTTAGC	222
FAS	TGAAGCGGAGGCAGAAA	CAAGCAGTGCGGTAAGG	175
ACC2	CGCCACTCTGACCTCAT	CGACTACCGTATCGCATC	224
VTG	TCCTGAGTTTGCCCATGATA	GATGAGAACCTTGCTGCTGA	118
POMC	GAACGGCGTGGAAGAGG	CCAGCGGAAATGGGTCA	151
PEPCK	GACTGGGCGCACGGAGTGTT	AAAGCCCGGCCACAGGA	246
INS	TGGGTTTCCTTCTCCT	CACTGCTCTACAATGCCTC	103
CAT	TGAGGCTGGGTCATCAGATA	AAAGACGGAACAGAAAGCGT	138
GST	CTGAGACATCTGGGTCGAAA	AGATCTTCAACTCCGTCGTTT	92

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 μ l Brilliant SYBR Green QPCR master mix (Applied Biosystems), 0.5 μ l of forward primer (10 μ M), 0.5 μ l of reverse primer (10 μ M), 2 μ l cDNA template, and 9.5 μ l nuclease-free water for a final volume of 25 μ l.

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 *z β A*. 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 *β A* gene promoter was isolated using genomic PCR. Potential transcription factor binding sites that may be involved in regulating the *β A* gene promoter were found (Fig. 1a). SL α -transgenic and SL β -transgenic zebrafish were produced by the coinjection of the transgenes *z β AzSL α* and *z β AhrGFP*, and *z β AzSL β* and *z β AhrGFP* 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 SL α -transgenic ones and 343 out of 1300 (26.4%) for the SL β -transgenic ones respectively. Among the 352 SL α -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 SL β -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 *z β AzGH* and *z β AhrGFP*. 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 *z β AzSL α* , *z β AzSL β* , and *z β AzGH* transgenes. In the PCR analysis, a 299 bp fragment was generated from SL α -transgenic G₀ zebrafish #14 and #28 using primers P1 and P2, and a 298 bp fragment was generated from SL β -transgenic G₀ zebrafish #5 and #11 using primers P3 and P4 (Fig. 3a). A 673 bp fragment was detected in the positive control

Figure 3 Identification of transgenic fish by genomic DNA PCR. (a) Positive transgenic fish were identified using primers P1 and P2 for SL α (299 bp) and primers P3 and P4 for SL β (298 bp). Lane 1, 1 kb Plus DNA Ladder (Invitrogen); lanes 2 and 3, SL α -transgenic samples #14 and #28; lanes 6 and 7, SL β -transgenic samples #5 and #11; lanes 4 and 5 and lanes 8 and 9, non-transgenic samples. (b) Positive transgenic fish were identified using primers P5 and P6 for GH (673 bp). Lane 1, 1 kb Plus DNA Ladder (Invitrogen); lanes 2 and 3, GH-transgenic samples #25 and #26; lanes 4 and 5, non-transgenic samples.

Figure 4 Southern blot analysis of genomic DNA samples isolated from SL-transgenic zebrafish. (a) Genomic DNA from SL α -transgenic lines digested with EcoRI. Lane A, z β AzSL α plasmid (uncut control); lanes B and C, SL α -transgenic G₀ samples #14 and #28; lanes D and E, non-transgenic samples. (b) Genomic DNA from SL β -transgenic lines digested with EcoRI. Lane A, z β AzSL β plasmid (uncut control); lanes B and C, SL β -transgenic G₀ samples #5 and #11; lanes D and E, non-transgenic samples. (c) Genomic DNA from SL α -transgenic lines digested with SmaI and XhoI. Lane A, z β AzSL α plasmid (uncut control); lanes B and C, SL α -transgenic G₀ samples #14 and #28; lanes D and E, SL α -transgenic G₁ samples #57 and #60; lanes F and G, non-transgenic samples. (d) Genomic DNA from SL β -transgenic lines digested with SmaI and XhoI. Lane A, z β AzSL β plasmid (uncut control); lanes B and C, SL β -transgenic G₀ samples #5 and #11; lanes D and E, SL β -transgenic G₁ samples #37 and #38; lanes F and G, non-transgenic samples. *The specific DIG-labeled probes used in the SL α -transgenic and SL β -transgenic lines are shown in the z β AzSL α and z β AzSL β constructs respectively (Fig. 1).

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 SL α -transgenic G₀ zebrafish #14 and #28 (Fig. 4a), one band in SL β -transgenic G₀ zebrafish #5 and two bands in SL α -transgenic G₀ zebrafish #11 in the digestion of EcoRI (Fig. 4b). It is suggested that the transgene z β AzSL α was integrated into three different sites in the genome of SL α -transgenic G₀ zebrafish #14 and #28, and the transgene z β AzSL β was integrated into only one site in SL β -transgenic G₀ zebrafish #5 and two

sites in SL β -transgenic G₀ zebrafish #11. Simultaneously, in the digestion of SmaI and XhoI, which cut through the whole length of SL α and SL β 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 z β AzSL α , z β AzSL β , and z β AzGH 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 SL α -transgenic G₀ line (Fig. 5). At the end of the growth experiment, the SL α -transgenic G₀ fish reached a final average body weight of 421.1 ± 78.3 mg; their SL β -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 SL α , SL β , and GH genes in 5-week-old transgenic zebrafish were investigated (Fig. 7). The results show a 24.3-fold induction of SL α in the SL α -transgenic fish without obvious up- or down-regulation of other members in the GH family.

Figure 5 Photographs showing 6-month-old zebrafish (*Danio rerio*) of transgenic zebrafish. (a) SL α -transgenic G₀ fish; (b) SL β -transgenic G₀ fish; (c) non-transgenic fish. The fish are a representative sample of the mean size of the three groups. The arrow indicates the special stripe phenotype of the SL α -transgenic G₀ fish. Full color version of this figure available via <http://dx.doi.org/10.1677/JME-09-0173>.

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; $N = 10$.

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: IGF2a and IGF2b. 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 GH-transgenic fish as positive controls. The level of *IGF2* was also found to increase in the transgenic fish: 4.38-, 4.35-, and 11.76-fold for *IGF2a* and 2.83-, 3.94-, and 13.40-fold for *IGF2b* in the SLα-transgenic, SLβ-transgenic, and GH-transgenic fish respectively. However, the amount of *IGF3* decreased in the SL-transgenic fish (0.57-fold in the SLα-transgenic and 0.54-fold in the SLβ-transgenic ones), but showed an increase of 5.41-fold in the GH-transgenic fish.

Regarding energy metabolism, key marker genes, such as *LEP*, insulin (*INS*), *PEPCK*, *SREBP*, *FAS*, and *ACC*, were selected to understand the effect of SL on glucose synthesis and lipid metabolism. The two types of *SREBP* in zebrafish, *SREBP1* and *SREBP2*, and only *ACC2* were considered in this experiment. The expression levels of leptin, insulin, and *PEPCK* were found to demonstrate a 2.58-, 2.96-, and 1.32-fold increase in the SLα-transgenic fish, a 2.14-, 1.92-, and 1.14-fold increase in the SLβ-transgenic fish, and a 1.89-, 1.95-, and 5.42-fold increase in the GH-transgenic fish as positive controls (Fig. 9). It is interesting to discover that the expression level of *SREBP1* was up-regulated 1.51- and 1.40-fold and that of *SREBP2* was down-regulated 0.51- and 0.53-fold in the SLα-transgenic and SLβ-transgenic lines respectively. GH-transgenic fish demonstrated a 2.13-fold increase in *SREBP1* expression and a 1.35-fold increase in *SREBP2* expression. *FAS* expression increased 5.10- and

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).

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

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 $\pm$ S.D. of the three replicates. Different letters indicate significant differences ($P < 0.05$) from each other (one-way ANOVA).

Figure 8 Insulin-like growth factor (*IGF1*, *IGF2a*, *IGF2b*, and *IGF3*) gene expression in the non-transgenic and different transgenic lines. Both SLs and GH induced different levels of IGFs *in vivo*. Each value represents the mean $\pm$ s.d. of the three replicates. Different letters indicate significant differences ($P < 0.05$) from each other (one-way ANOVA).

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 G_0 generation. One week after microinjection, 66.5% (SL α) and 63% (SL β) of the fish embryos expressed

Figure 9 Leptin (*LEP*), insulin (*INS*), and phosphoenolpyruvate carboxykinase (*PEPCK*) gene expression in the non-transgenic and different transgenic lines. Both SLs and GH regulated the expression of *LEP*, *INS*, and *PEPCK* in different ways *in vivo*. Each value represents the mean $\pm$ s.d. of the three replicates. Different letters indicate significant differences ($P < 0.05$) from each other (one-way ANOVA).

Figure 10 Sterol regulatory element binding protein (*SREBP1* and *SREBP2*), fatty acid synthase (*FAS*), and acetyl-CoA carboxylase 2 (*ACC2*) gene expression in the non-transgenic and different transgenic lines. Both SLs and GH regulated the expression of *SREBP*, *FAS*, and *ACC* in different ways *in vivo*. Each value represents the mean $\pm$ s.d. of the three replicates. Different letters indicate significant differences ($P < 0.05$) from each other (one-way ANOVA).

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% (SL α) and 5.5% (SL β) 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 G_0 -transgenic zebrafish and >3% of the fish with strong GFP expression reported by Gibbs & Schmale (2000) and Thermes *et al.* (2002) for G_0 -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 β A).

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 SL α -transgenic G_0 sample #116 and #117, whereas only one band and two bands appeared in SL β -transgenic G_0 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 β AzSL α and β AzSL β

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 $\pm$ s.d. of the three replicates. Different letters indicate significant differences ($P < 0.05$) from each other (one-way ANOVA).

transgenes produced high levels of ubiquitous active hormone mRNAs, with an increase of 24.3-fold for SL α and 14.5-fold for SL β , 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

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 $\pm$ 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 $\pm$ s.d. of the three replicates. Different letters indicate significant differences ($P < 0.05$) from each other (one-way ANOVA).

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 SL α -transgenic and the SL β -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). SL α and SL β 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

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 $\pm$ 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 (Mommensen & 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 SL α could induce more mRNAs of insulin (2.96-fold), and thus PEPCK (1.32-fold), than SL β 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, Brownsey *et al.* 2006). The overexpression of both SL α and SL β increased SREBP1 expression but inhibited SREBP2 expression in zebrafish. The abundant SL α resulted in more fatty acids through activation of a higher level of FAS expression than did SL β (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 SL α -transgenic and SL β -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 SL α and SL β *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 granulosa 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-lf* 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 *lf* 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

SL α -transgenic and the SL β -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 SL α -transgenic fish (Fig. 5). The molecular mechanism of SL α 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 SL α -transgenic and the SL β -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 SL α and SL β 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.

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