Evidence for the existence of a local activin–follistatin negative feedback loop in the goldfish pituitary and its regulation by activin and gonadal steroids

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Abstract

Activin is an important regulator of gonadotropin expression and production in the vertebrate pituitary, and its activity is fine-tuned by its binding protein follistatin. In the present study, a full-length cDNA for follistatin was cloned in the goldfish, which shows 74% amino acid sequence identity with that of mammals. Recombinant goldfish follistatin expressed in the Chinese hamster ovary cells significantly blocked activin-induced F5–5 cell differentiation. Goldfish follistatin is expressed in a wide range of tissues including the brain, pituitary, ovary, and testis. The expression of follistatin mRNA in the pituitary is regulated by both activin and gonadal steroids *in vitro*. Treatment with goldfish activin B for 48 h significantly up-regulated follistatin expression in cultured pituitary cells, suggesting a closed activin–follistatin feedback loop in the pituitary. In agreement with this, both

human and goldfish follistatin down-regulated the expression of follistatin itself, probably due to the neutralization of endogenous activin. Examination of FSH β and LH β expression in the same samples supports the role of activin and follistatin in the differential regulation of FSH and LH as demonstrated previously. Since the expression level of activin β_B in the pituitary is rather stable both *in vitro* and *in vivo*, it is conceivable that follistatin may play a pivotal regulatory role in the intra-pituitary activin system. Both estradiol and testosterone up-regulated follistatin expression *in vitro*, suggesting a mediating role for follistatin in steroid feedback on pituitary hormones. These results provide clues to the potential physiological roles of activin–follistatin system in the regulation of gonadotropin biosynthesis in teleosts.

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Introduction

Gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), are the primary hormones governing gonadal development and function in vertebrates including teleosts (Gharib *et al.* 1990, Swanson *et al.* 2003, Weltzien *et al.* 2004). Similar to those in mammals (Gharib *et al.* 1990, Burger *et al.* 2004), the secretion and expression of fish gonadotropins are controlled by both neuroendocrine factors from the hypothalamus such as gonadotropin-releasing hormone (GnRH; Peter *et al.* 1986, Weltzien *et al.* 2004, Ando & Urano 2005) and steroids from the gonads (Kobayashi *et al.* 1987, 1989, Dickey & Swanson 1998). Our previous study in the goldfish demonstrated that in addition to the neuroendocrine and endocrine regulation, pituitary gonadotropins are also subject to local paracrine regulation by activin (Yam *et al.* 1999a, Yuen & Ge 2004), similar to the situation in mammals (Roberts *et al.* 1989, Corrigan *et al.* 1991, Bilezikjian *et al.* 2004).

Activin ($\beta_A\beta_A$, $\beta_A\beta_B$, and $\beta_B\beta_B$) was first identified as an ovarian factor in mammals that specifically stimulated pituitary FSH secretion without any effect on LH (Ling *et al.*

1986, Vale *et al.* 1986). Its subunits β_A and/or β_B have later been demonstrated to be expressed in a variety of extra-gonadal tissues including the pituitary in both mammals and fishes (Meunier *et al.* 1988, Roberts *et al.* 1989, Lau & Ge 2005), suggesting a wide range of biological activities at various sites. The functionality of the pituitary-derived activin in regulating mammalian FSH secretion has been demonstrated by immunoneutralization of activin both *in vitro* (Corrigan *et al.* 1991) and *in vivo* (DePaolo *et al.* 1992). The activities of activin are modulated or fine-tuned by its potent binding protein follistatin, which serves as a local modulator of activin activities in various tissues including the pituitary (Welt *et al.* 2002). In the goldfish, the treatment of cultured pituitary cells with follistatin caused a decrease in FSH β but an increase in LH β expression (Yuen & Ge 2004), which were opposite to the effects of activin (Yam *et al.* 1999a, Yuen & Ge 2004), suggesting an intra-pituitary activin-mediated autocrine/paracrine system in this species (Yuen & Ge 2004).

The potent modulatory effect of follistatin on activin and their co-expression in the pituitary suggest that the pituitary-derived follistatin may represent a regulatory point subject to the influence by both neuroendocrine and endocrine inputs,

which in turn influences gonadotropin biosynthesis by suppressing activin activities. This hypothesis is supported by several studies in mammalian models. In the rat, a pulsatile application of GnRH at high frequency inhibited FSH β by stimulating pituitary follistatin, leading to a decreased activin activity (Kirk *et al.* 1994, Besecke *et al.* 1996). Follistatin expression was noted to precede that of FSH β in the rat pituitary during its estrous cycle but dropped drastically when FSH expression reached the maximal level (Halvorson *et al.* 1994), suggesting that follistatin expression may also be influenced by gonadal sex steroids. The effects of sex steroids on follistatin have been evidenced by the observation that gonadectomy stimulated pituitary follistatin in both male and female adult rats (Kaiser & Chin 1993). In support of this, treatment with testosterone suppressed follistatin expression in the rat pituitary *in vitro* (Bilezikjian *et al.* 1996). In contrast, the expression of follistatin in the pituitary of rhesus monkey was stimulated by *in vitro* testosterone treatment (Kawakami *et al.* 2002). This discrepancy may account for the difference in the post-gonadectomy increase of FSH β expression and FSH secretion between the rats (2- to 3-fold; Gharib *et al.* 1987) and primates (40-fold; Attardi *et al.* 1992). Interestingly, activin has been reported to stimulate follistatin mRNA expression (Bilezikjian *et al.* 1996), synthesis (Farnworth *et al.* 1995), and release (Bilezikjian *et al.* 1993) from cultured pituitary cells, and the effects could be abolished by follistatin itself. These results point to the presence of a local regulatory feedback loop involving activin and follistatin in the pituitary (DePaolo *et al.* 1991), and this regulatory loop is subject to the regulation by external hormonal factors.

In the goldfish, both activin β_A and β_B have been cloned by our laboratory (Ge *et al.* 1997, Yam *et al.* 1999a), and our previous work has demonstrated that different from the situation in mammals, activin differentially stimulates goldfish FSH β but suppresses LH β expression, and its effects can be reversed by follistatin (Yam *et al.* 1999a, Yuen & Ge 2004). The inverse effects of activin on the expression of the two gonadotropins make goldfish a unique model for studying the role of activin in the differential regulation of FSH and LH. Similar to that in mammals, we have also shown that the transcript of activin β_B subunit, but less likely β_A , is expressed in the goldfish pituitary (Ge *et al.* 1997, Yam *et al.* 1999b, Lau & Ge 2005). This, together with the reports in mammals that follistatin is also expressed in the pituitary (Besecke *et al.* 1996, Bilezikjian *et al.* 1996), has led us to hypothesize that there may exist a local paracrine regulatory system involving activin and follistatin in the goldfish pituitary, and this system may serve as a regulatory point that relays various neuroendocrine and endocrine inputs to the pituitary to control pituitary hormone biosynthesis, especially that of gonadotropins. In support of this hypothesis would be the evidence for activin and follistatin response to potential factors that control pituitary function. The present study was therefore undertaken to characterize the activin–follistatin system in the goldfish pituitary, with particular emphasis on its regulation.

Materials and Methods

Experimental animal

Goldfish (*Carassius auratus*) of about 4–5 inches in body length were purchased from the local pet market and reared in 1000 l flow-through tanks with ample aeration for at least 1 week before use. The conditions for maintaining the fish were at 22 °C water temperature and 14 h light:10 h darkness photoperiod. All experiments were performed under the license from the Government of the Hong Kong Special Administrative Region and endorsed by the Animal Experimentation Ethics Committee of the Chinese University of Hong Kong. All fishes were anesthetized with tricaine methanesulfonate (MS222) before killing.

Reagents

Chemicals and enzymes were purchased from Sigma and Promega respectively unless otherwise specified. The medium M199 used in cell culture was obtained from Gibco Invitrogen. The Flp-In CHO cells for recombinant goldfish follistatin production were purchased from Invitrogen. Recombinant human follistatin was kindly provided by Dr A F Parlow (National Hormone and Peptide Program, National Institute of Diabetes & Digestive & Kidney Disease, CA, USA). Recombinant goldfish activin B was produced in our laboratory from an established CHO cell line, and one unit (U) is defined as the amount per ml that induces half maximal erythroid differentiation of F5-5 cells (ED₅₀). Estradiol (E₂) and testosterone (T) were purchased from Sigma. All drugs were first prepared as stocks and then diluted to specific working concentrations in culture medium before use.

Extraction of total RNA and reverse transcription

Goldfish tissues were collected and the total RNA from each tissue (10–30 mg) extracted in a 1.5 ml microtube containing 200 μ l TRI Reagent (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer's protocol with some modifications. Following homogenization of the tissue, chloroform (50 μ l) was added to each microtube, vortexed, and left at room temperature for 15 min. After centrifugation at 4 °C for 20 min, the aqueous supernatant was transferred to another tube containing 100 μ l isopropanol for precipitation (–20 °C, 10 min). The pellet was washed with 75% ethanol prepared with diethylpyrocarbonate (DEPC)-treated water and dissolved in 4 μ l RNase-free water. For RNA extraction from single pituitary, the same procedure was followed after the pituitary was homogenized in 200 μ l TRI Reagent. The RNA (1 μ g) was then subjected to reverse transcription in a total volume of 10 μ l containing 1 $\times$ Moloney murine leukemia virus (MMLV) first-strand buffer, 0.5 mM each dNTP, 0.5 μ g oligo-dT, and 100 U MMLV reverse transcriptase (Promega) at 42 °C for 2 h.

Cloning and sequencing of full-length cDNA encoding goldfish follistatin

A pair of degenerate primers (Primer 1 and Primer 2; Table 1) was designed based on the conserved amino acid sequences of follistatin from other species. A goldfish ovarian cDNA library was used as template for PCR amplification, which was performed in 1× PCR buffer, 0.2 mM each dNTP, 2.5 mM MgCl₂, 0.6 U *Taq* polymerase, and 0.2 μM each primer in a total reaction volume of 25 μl. The reaction was run for 31 cycles with the profile of 30 s at 94 °C, 30 s at 56 °C, and 1 min at 72 °C.

The fragment amplified from goldfish ovary was purified and cloned into pBluescript II KS (+) (Stratagene, La Jolla, CA, USA) by T/A cloning and sequenced. An antisense gene-specific primer (Primer 3; Table 1) was then designed to amplify the 5'-region of the cDNA from the goldfish brain using SMART RACE cDNA Amplification Kit (Clontech Laboratories). The RACE PCR was performed according to the manufacturer's instructions. The amplified 5'-cDNA end was cloned as described above and sequenced. A sense gene-specific primer (Primer 4; Table 1) was then designed based on the 5'-end sequence of the cloned 5'-RACE product to amplify and clone the full-length cDNA by 3'-RACE.

The sequences of both strands of the cloned cDNA were obtained by exonuclease III and mung bean nuclease deletion method. All the sequencing reactions were performed on Peltier Thermal Cycler 100 (MJ Research, San Francisco, CA, USA) using dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer, Foster City, CA, USA). The sequence was analyzed by ABI PRISM 310 Genetic Analyzer (Perkin-Elmer) followed by GenBank database search.

Production of recombinant goldfish follistatin

The Flp-In CHO Expression System (Invitrogen) was used to produce recombinant goldfish follistatin. Briefly, a pair of primers (Primer 5 and Primer 6; Table 1) was designed based on the sequence of the cloned cDNA, which flank the open reading frame (ORF) of goldfish follistatin. Two restriction sites, *Hind*III and *Bam*HI, were added to the two primers respectively for cloning into the expression vector, pcDNA/FRT. A Kozak sequence (CCACC) was included between *Hind*III and the initiation codon ATG to improve translation

efficiency (Kozak 1984). The protocol used for amplifying ORF was the same as described above except that *Pfu* polymerase (1.5 U) instead of *Taq* was used to increase fidelity of the reaction. The purified amplicon was digested with *Hind*III and *Bam*HI and subcloned into the double-digested pcDNA/FRT to obtain the expression construct pcDNA/FRT/goldfish follistatin (gfFS). To facilitate future purification of recombinant follistatin, we also constructed an expression plasmid that contained an insert with six His residues added at the carboxyl terminal (pcDNA/FRT/gfFS-His). The construction of pcDNA/FRT/gfFS-His was the same as that of pcDNA/FRT/gfFS except that the antisense primer had the codons for His residues incorporated.

The cultured Flp-In CHO cells at ~40% confluence were co-transfected with pcDNA/FRT/gfFS or pcDNA/FRT/gfFS-His and pOG44 at the ratio of 1:9 by Lipofectamine (Invitrogen). The plasmid pOG44 provides the recombinase essential for genomic integration. The transfected cells were cultured to confluence in Ham F12 medium containing 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum (HyClone Laboratories, Logan, UT, USA) at 37 °C with 5% CO₂. The cells were then trypsinized, washed, and plated in 10 cm culture dishes at the density of 1×10⁴/ml for selection with 450 ng/ml hygromycin B (Invitrogen). Individual clones were isolated by limited dilution in 96-well plates. The positive clones were identified by PCR on the genomic DNA and RT-PCR on the total RNA isolated from each clone.

Functional assay for recombinant goldfish follistatin

The biological activity of recombinant goldfish follistatin was assessed based on its inhibition of activin-induced differentiation of F5-5 cells, a mouse erythroleukemia cell line that responds to activin by differentiating into hemoglobin-producing cells (Eto *et al.* 1987, Schwall & Lai 1991). The assay protocol was according to Machida *et al.* (2000) with some modifications. Briefly, the F5-5 cells were cultured at 37 °C with 5% CO₂ in Ham F12 medium containing 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum (HyClone Laboratories). The cells were plated at a density of 2000 cells/400 μl/well in a 48-well plate. Hemoglobin production reached maximal level after incubation for 6 days with recombinant goldfish activin B. To assay the activity of follistatin produced, the conditioned media collected from

Table 1 Primers used in cloning goldfish follistatin and constructing pcDNA/Flp/rgFS

No.	Use	Strand	Sequence
1	Degenerate PCR	Sense	GAIGAIGAIGTICCIAA
2		Antisense	TCIGGIGCICAIACICA
3	5'-RACE	Antisense	CGCAGACGCAGCGAGGCTTACTCCT
4	3'-RACE	Sense	GGTTCATTGAGCACTGCCTGAGTA
5	Recombinant expression	Sense	CCCAAGCTTCCACCATGCAAAGGATGCTAAAGC
6		Antisense	CGCGGATCCTTACTTACAGTTGCAAGATCCTG'

untransfected and transfected CHO cells as well as individual clones at 80% confluence were added to the culture wells in the presence or absence of activin B (3 U/ml). Recombinant human follistatin was used as the positive control. After incubation for 6 days, the cells from each well were suspended, transferred to a 1.5 ml microtube, and stained by O-dianisidine/H₂O₂ solution for 15 min. After centrifugation to remove the supernatant, the pelleted cells were lysed with 100 µl H₂O followed by freezing at -80 °C for 1 h and further solubilized with 10% SDS at 37 °C for 30 min. The lysates were transferred to a 96-well plate and the absorbance at the wavelength of 405 nm was measured on SpectraMax 250 microplate spectrophotometer (GMI Inc., Albertville, MI, USA).

Primary culture of dispersed pituitary cells

The primary culture of goldfish pituitary cells was performed as described previously (Yam *et al.* 1999a). Briefly, the pituitaries were collected from both males and females, washed, minced into small pieces, and trypsinized to disperse the cells. The dispersed cells were seeded in 48-well plates at a density of 0.4–0.6 × 10⁶ cells/0.5 ml/well in 70% M199 supplemented with Earle’s salt and 10% fetal calf serum (Hyclone Laboratories) and incubated at 28 °C with 5% CO₂. The plates were pre-coated with 0.01% poly-D-lysine to enhance cell attachment. After pre-incubation for 24 h, the medium was changed before drug treatment.

Real-time RT-PCR assays

Total RNA from the cultured pituitary cells or individual pituitary gland was extracted with Tri Reagent and reverse transcribed as described above. The levels of gene expression were assayed with real-time RT-PCR. All primers used in the assays are listed in Table 2. To prepare standard for real-time

PCR assay, the cDNA fragment of each target gene was amplified and purified by phenol/chloroform extraction and ethanol precipitation. The amplicon was then quantified by electrophoresis on a 1.8% agarose gel together with serially diluted MassRuler DNA Marker (MBI Fermentas, Hanover, MD, USA). The analysis and quantification were carried out on the Gel-Doc 1000 system with Molecular Analyst software (Bio-Rad).

The real-time PCR assay was performed on the iCycler iQ Real-time PCR Detection System (Bio-Rad) in a volume of 30 µl containing varying amounts of RT reaction product, 1 × PCR buffer, 0.2 mM each dNTP, 2.5 mM MgCl₂, 0.2 µM each primer, 0.75 U Taq polymerase, SYBR Green I (1:35 000, Molecular Probes, Leiden, The Netherlands), and 20 nM fluorescein (Bio-Rad). The PCR profile consisted of an initial denaturation at 95 °C for 2 min, followed by 40 cycles of denaturation for 20 s, annealing at 56 °C for 30 s, and elongation at 72 °C for 30 s. An extra step (80 °C for 7 s) was inserted in each PCR cycle for signal detection. A melt-curve analysis was performed at the end of the reaction to assess assay specificity, which was also confirmed by electrophoresis. A standard curve was always included for monitoring assay quality and calculating the levels of gene expression in the samples.

Data analysis

Each experiment was repeated at least twice with each treatment performed in triplicate. Data of follistatin bioassays were expressed as absorbance at OD₄₀₅ and analyzed with one-way ANOVA followed by Dunnett’s test. For the experiments on gene expression, the expression levels of goldfish follistatin, activin β_B, FSHβ, LHβ, and growth hormone (GH) were normalized to β-actin, the internal control for RNA loading. Statistical analysis was performed with one-way ANOVA followed by Dunnett’s test or Student’s *t*-test using Prism 4.0 on Macintosh (GraphPad, San Diego, CA, USA). The differences were considered significant at *P* < 0.05 and very significant at *P* < 0.001.

Results

Cloning and sequence analysis of goldfish follistatin

A full-length cDNA (1228 bp) encoding goldfish follistatin was cloned by RACE. ORF analysis reveals that the cloned cDNA encodes a precursor protein with 323 amino acid residues (Fig. 1). GenBank database search showed that the deduced amino acid sequence had the highest homology with zebrafish follistatin (accession number: NM_131037; Fig. 1). The cloned goldfish follistatin lacks 27 conserved amino acids at the carboxyl terminal, similar to that of *Xenopus* and zebrafish, which is similar to the form of FS288 but different from FS315 reported in birds and mammals. The secretory nature of goldfish follistatin was suggested by the presence of a potential signal peptide at the amino terminal of the protein,

Table 2 Primers used in real-time PCR assays

No.	Gene	Strand	Sequence
7	β-actin (<i>bactin</i>)	Sense	TGCGGAATATCATCTGCCTGTA
8		Antisense	CAACAACCACTGCGGCAAT
9	Follistatin (<i>fst</i>)	Sense	CCACGTGCTTGCTTGGTAGA
10		Antisense	CCACGCTGCAATGGATATCA
11	Activin β _B (<i>inhbb</i>)	Sense	GACAAGACCTCGACATTCAGT
12		Antisense	CAGGAATGGACGGTGTGAGG
13	FSHβ (<i>fsbh</i>)	Sense	GCAAGTGTAAGTCTGACATCACAG
14		Antisense	GTTCCTTGCTAATGTGCATTGC
15	LHβ (<i>lhb</i>)	Sense	AGAGCCCATTTCCACTGTCTAC
16		Antisense	GAGAGCCACAGGTTAGGTGAT
17	GH (<i>gh</i>)	Sense	TACCCAGCCAGACCCTGAG
18		Antisense	CCATCGAGACTTCCCTTAATGAGC

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goldfish 1 MQRMLKROLLHSRMILL-LWLCYLTEDQKVQA GNCWLQQGKNGRCQVLYMPGMSREECC
zebrafish .L....Q..PG....-F....I.....
Xenopus ---.N-ERIQPG..F..TVS..HFM.YRA.....S.....RTEL.K....
chicken ---.NQRIHPGML-V..-MF.YHFM..HTA.....R.AR.....KTDL.K....
bovine ---.ARPRHQPGGLC...-L..QFM..RSA.....R.A.....KTEL.K....
horse ---.VRPRHQPGGLC...-L..QFM..RSA.....R.A.....KTEL.K....
mouse ---.VCARHQPGGLC...-L..QFM..RSA.....R.A.....KTEL.K....
human ---.VRARHQPGGLC...-L..QFM..RSA.....R.A.....KTEL.K....
* * * * *

goldfish 61 RSGRLGTSWTEEDVENSTLFRWMI FNGGAPNCIPCKETCDNVDCGPGCKKKCKMNRRSKPRC
zebrafish .....R.....
Xenopus KT.....K...H...H.....E.....KKN...
chicken K...T.....NDN...K.....E.....KKN...
bovine ST...S.....NDN...K.....E.....R..KKN...
horse ST...S.....NDN...K.....E.....R..KKN...
mouse ST...S.....NDN...K.....E.....R..KKN...
human ST...S.....NDN...K.....E.....R..KKN...
* * * * *

goldfish 121 VCAPDCSNITWKGSVCGSDGKTYRDECALLKSKCKGHPDLEVQYQGKCKKACHDVMCPGS
zebrafish .....V...P.....T.R..L...
Xenopus .....I....K.....A...V.E.D.....T.R..L...
chicken .....P...L.....N.....AR..EQ.E.....T.R..L...
bovine .....P...L.....N.....AR..EQ.E.....T.R..F...
horse .....P...L.....N.....AR..EQ.E.....T.R..N...
mouse .....P...L.....N.....AR..EQ.E.....T.R..F...
human .....P...L.....N.....AR..EQ.E.....R...T.R..F...
* * * * *

goldfish 181 STCVVDQTNNAVCVTCNCRICPEVTSPDSYLCGNDGIVYANACHLRATCLLGRSISVAYE
zebrafish .....M...Q.....I..S.....
Xenopus .S.....P...Q.....T.GS....K.....L...
chicken .....P...EQ.....T..S....K.....L...
bovine .....P...SEQ.....VT.PS....K.....L...
horse .....P...SEQ.....VT.SS....K.....L...
mouse .....PS.SEQ.....VT.SS....K.....L...
human .....PA.SEQ.....VT.SS....K.....L...
* * * * *

goldfish 241 GKCIKAKSCNDIHC SVGKKCLWDSKMGRGRCAVCMESCPESRSEAEVASCADNTTYPSECA
zebrafish .....D....A.....A..S.....A.....
Xenopus .....E..Q..A.....RV.....L.DDL.G..K.DDT.....
chicken .....E..Q..A.....F.V.....L.D.L...K.D.....
bovine .....D..Q.TG.....F.V.....SL.G.L...K...P....A..A...
horse .....E..Q.TG.....F.V.....SL.D.L..D.K..P....A..A...
mouse ....-T...E..Q.GG.....V.....SL.D.L..D.K.D.P....A..A...
human .....E..Q.TG.....F.V.....SL.D.L..D.K.D.P....A..A...
* * * * *

goldfish 301 MKQAACSLGVLLEVKHSGSCNCK-----
zebrafish .....
Xenopus .....T.I.....
chicken ..E...M.....SINEDPEEEEEDEDQDYSFPISILEW
bovine ..E...S.....SISEDTEEEEEDEDQDYSFPISILEW
horse ..E...S.....SISEDTEEEEEDEDQDYSFPISILEW
mouse ..E...S.....SISEETEEEEDEDQDYSFPISILEW
human ..E...S.....SISEDTEEEEEDEDQDYSFPISILEW
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Figure 1 Deduced amino acid sequence of goldfish follistatin and its comparison with the homologous protein from representative species of amphibians, birds, and mammals. The potential signal peptide, lysine-rich heparan-binding site, and two predicted *N*-glycosylation sites (NST, NIT) are boxed. The asterisks (*) indicate sequence identity and the dashes (—) represent the gaps inserted for better sequence alignment.

which contains a leucine-rich hydrophobic region (ILL-LLWLICYL) and a hydrophilic region (EDQKVQ) located just prior to the putative cleavage site (A/G). The possible cleavage site was identified between residues 33 and 34, rendering the secreted mature peptide 290 amino acids.

Mature goldfish follistatin shows 75% of overall sequence identity with the molecule from other vertebrates (accession numbers: *Xenopus*, A53502; chicken, S55369; cow, I45894; horse, BAA25699; mouse, NP_032072; and human, AAH04107; Fig. 1). The arrangement of 36 cysteine residues (Shimasaki *et al.* 1988a), which is critical in determining the 3D structure of follistatin by forming intra-molecular disulfide bonds, is fully conserved among all vertebrate species compared. A potential lysine-rich binding site (KKCKMNRRSK) for heparan sulfate proteoglycan on target cells (Inouye *et al.* 1992, Sumitomo *et al.* 1995) is present at the 76th residue of the mature protein. Two potential *N*-glycosylation sites are present at asparagines 43 (NST) and 92 (NIT) of the mature peptide, based on the consensus Asn-Xaa-Ser/Thr sequence, where Xaa can be any amino acid besides Pro and Asp (Walmsley & Hooper 2003).

Tissue distribution of follistatin expression in the goldfish

To examine the expression of follistatin in the goldfish, primers specific for follistatin were designed based on the sequence of the cloned cDNA. As shown in Fig. 2, a specific band of expected size (432 bp) was detected in all the tissues of the brain–pituitary–gonadal axis (brain, pituitary, ovary, and testis) although the expression level was relatively low in the pituitary. Expression of follistatin was also noted in the kidney and gill, but not in the liver. No product was observed in RT negative controls, which were the total RNA samples without being reverse transcribed. The expression of β -actin as the internal control could be detected in all the tissues tested.

Functional analysis of recombinant goldfish follistatin

After pcDNA/FRT/gfFS or pcDNA/FRT/gfFS-His was transfected into the Flp-In CHO cells, positive cells were selected and cloned in the presence of hygromycin B. The production and functional identity of goldfish follistatin were examined by the inhibition of activin activity in the erythroid differentiation factor (EDF) bioassay using F5-5 cells. One of the clones (gfFS-C3) that exhibited the best performance in suppressing activin activity was identified and expanded. The conditioned medium from gfFS-C3 significantly suppressed the activin effect on F5-5 cells with an ED_{50} of about 1.8 μ l/ml (Fig. 3). As the positive control, recombinant human follistatin (FS288) also suppressed activin activity with an ED_{50} of about 3.1 ng/ml (Fig. 3). The amount of recombinant goldfish follistatin in the medium collected after 2-day incubation was therefore equivalent to 1.7 ng/ μ l human follistatin. Interestingly, addition of the His-tag at the carboxyl terminal appeared to abolish the activity of the recombinant protein because the selected pcDNA/FRT/

gfFS-His-transfected cells produced no bioactivity of suppressing activin although the mRNA was abundantly transcribed from the insert (data not shown).

Activin regulation of follistatin expression in the pituitary

Follistatin is a potent activin-binding protein and its expression has been demonstrated to be regulated by activin in a few systems (Farnworth *et al.* 1995, Dalkin *et al.* 1996, Besecke *et al.* 1996, Bilezikjian *et al.* 1996). To examine whether activin has any influence on the expression of follistatin in the goldfish pituitary, the cultured pituitary cells were exposed to different concentrations of goldfish activin B for 48 h after 24-h pre-incubation as described previously (Yuen & Ge 2004). Activin B significantly stimulated the expression of follistatin in a dose-dependent manner, and this effect could be blocked by co-incubation with recombinant human follistatin (Fig. 4A). Furthermore, treatment with human follistatin alone at 100 ng/ml nearly abolished the basal expression of goldfish follistatin in cultured pituitary cells (Fig. 4A).

We also analyzed the expression responses of FSH β and LH β in the same samples. As shown in Fig. 4B, activin significantly up-regulated FSH β but inhibited LH β expression, while treatment with human follistatin resulted in opposite effects on the expression of the two gonadotropin subunits, consistent with our previous report (Yuen & Ge 2004). The conditioned medium from gfFS-C3 cells (recombinant goldfish follistatin) and recombinant human follistatin showed the same effects on the expression of FSH β , LH β , and follistatin itself (Fig. 5).

Effects of sex steroids on activin and follistatin expression in the pituitary

The dispersed goldfish pituitary cells were treated with various doses of testosterone or E_2 for 48 h after pre-incubation. Treatment with testosterone (1–100 nM) stimulated pituitary follistatin expression in a dose-dependent manner with the maximal response observed at 100 nM. Similarly, E_2 also stimulated follistatin expression. Interestingly, none of the steroids had any effect on the expression of activin β_B (Fig. 6A). We also examined the responses of FSH β and LH β expression to the steroids in the same samples. The expression of LH β was significantly up-regulated by both testosterone and E_2 with the maximal stimulation being observed at the highest concentration (100 nM). However, the expression of FSH β showed little response to either testosterone or E_2 (Fig. 6B).

Annual expression profiles of follistatin, activin β_B , FSH β , LH β and GH in the pituitary

The results described above suggested that there existed an activin–follistatin system in the goldfish pituitary and that follistatin could be the point of the system that is subject to regulation by endocrine and paracrine inputs. To provide

Figure 2 Tissue distribution of follistatin expression in the goldfish. Follistatin is expressed in all the tissues of the hypothalamic-pituitary-gonadal axis. +, reverse transcription with MMLV; -, reverse transcription without MMLV.

further evidence for this hypothesis, we examined the annual expression profiles of follistatin, activin β_B , FSH β , and LH β in the individual pituitary glands during a 12-month reproductive cycle. Since GH may have potential roles in vertebrate reproduction (Hull & Harvey 2002), we also included GH in this study. In the spawning period (February and March), both FSH β and LH β significantly increased their expression

Figure 3 Detection of recombinant goldfish follistatin with EDF assay. The F5-5 cells were induced to differentiate into hemoglobin-producing cells by recombinant goldfish activin B (3 U/ml). The differentiation was suppressed by the conditioned medium from rgFS-C3 (A) or recombinant human follistatin (B) in a dose-dependent manner. The ED₅₀ was estimated to be 3.1 ng/ml and 1.8 µl/ml for human follistatin and rgFS-C3 conditioned medium respectively. ***P* < 0.001 versus control.

Figure 4 Effects of activin and follistatin on the expression of activin β_B (*inhbb*), follistatin (*fst*), FSH β (*fshb*), and LH β (*lhb*) in cultured goldfish pituitary cells. The cells were pre-incubated for 24 h followed by 48-h treatment. The expression level of each gene was assayed by real-time RT-PCR and is expressed as the percentage of control (% control) after normalization with β -actin (*bactin*). Each value represents the mean $\pm$ s.e.m. of independent samples in four replicates. **, ##*P* < 0.001 versus control.

levels, which remained high in the post-spawning period (April and May). Their expression levels dropped abruptly in June and remained low in the seasons afterward. These profiles were similar to those reported previously in the same

Figure 5 Effects of recombinant human follistatin (100 ng/ml) and recombinant goldfish follistatin (50 µl/ml) on the expression of follistatin, FSHβ, and LHβ in the cultured pituitary cells. The cells were pre-incubated for 24 h before 48-h treatment. The expression level is expressed as the percentage of control (% control) after normalization with β-actin. Each value represents the mean ± S.E.M. of independent samples in four replicates. **P* < 0.05 versus control; ***P* < 0.001 versus control.

species (Sohn *et al.* 1999). The GH showed relatively high expression levels in both the spring and autumn and low levels in the summer (June to August); however, the differences were not statistically significant. As demonstrated *in vitro*, the expression level of activin βB remained rather stable throughout the reproductive cycle with no significant trend of changes. In contrast, follistatin expression in the pituitary showed significant variation in the annual reproductive cycle with the highest levels detected from August to November (Fig. 7).

Discussion

A full-length cDNA encoding follistatin has been cloned from the goldfish using PCR and RACE approach. Goldfish follistatin exhibits as much as 95% sequence identity with that of zebrafish (Bauer *et al.* 1998) and about 75% with the homologous protein of other vertebrates (Inouye *et al.* 1991, Tashiro *et al.* 1991, Connolly *et al.* 1995), suggesting conserved functions of this protein in vertebrates. In mammals and birds,

Figure 6 Effects of estradiol and testosterone on the expression of activin βB, follistatin, FSHβ, and LHβ in cultured goldfish pituitary cells. The cells were pre-incubated for 24 h followed by 48-h treatment. The expression level of each gene is expressed as the percentage of control (% control) after normalization with β-actin. Each value represents the mean ± S.E.M. of independent samples in four replicates. **P* < 0.05, ***P* < 0.001 versus control.

Figure 7 Seasonal expression profiles of pituitary FSH β , LH β , GH, activin β_B , and follistatin in female goldfish. Pituitary glands were collected each month for real-time RT-PCR analysis. The expression levels were normalized with β -actin. The annual profile of gonadosomatic index (GSI) is shown at the bottom to indicate the ovarian stage of the fish sampled. Each value represents the mean $\pm$ S.E.M. ($n=9-16$). ** $P<0.001$ versus January.

two differentially spliced forms of follistatin, FS315 and FS288, have been reported (Shimasaki *et al.* 1988a,b, Michel *et al.* 1990, Inouye *et al.* 1991), and the short form, FS288, which lacks 27 amino acids at the carboxyl terminus binds activin with 8–10 times higher potency than its full-length counterpart, FS315 (Inouye *et al.* 1991). The cloned goldfish follistatin also lacks

these 27 amino acids at the carboxyl terminus compared with the form of FS315 in birds and mammals but possesses two extra amino acids (CK). We attempted to illustrate the presence of goldfish counterpart of FS315 using a pair of gene-specific primers designed at the carboxyl terminus flanking the location of the 27 residues; however, no specific product could be amplified (data not shown), which may suggest the lack of FS315 form in teleosts.

The functional identity of the cloned goldfish follistatin homolog was confirmed by establishing a stable CHO cell line (gfFS-C3) that expressed the protein. The conditioned medium from the cloned gfFS-C3 cells significantly suppressed activin-induced F5-5 cell differentiation, while the control CHO cells produced no follistatin activity (data not shown).

Although follistatin was first isolated from the follicular fluid (Esch *et al.* 1987, Robertson *et al.* 1987, Ueno *et al.* 1987), its expression has been shown in other locations of the brain–pituitary–gonadal axis as well as in a variety of nonreproductive tissues (Michel *et al.* 1990). RT-PCR analysis in the present study showed that goldfish follistatin also had widespread tissue distribution except for the liver, which correlates well with the ubiquitous expression of activin subunits in the goldfish (Lau & Ge 2005). The co-expression of follistatin and activin in various tissues supports the idea that follistatin may be a critical factor in fine-tuning local activities of activin in the target tissues. It is noteworthy that although activin subunits and its receptors were detected in the goldfish liver in our previous studies (Ge *et al.* 1997, Yam *et al.* 1999b), the expression of follistatin was undetectable there, which agrees well with a previous study in the rat (Michel *et al.* 1990).

Similar to reports in mammals (Gospodarowicz & Lau 1989, Kogawa *et al.* 1991), the present and our previous studies also demonstrated co-expression of activin and follistatin in the goldfish pituitary (Lau & Ge 2005), suggesting an intra-pituitary activin–follistatin regulatory loop across vertebrates. Since activin stimulates goldfish FSH β but inhibits LH β expression in the goldfish (Yam *et al.* 1999a, Yuen & Ge 2004), the co-expression of activin and follistatin in the pituitary has led us to hypothesize that the relative expression levels of FSH and LH are likely influenced by the balance between activin and follistatin in the pituitary, and any factors that disturb such balance would potentially regulate the expression of the two gonadotropins. An interesting question that follows would be how activin subunits and follistatin in the goldfish pituitary are regulated.

To address this question, we first investigated the effect of activin on follistatin expression in cultured goldfish pituitary cells. In agreement with our previous finding (Yam *et al.* 1999a), goldfish activin B exhibited differential effects on FSH β and LH β expression. In contrast, both human follistatin and the conditioned medium of gfFS-C3 cells that produced recombinant goldfish follistatin caused opposite responses of FSH β and LH β , and these effects were likely due to the

neutralization of endogenous activin in the pituitary. Interestingly, treatment with activin also significantly increased the expression of pituitary follistatin, therefore supporting the existence of a closed activin–follistatin regulatory loop in the pituitary (Fig. 8). The increased follistatin expression in response to activin may help keep the local activities of activin in check. In mammals, it has also been reported that addition of activin to cultured pituitary cells induced parallel increases of follistatin and FSH secretion in the rat (Bilezikjian *et al.* 1993) and sheep (Farnworth *et al.* 1995). The stimulatory effect of activin on follistatin has also been demonstrated at the transcriptional level (Besecke *et al.* 1996, Bilezikjian *et al.* 1996, Dalkin *et al.* 1996).

As a binding protein that modulates the activities of activin in various tissues, increasing evidence points to follistatin as a critical target of the activin–follistatin system that is subject to endocrine and neuroendocrine regulation. To test this hypothesis, we went on to examine the effects of gonadal steroids (testosterone and E₂) on the expression of follistatin and activin β_B , which is the main form of activin subunit expressed in the goldfish pituitary (Ge *et al.* 1997, Yam *et al.* 1999b, Lau & Ge 2005). Both testosterone and E₂ significantly increased follistatin and LH β expression in cultured pituitary cells within the physiological range of concentration. However, neither testosterone nor E₂ had any effect on the expression of activin β_B and FSH β . Similar studies have also been performed in some

mammalian models, but the results have somehow been inconsistent. Testosterone treatment stimulated follistatin expression in the pituitary of rhesus monkey, *Macaca mulatta* (Kawakami *et al.* 2002), whereas its expression was suppressed by steroids in the rat (Besecke *et al.* 1996, Leal *et al.* 2002).

The increased expression of follistatin but not activin β_B in response to steroid treatments suggests that it is likely follistatin that serves as a regulatory point of the activin–follistatin system in the pituitary. Although activin β_B level remains rather constant, the activities of activin are expected to be fine-tuned by the local level of follistatin in the pituitary. In support of this hypothesis is the *in vivo* evidence that similar to FSH β and LH β , follistatin in the female goldfish pituitary exhibited significant changes in expression during the annual reproductive cycle whereas activin β_B showed no evident trend of variation. Although it is difficult to assess the importance of follistatin variation in the annual expression profiles of gonadotropins (FSH and LH) at this moment because of the multifactorial nature of FSH and LH regulation, this result at least lends further weight to the hypothesis that follistatin in the pituitary may represent a critical regulatory point.

Both testosterone and E₂ stimulated LH β expression but lacked effect on FSH β expression in the present study. In the goldfish, previous studies using incubated pituitary fragments demonstrated stimulatory effects of testosterone and E₂ on the expression of both FSH β and LH β ; however, FSH β appeared to be less responsive to E₂ treatment than LH β (Huggard *et al.* 1996, Habibi & Huggard 1998, Huggard-Nelson *et al.* 2002). The *in vitro* effect of E₂ on gonadotropin subunit expression, especially that of FSH β , seemed to be dependent on the stage of sexual maturity. E₂ significantly stimulated FSH β expression in cultured pituitary fragments from early but not late recrudescence (vitellogenic) fish (Huggard-Nelson *et al.* 2002). This stage dependence could also be the reason for the lack of effects of steroids on FSH β in the present study because we performed the experiments in February, when the goldfish in Southern China was near the peak spawning period. Indeed, in a previous unpublished study using slot blot hybridization, we did observe significant stimulatory effects of testosterone and E₂ on both FSH β and LH β expression in cultured pituitary cells from sexually regressed goldfish (Yuen & Ge unpublished data). In contrast to the variable response of FSH β to steroids, both testosterone and E₂ consistently stimulated goldfish LH β expression in the present as well as our previous unpublished studies. Although these stimulatory effects could be due to direct actions of these steroids on LH cells, the increase might also be linked to the increased expression of follistatin, which could in turn reduce the inhibitory effect of activin on LH β expression. The same speculation does not seem to be applicable to FSH β , whose expression would be expected to decrease in response to an increased follistatin expression. In mammals, the expression level of FSH β has been reported to correlate well with that of pituitary follistatin (Bilezikjian *et al.* 1996, Kawakami *et al.* 2002, Leal *et al.* 2002). One possibility is that goldfish FSH β

Figure 8 Hypothetical model for the role of follistatin in the goldfish pituitary. As an activin-binding protein that fluctuates during annual reproductive cycle and responds to both activin and gonadal steroids, follistatin is likely a critical regulatory point that receives and relays both neuroendocrine and endocrine inputs in the pituitary. Its level in turn fine-tunes the bioavailability of activin that influences multiple pituitary hormones including FSH and LH.

may be subject to dual controls by both the intra-pituitary activin–follistatin system and gonadal steroids, and the relatively stable FSH β expression in response to steroidal treatments may reflect the net effect of reduced activin stimulation due to the increased follistatin and an increased steroidal stimulation. What is intriguing is that in addition to activin, follistatin has also been reported to bind some members of the bone morphogenetic protein (BMP) family such as BMP-4 and BMP-7 (Iemura *et al.* 1998), and some BMP members such as BMP-6 and BMP-7 have also been shown to exert a paracrine effect in the pituitary of mammals to regulate gonadotropin expression (Huang *et al.* 2001). How these BMP ligands fit into the activin–follistatin system in the goldfish pituitary remains to be elucidated.

To summarize, a full-length cDNA for goldfish follistatin was cloned and its expression demonstrated in the pituitary. Further experiments showed that in addition to the differential effects on the expression of goldfish FSH β and LH β , activin also stimulated the expression of its binding protein follistatin within the pituitary, which may represent a local closed-loop negative feedback mechanism to modulate the activities of activin. This mechanism seems to be conserved across vertebrates, suggesting physiological importance of the intra-pituitary activin–follistatin system in controlling pituitary hormone biosynthesis and secretion. Furthermore, the expression of follistatin but not activin itself was regulated by sex steroids, suggesting that it is likely follistatin that serves as the point of the system that is subject to the endocrine or neuroendocrine regulation (Fig. 8). This idea is further supported by the *in vivo* evidence that the expression of follistatin in the pituitary varied significantly, similar to that of FSH β , LH β , and GH, whereas activin β_B expression remained rather constant during the annual reproductive cycle.

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