Characterization of inhibin α subunit (*inha*) in the zebrafish: evidence for a potential feedback loop between the pituitary and ovary

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Abstract

Inhibin and activin are closely related disulphide-linked dimers that belong to the transforming growth factor β superfamily. Although inhibin has been extensively studied in mammals, the information about its existence and function in lower vertebrates is very scarce. Using zebrafish as a model, the present study demonstrated that the inhibin-specific α subunit (*inha*) was predominantly expressed in the gonads and no transcript could be detected in other tissues including the pituitary and brain. In the ovary, the expression of *inha* was restricted to the somatic follicle cells surrounding the oocyte, together with the β subunits (*inhbaa* and *inhbb*). This was further supported by the absence of its expression in the ovulated unfertilized eggs. During folliculogenesis, *inha* expression in the follicles slightly but steadily increased from primary growth to the mid-vitellogenic stage; however, its expression surged dramatically at the full-grown stage. Interestingly, the expression level of *inha* decreased significantly in the follicles whose oocytes were undergoing spontaneous maturation or germinal vesicle breakdown. When tested on cultured ovarian fragments, both goldfish pituitary extract and forskolin significantly stimulated *inha* expression. Further experiments showed that recombinant zebrafish FSH but not LH significantly increased *inha* expression in the same assay system. When tested *in vitro*, human inhibin A exhibited a slight but significant inhibitory effect on 17 α , 20 β -dihydroxyprogesterone-induced oocyte maturation after 4 h incubation. The stimulation of *inha* expression by FSH and the potential inhibition of FSH by inhibin suggest a possible existence of a negative feedback loop between the pituitary and ovary in the zebrafish.

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Introduction

In the past two decades, the characterization of the inhibin–activin–follistatin system has been considered one of the most important developments in the field of reproductive endocrinology. Inhibin was first purified in 1985 from the ovarian follicular fluid of mammals for its specific inhibitory effect on the secretion of FSH but not LH in the pituitary gland (Ling *et al.* 1985, Miyamoto *et al.* 1985, Rivier *et al.* 1985, Robertson *et al.* 1985). This was followed by the identification of activin in the ovary in 1986 (Ling *et al.* 1986, Vale *et al.* 1986) and follistatin in 1987 (Ueno *et al.* 1987). While follistatin was first found to have a similar inhibin-like effect on FSH secretion, activin exhibited an opposite effect by specifically stimulating FSH but not LH release *in vitro* (Ying 1988). Subsequent studies have provided extensive evidence that inhibin, activin, and follistatin function as an integral regulatory system with activin being the central player of the system whereas inhibin and follistatin serving as its antagonist and binding protein respectively (Ying 1988, Ge 2000, de Kretser *et al.* 2002, Welt *et al.* 2002).

Inhibin and activin are both members of the transforming growth factor β (TGF β) superfamily and they are structurally related by sharing a common β subunit (mostly β A or β B). Inhibin is a heterodimer consisting of a β subunit and a specific α subunit, whereas activin is a homo- or heterodimer of two β subunits (Ying 1988). It is generally accepted now that inhibin functions as a classical endocrine hormone from the gonads to regulate FSH secretion at the pituitary, whereas activin is mainly produced as a local paracrine factor in multiple tissues (Wiater & Vale 2008). Activin acts by first binding to a specific type II receptor (II or IIB) followed by recruitment of a type I receptor (I or IB) for signaling (Pangas & Woodruff 2000). In contrast, inhibin does not have its own signaling receptor and it works largely as an antagonist to compete with activin for the type II activin receptors (Wiater & Vale 2008). The high potency of inhibin in competing for activin receptors is due to the involvement of an inhibin co-receptor, betaglycan (also termed TGF β type III receptor), which helps to deliver inhibin to activin type II receptors (Lewis *et al.* 2000).

With the action mode of the inhibin–activin–follistatin system elucidated, studies on this important regulatory system have been largely focusing on activin, covering a wide range of organisms including fish (Ge *et al.* 1997b, Ge 2000), amphibians (Asashima 1994, Fukui *et al.* 1994), birds (Davis *et al.* 2001, Schmierer *et al.* 2003), and mammals (Ying 1987, Bilezikjian *et al.* 2004). Activin has even been shown to exist in invertebrates as well, such as *Drosophila* (Haerry & O'Connor 2002). In contrast, most studies on inhibin have been restricted to mammals, particularly the rodents. In lower vertebrates, particularly fishes whose reproductive physiology is best studied in non-mammalian vertebrates, inhibin has been largely ignored despite its functional importance in mammalian reproduction.

In fish, there have been several studies exploring the existence of inhibin and its actions in reproduction. However, all these studies used heterologous inhibins from mammals, and the results were somehow confusing and inconsistent. In 1992, we first reported a stimulatory effect of porcine inhibin on the secretion of goldfish LH (GTH-II) *in vitro* (Ge *et al.* 1992). A recent study in the zebrafish showed that human inhibin could stimulate final oocyte maturation *in vitro*, similar to the effect of activin (Wu *et al.* 2000). In contrast, porcine inhibin suppressed oocyte maturation in the killifish, which was opposite to the effect of activin (Petrino *et al.* 2007). Other than these bioassay studies, there has been no systematic characterization of inhibin molecule itself or its specific α subunit in any fish species. We made several attempts to clone and characterize the inhibin-specific α subunit in both goldfish and zebrafish, but to no avail, largely because of the high sequence variation of the α subunit. Tada *et al.* (2002) reported, for the first time, the cloning of a cDNA coding for an α -like molecule in the rainbow trout; however, there has been no follow-up study to further characterize inhibin in this species.

Recently, with the zebrafish genome project close to completion, we have identified a homolog of inhibin α subunit (*inha*) in the zebrafish genome database by *in silico* data mining. Based on this sequence, we undertook the present study with the aim of characterizing *inha* in the zebrafish ovary in terms of its spatial and temporal expression profiles, regulation of expression, and potential function.

Results

Sequence characterization of zebrafish *inha*

The precursor of zebrafish *inha* consists of 347 amino acids and the mature peptide is located at the C-terminus with 111 amino acids, which shares 42% sequence identity with its counterparts in other vertebrates (mammals and chicken). Among the

three fish species whose *inha* sequences are available, the amino acid sequence identity over the mature region is 60%. All seven cysteine residues characteristic of inhibin α subunit are fully conserved across vertebrates. Interestingly, a 17-amino acid sequence present in mammalian inhibin α subunits (69–85 in humans) is deleted in fish and chicken molecules. Also, in the zebrafish and killifish, a conserved N-linked glycosylation site (36 in humans) is not present; instead, a new potential glycosylation site appears at position 56 in the zebrafish and 61 in the killifish (Fig. 1).

Figure 1 Comparison of zebrafish *Inha* amino acid sequence with those of other vertebrates. (A) Sequence alignment over the mature region. The amino acids conserved in most species compared are boxed in dark whereas the similar amino acids are boxed in grey. The seven characteristic cysteine residues and the N-linked glycosylation sites are marked by asterisk (*) and symbol # respectively. (B) Phylogenetic relationship of zebrafish *inha* with its counterparts in selected vertebrate species (rainbow trout, NP_001117672; killifish, AW02847; chicken, NP_001026428; mouse, NP_034694; rat, NP_036722; human, NP_002182). Zebrafish inhibin/activin β A subunit (*inhbaa*; BC066402) was used as the outlier. The analysis was performed by MacVector using the UPGMA method based on the entire sequence of the precursor proteins. The numbers at the forks are the bootstrap proportions.

Tissue distribution of *inha* expression

It is well documented in both mammals (Meunier *et al.* 1988) and fish (Ge *et al.* 1997a, Lau & Ge 2005) that the β subunits (β A and β B) of activin and inhibin are expressed in a wide range of gonadal and extragonadal tissues. To localize the production of inhibin in the zebrafish, we examined a variety of tissues, including the pituitary, brain, gill, ovary, testis, liver, kidney, and muscle, for *inha* expression using semi-quantitative qPCR assay. Unlike β A (*inhbaa*, one of the two β A isoforms in the zebrafish) and β B (*inhbb*) subunits, *inha* was found to be predominantly expressed in the ovary and testis using 26 cycles of amplification, and no signal could be detected in any extragonadal tissues examined under the same experimental condition (Fig. 2A).

Localization of *inha* expression within the ovarian follicle

Similar to those in other teleosts, the ovarian follicle of the zebrafish has a thin layer of somatic follicle cells surrounding the oocyte, which presumably include both granulosa and theca cells. This makes it extremely difficult to separate the follicle layer from the oocyte. As the granulosa cells are the main production sites of inhibin in most mammals based on mRNA and protein localization (Findlay *et al.* 2001), it would be interesting to examine whether this also holds true in the zebrafish. To detect the expression of *inha* in different compartments of the follicle, we managed to separate the somatic follicle layer from the full-grown (FG) oocyte followed by RNA extraction from each compartment for RT-PCR detection.

To ensure a clean separation of follicle layers from oocytes, we used LH receptor (*lhcr*) and growth differentiation factor 9 (*gdf9*) as markers for the follicle layers and oocytes, respectively, as we previously demonstrated (Wang & Ge 2003, Liu & Ge 2007). As predicted, signals of *lhcr* and *gdf9* were only detected in the follicle layers and oocytes, respectively, indicating no cross contamination of the two follicular compartments. RT-PCR amplification of *inha* transcript demonstrated the expression of the molecule in the somatic follicle layers but not the oocytes under the experimental condition described in the Materials and Methods (Fig. 2B).

Further evidence for *inha* mRNA localization in the follicle layer came from the experiment on the ovulated oocytes (Fig. 2C). During the process of natural ovulation, the oocytes were freed from the surrounding somatic follicle layers through proteolytic enzymes-induced follicle rupture and released into the ovarian cavity in teleosts (Ogiwara *et al.* 2005). Both real-time (Fig. 2D) and semi-quantitative (Fig. 2E) qPCR on these ovulated oocytes freshly isolated from the ovary showed no expression of *inha*, in contrast to the intact follicles with follicle layers.

Figure 2 Spatial distribution of zebrafish *inha* expression.

(A) Semi-quantitative detection of *inha* expression in the pituitary, brain, gill, ovary, testis, liver, kidney, and muscle in the zebrafish. +, RT with reverse transcriptase; -, RT without reverse transcriptase. (B) Localization of *inha* expression within the FG follicle. The somatic follicle layer was separated from the oocyte followed by RNA extraction and semi-quantitative PCR detection in the two compartments (somatic follicle layer versus denuded oocyte). Each sample represents the total RNA pooled from 5 to 10 follicles. (C) Immature full-grown follicles (FG) and ovulated mature eggs (OE). (D) Real-time qPCR measurement of *inha* expression levels in FG follicles and ovulated eggs. ** $P < 0.01$ versus FG follicle. (E) Semi-quantitative qPCR on immature FG follicles and ovulated eggs. The housekeeping gene *gapdh* was used as the internal control for all samples, whereas *gdf9* and *lhcr* were used as the markers for denuded oocytes and somatic follicle layers respectively.

Stage-dependent expression of *inha* in the ovarian follicles

Real-time and semi-quantitative qPCR were performed to assess the relative levels of *inha* expression in the ovarian follicles of different developmental stages from primary growth (PG) to FG. The expression of *inha* was undetectable or extremely low in the follicles of the PG stage, but it started to appear at the pre-vitellogenic (PV) stage and progressively increased through the mid-vitellogenic (MV) stage. Interestingly, its level surged dramatically at the FG stage prior to final oocyte maturation (Fig. 3A).

Figure 3 Temporal expression profiles of *inha* during folliculogenesis and *in vitro* incubation. (A) Stage-dependent expression of *inha* in follicle development. The expression levels at different developmental stages were determined by real-time qPCR (mean $\pm$ S.E.M., $n=3$). Different letters indicate statistical significance ($P<0.05$). The gel image for semi-quantitative qPCR detection is shown on the top. PG, primary growth (Stage I); PV, pre-vitellogenic (Stage II); EV, early-vitellogenic (early Stage III); MV, mid-vitellogenic (mid-Stage III); FG, full-grown stage (late Stage III). (B) Expression of *inha* in spontaneously mature FG follicles *in vitro*. The immature FG follicles (GVBD (-)) and follicles undergoing germinal vesicle breakdown (GVBD (+)) after 6 h incubation were isolated followed by real-time qPCR quantitation of *inha* expression (mean $\pm$ S.E.M., $n=5$). ** $P<0.01$ versus immature follicle. (C) Temporal change of basal *inha* expression in the ovarian fragments cultured *in vitro*. The ovarian fragments were sampled at different times of incubation up to 24 h followed by real-time qPCR quantitation of *inha* expression (mean $\pm$ S.E.M., $n=3$). Different letters indicate statistical significance ($P<0.05$).

We also examined the change of *inha* expression during the final stage of follicle development, i.e. the maturation or meiotic resumption. In this experiment, the immature FG follicles were incubated *in vitro* for 6 h during which some follicles underwent spontaneous maturation or germinal vesicle breakdown (GVBD). These spontaneously matured but non-ovulated follicles were collected for analyzing *inha* expression as compared with that in the immature follicles. Interestingly, the expression of *inha* in the mature follicles undergoing GVBD dropped significantly by about 70% (Fig. 3B).

Temporal change of basal *inha* expression in cultured ovarian fragments

As illustrated above, zebrafish *inha* expression showed a significant trend of increase during follicle development with the highest level detected in the FG stage. This observation prompted us to hypothesize that *inha* expression must be vigorously regulated during folliculogenesis. To develop an *in vitro* bioassay for studying *inha* regulation, we incubated zebrafish ovarian fragments of mixed stages for 5 days without any treatment to monitor the change of basal *inha* expression during the incubation. We adopted ovarian fragment incubation because it minimized the disruption of the ovarian structure and therefore maintained the original spatial relationship of follicles of different stages. Interestingly, *inha* expression plunged dramatically to a very low level after 1 day incubation and continued to decline afterwards to barely detectable level (data not shown). Further experiments showed that within a 24-h incubation period, *inha* expression dropped significantly by nearly 80% in the first 3 h (Fig. 3C). Based on this result, all the following experiments on *inha* regulation using ovarian fragments were carried out within the first 24 h of incubation.

Effects of pituitary extract on the expression of *inha*

The expression profile of zebrafish *inha* during folliculogenesis showed a similar change to those of gonadotropin receptors (*fshr* and *lhcr*; Kwok *et al.* 2005), which prompted us to speculate that the pituitary gonadotropins are probably involved in its regulation. To investigate the effects of gonadotropins on *inha* expression, we adopted the *in vitro* ovarian fragment incubation described above and real-time qPCR to assess the response of *inha* expression. In the time course experiment, all groups of ovarian fragments were cultured for the same period of 24 h. The goldfish pituitary extract was added at different times towards the end of incubation. Different groups therefore received different durations of treatment before sampling at the end of the 24-h incubation period while the

Figure 4 Effect of goldfish pituitary extract on the expression of *inha* in cultured ovarian fragments containing all stages of follicles. (A) Time course of *inha* response to the pituitary extract (30 µg/ml). The ovarian fragments were incubated for 24 h for all groups; however, the pituitary extract was added at different times towards the end of incubation. Therefore, all samples were incubated for the same period while exposed to the treatment for different times. (B) Dose-response of *inha* stimulation by the pituitary extract. The ovarian fragments were treated by different doses of the extract for 24 h followed by RNA extraction and real-time qPCR quantitation. The values are the mean $\pm$ S.E.M. ($n=3-4$), and different letters indicate statistical significance ($P<0.05$).

control group received no treatment. The goldfish pituitary extract (30 µg/ml) stimulated *inha* expression effectively in a time-dependent manner with the maximal effect achieved at 24 h of treatment (Fig. 4A). In the dose-response experiment, treatment of the ovarian fragments with pituitary extract for 24 h upregulated the expression of *inha* in a clear dose-dependent manner, with the significant effect reached at 30 µg/ml (Fig. 4B). Although *inha* had the highest expression at FG stage, the effect of pituitary extract did not seem to be restricted to FG follicles because ovarian fragments with the FG follicles removed also responded significantly to pituitary extract treatment (data not shown).

Effects of recombinant zebrafish FSH and LH on *inha* expression

The experiment with goldfish pituitary extract strongly implicated gonadotropins in the upregulation of *inha* expression. To identify the gonadotropin involved, we further investigated the effects of recombinant zebrafish FSH (zfFSH) and LH (zfLH) as well as the LH analog

human chorionic gonadotropin (hCG) on *inha* expression. As shown in Fig. 5A, treatment with zfFSH (160 µl/ml) for 24 h significantly stimulated *inha* expression, similar to the effect of pituitary extract (30 µg/ml). In contrast, neither zfLH (160 µl/ml) nor hCG (20 IU/ml) had any significant effect (Fig. 5A). In the dose-response experiment, treatment of ovarian fragments with zfFSH for 24 h, consistently upregulated the expression of *inha* in a clear dose-dependent manner (Fig. 5B). To ensure that the two gonadotropins (zfFSH and zfLH) used were equivalent in their bioactivities, we also examined them in a parallel test with the receptor-based reporter assays as previously published (Kwok *et al.* 2005, So *et al.* 2005). As shown in Fig. 5C, both zfFSH and zfLH could stimulate their cognate receptors to the same extent (Fig. 5C).

Figure 5 Gonadotropin regulation of *inha* expression in cultured ovarian fragments. (A) Real-time qPCR quantitation of the effects of zfFSH (160 µl/ml), zfLH (160 µl/ml), human chorionic gonadotropin (hCG; 20 IU/ml) and pituitary extract (PE; 30 µg/ml) on *inha* expression after 24-h incubation (mean $\pm$ S.E.M., $n=3$). (B) Dose-response of zfFSH effect on *inha* expression *in vitro*. The ovarian fragments were treated with zfFSH at different doses for 24 h followed by RNA extraction and real-time qPCR quantitation (mean $\pm$ S.E.M., $n=3$). (C) Confirmation of the bioactivities of zfFSH and zfLH by the receptor-based assays. Briefly, the zebrafish receptors for FSH (zfFshr) and LH (zfLhcgr) were transiently overexpressed in the COS cells together with a cAMP-responsive reporter secreted alkaline phosphatase (SEAP). The cells were treated with the conditioned media containing zfFSH and zfLH as well as hCG as the control followed by measurement of SEAP activity in the medium. (D) Effects of forskolin (FK) on *inha* expression *in vitro*. The ovarian fragments were treated with forskolin (10 µM) and pituitary extract (PE, positive control) for 24 h followed by RNA extraction and real-time qPCR quantitation (mean $\pm$ S.E.M., $n=4$). Different letters indicate statistical significance ($P<0.05$). ** $P<0.01$ versus control.

Effect of forskolin on the expression of *inha*

cAMP is well known to be the major second messenger involved in gonadotropin signal transduction (Leung & Steele 1992). To test if cAMP could be involved in zFfSH and pituitary extract-stimulated *inha* expression, we examined the response of *inha* expression to forskolin, an adenylate cyclase activator that increases intracellular cAMP level. Similar to the effects of zFfSH and pituitary extract, forskolin (10 μ M) also significantly stimulated *inha* expression in cultured ovarian fragments at 24 h of treatment (Fig. 5D).

Effect of inhibin on final oocyte maturation

It is well known that inhibin antagonizes various activin actions in many mammalian systems. Previous studies in the zebrafish have shown that activin promotes final oocyte maturation either directly (Pang & Ge 1999, Wu *et al.* 2000) or by stimulating the acquisition of oocyte maturation competence (responsiveness of oocytes to the maturation-inducing hormone (MIH) 17 α , 20 β -dihydroxyprogesterone; DHP; Pang & Ge 2002a). To test if inhibin also influences oocyte maturation, we examined the effect of recombinant human inhibin A on the basal and DHP-induced GVBD *in vitro*. We used Cortland's solution in this experiment instead of Leibovitz L15 medium as we used in previous studies (Pang & Ge 1999, 2002a) because of the lower rate of spontaneous maturation in this solution. The result showed that human inhibin A (10 ng/ml) had little effect on basal maturation rate; however, it exhibited a slight but significant inhibitory effect on the rate of DHP-induced (5 ng/ml) oocyte maturation after 4 h treatment (Fig. 6).

Figure 6 Effect of human inhibin A on the basal and 17 α , 20 β -dihydroxyprogesterone (DHP)-induced final oocyte maturation. The FG follicles were incubated with inhibin A (10 ng/ml), DHP (5 ng/ml) or both in combination for 4 h *in vitro* followed by scoring the follicles that had undergone GVBD. % GVBD = No. of GVBD follicles $\times$ 100 / (Total no. of follicles – No. of dead follicles).

Discussion

Very limited information is available in teleosts about inhibin and its production site in the gonads despite the well-recognized importance of this molecule in mammalian reproduction. Using zebrafish as the model, the present study demonstrated a predominant expression of inhibin-specific α subunit (*inha*) in the gonads. This result is different from a previous report in the rat that inhibin α subunit was expressed in a wide range of extragonadal tissues including the pituitary and brain in addition to the gonads (Meunier *et al.* 1988), strongly suggesting that the function of inhibin is more restricted to reproduction in the zebrafish.

Inside the zebrafish ovary, the expression of *inha* was mainly localized in the somatic follicle cells with no signal detected in the oocyte. This was further supported by the lack of *inha* expression in the ovulated oocytes that are free of the follicle cells. The spatial distribution of *inha* expression within the follicle agrees well with the expression of β A (*inhbaa*) and β B (*inhbbb*) subunits as demonstrated by our previous study (Wang & Ge 2003), suggesting that the somatic follicle layer is the production site of both inhibin and activin. What remains unknown is the cell type that expresses *inha* in the follicle layer of zebrafish ovary. In the rainbow trout, *inha* expression was detected in the granulosa cells by *in situ* hybridization (Tada *et al.* 2002), in agreement with the situation in mammals whose main source of circulating inhibin is also the granulosa cells in the ovary (Erickson & Hsueh 1978, Bicsak *et al.* 1986, Hillier *et al.* 1989).

Zebrafish *inha* increased its expression dramatically during folliculogenesis with the maximal level reached at the FG stage, which is similar to that in mammals. In humans and monkeys, the pre-antral and small antral follicles express mostly β B with little α mRNA. As follicles increase in size, they express more and more α as well as β A subunit and follistatin mRNA, whereas the β B transcript level seems to remain relatively constant (Schwall *et al.* 1990, Roberts *et al.* 1993). These results indicate that activin B and later inhibin B may be produced in small developing follicles, which are gradually replaced by activin A and inhibin A as follicles develop into pre-ovulatory stage.

A previous study in our laboratory has also reported the expression profiles of the common β A (*inhbaa*) and β B (*inhbbb*) subunits of inhibin and activin during folliculogenesis in the zebrafish (Wang & Ge 2004). Similar to *inha*, *inhbaa* also has an extremely low level of expression at the PG stage, but its expression level increases progressively and significantly after the follicles are recruited into the vitellogenic growth at PV stage. However, after reaching the peak level at the MV stage, the expression of *inhbaa* dropped slightly but significantly at the FG stage. In contrast, the expression level of *inhbbb* remains more or less constant across all stages except a sudden surge at 0700 h in the morning

when the follicles undergo final maturation and ovulation (Wang & Ge 2004). The differential temporal expression profiles of the three subunits may imply distinctive secretion patterns of activin and inhibin molecules during folliculogenesis. With relatively high expression levels of *inhbaa* and *inhbb* but a low level of *inha* in early stages of folliculogenesis, it is conceivable that the predominant product synthesized from PV to MV stage is likely activin, which is probably involved in promoting follicle growth and vitellogenesis. However, as *inha* expression surges at the FG stage, the follicle may shift from synthesizing activin to producing inhibin, which may act as an endocrine hormone of ovarian origin to trigger the reproductive axis for final oocyte maturation and ovulation. This speculative idea is partly based on our recent finding that different from its specific stimulation of FSH secretion in mammals, activin in fish stimulates *fshb* (FSH β) but suppresses *lhb* (LH β) expression in the pituitary in a paracrine manner. We first demonstrated this in the goldfish (Yam *et al.* 1999, Yuen & Ge 2004) and recently also confirmed it in the zebrafish (Lin & Ge 2009). If inhibin also acts as a potent antagonist of activin as it does in mammals, it may reverse the opposing effects of activin on FSH and LH at the pituitary level. At the preovulatory stage, the surge increase in circulating inhibin may cause a decrease of FSH but an increase of LH synthesis and secretion by blocking the actions of activin in the pituitary. One of our earlier studies in the goldfish did show that mammalian inhibin could stimulate LH secretion *in vitro* (Ge *et al.* 1992). The increased production of LH, whose receptor (Lhcgr) reaches the highest expression level in the FG follicles (Kwok *et al.* 2005), is in turn responsible for inducing final oocyte maturation and ovulation. The mass production of inhibin by the FG follicles as shown in the present study may well serve as an important signal from the ovary to the pituitary for the readiness of the follicles to mature and ovulate (Fig. 7). This hypothesis needs to be further tested in the future.

As we have demonstrated before, *inhbb* is constantly expressed in the ovary during zebrafish folliculogenesis; however, its expression level increases sharply at 0700 h when GVBD occurs (Wang & Ge 2004), suggesting an important role for this subunit in GVBD or ovulation. However, since no data is available about the expression level of *inha* at this time, it remains unknown whether the increased *inhbb* leads to an increased activin B or inhibin B or both. This will be an interesting issue to address in the future.

In addition to the expression profile during folliculogenesis, we also determined the expression level of *inha* during final oocyte maturation. Interestingly, although the expression of *inha* reached the highest at the prematurational FG stage, it dropped significantly in the maturing follicles that were undergoing GVBD *in vitro* compared with the immature FG follicles. According to a previous study of our laboratory,

Figure 7 Hypothetical model for the potential role of inhibin in signaling and coordinating the reproductive axis for oocyte maturation and ovulation. The full-grown follicles may trigger the upper level of the reproductive axis, i.e. the pituitary, by releasing inhibin. As the antagonist of activin, inhibin may act at the pituitary level to block the actions of intrapituitary activin, which stimulates FSH but suppresses LH production. The action of inhibin in the pituitary would therefore lead to an increased LH secretion but a reduced FSH production, which reflects the relative levels of their receptors at the FG stage.

follistatin (*fst*) also reduces its expression in the follicle during spontaneous maturation *in vitro* whereas *inhbb* expression significantly increases (Wang & Ge 2004). The increased expression of *inhbb*, together with the simultaneous drop of inhibin (activin antagonist) and follistatin (activin-binding protein) production during oocyte maturation, would imply an increased output of activin within the follicle, which may be one of the stimuli for the spontaneous oocyte maturation. Previous studies in our laboratory and others have demonstrated a stimulatory role for activin in promoting oocyte maturation (Pang & Ge 1999, Wu *et al.* 2000) or increasing oocyte maturational competence (Pang & Ge 2002a) in the zebrafish, and its role in spontaneous maturation is evidenced by the suppressive effect of follistatin on oocyte maturation (Wang & Ge 2004).

To provide further evidence for the role of inhibin in oocyte maturation, we investigated whether inhibin has a direct effect on oocyte maturation *in vitro*. Our results demonstrated that recombinant human inhibin A had a slight but significant inhibitory effect on the final oocyte maturation induced by DHP, the most potent oocyte MIH in teleosts including the zebrafish. This is consistent with a recent study in *Fundulus heteroclitus* that also demonstrated an inhibitory effect of inhibin on DHP-induced final oocyte maturation (Petrino *et al.* 2007), but somehow different from a previous study in the zebrafish (Wu *et al.* 2000). More experiments are needed to define the role of activin–inhibin–follistatin system in follicle growth and maturation.

The robust surge expression of *inha* at the late stage of follicle growth before final oocyte maturation and ovulation immediately raises an interesting question about its regulation during folliculogenesis. It is well accepted in mammals that the expression of *inha* is mainly regulated by FSH from the pituitary (Woodruff *et al.* 1996, Welt *et al.* 1997). In order to demonstrate whether *inha* expression is also regulated by endocrine hormones in teleosts, we first monitored the change of its basal expression level in cultured ovarian fragments *in vitro*. Interestingly, the basal expression level of *inha* dropped dramatically within a few hours of incubation, indicating that its expression *in vivo* must be tightly controlled by endocrine hormones. Further experiments showed that the goldfish pituitary extract significantly stimulated *inha* expression after 24 h treatment. To identify the pituitary gonadotropin(s) that was involved in stimulating *inha* expression, we went on to examine the effects of recombinant zfFSH and zfLH as well as hCG individually. Interestingly, the *inha* expression was significantly stimulated by zfFSH but not zfLH and hCG in cultured ovarian fragments, and the effect could be mimicked by forskolin, an adenylate cyclase activator. This result provides an important piece of evidence for a specific effect of FSH in fish gonads, which represents one of the very few studies on the differential functions of pituitary FSH and LH in teleosts, especially the cyprinids. Like the situation in mammals, the pituitary FSH and ovarian inhibin may also form a negative feedback loop between the two organs of the zebrafish.

In conclusion, the present study systematically characterized inhibin in the zebrafish in terms of its expression, regulation, and function. The expression of *inha* expression was gonad-specific. In the ovary, *inha* was predominantly expressed in the somatic follicle cells, and its expression was highly responsive to the stimulation by FSH but not LH from the pituitary. During folliculogenesis, the expression of *inha* rose slightly and progressively after the follicles entered the vitellogenic stage, but increased dramatically in the FG follicles, suggesting a potential role for ovarian inhibin in signaling the upper level of the reproductive axis for maturation and ovulation.

Materials and Methods

Chemicals and hormones

All chemicals were obtained from Sigma–Aldrich unless otherwise stated. All PCR primers were synthesized by Integrated DNA Technologies (IDT; Coralville, IA, USA). hCG, DHP, and forskolin were purchased from Sigma–Aldrich. Recombinant human inhibin A was generously provided by Dr A F Parlow (National Hormone & Pituitary Program, National Institute of Diabetes and Digestive and Kidney Diseases, Torrance, CA, USA). Recombinant zfFSH and zfLH were produced in our laboratory by established Chinese hamster ovary (CHO) cell lines as we previously reported (So *et al.* 2005). The conditioned serum-free media from these cell lines were concentrated 200-folds by ultrafiltration before use. The control medium was collected from a CHO cell line transfected with the expression vector only. For stock preparation, hCG was dissolved in water, DHP in ethanol, and forskolin in DMSO. All these chemicals and hormones were further diluted to the desired concentrations with the medium before use. Modified Cortland's solution (7.25 g NaCl, 0.38 g KCl, 0.23 g CaCl₂·2H₂O, 1 g NaHCO₃, 0.41 g NaH₂PO₄·H₂O, 0.23 g MgCl₂·6H₂O, 0.23 g MgSO₄·7H₂O, 1 g BSA, 5.2 g HEPES, 100 000 U penicillin and 100 000 µg streptomycin in 1 l H₂O, pH 7.7; Wolf & Quimby 1969) was used as the incubation medium for all experiments.

Animals

Zebrafish (*Danio rerio*) were purchased from local tropical fish market and acclimated without separation of males and females in flow-through aquaria (36 l) at 28±1 °C. The photoperiod of the aquaria is 14 h light per day with the light on at 0800 and off at 2200. The fish were fed twice a day with commercial tropical fish food. The animals were maintained under these conditions for at least 4 weeks before experiments. All experiments were performed under 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. The fish were anesthetized with ice shock before use.

Preparation of goldfish pituitary extract

Five pituitaries from sexually mature goldfish were removed and homogenized in 1 ml PBS (0.2 g KH₂PO₄, 1.15 g Na₂HPO₄·2H₂O, 0.2 g KCl and 8 g NaCl in 1 l H₂O, pH 7.0) at 4 °C. The extract was then centrifuged at 20 000 g for 30 min at 4 °C followed by filtering the supernatant with a 0.22 µm filter (Millipore, Bedford, MA, USA). The protein concentration was determined with the Protein Assay Kit (Bio-Rad), and the extract was stored at –20 °C before use.

Separation of oocytes and follicle layers

The somatic follicle layers were carefully separated from the FG oocytes according to our recent report (Liu & Ge 2007) for RT-PCR determination of *inha* expression in the two

compartments. The isolated follicle layers and denuded but intact oocytes from 5 to 10 follicles were pooled and subjected to RNA extraction respectively.

Isolation of ovarian follicles

The ovaries were removed from 15 to 20 female zebrafish at noon after anesthetization and decapitation, and placed in a 100 mm culture dish containing Cortland's solution. The follicles of different stages were manually isolated under a dissecting microscope with fine forceps and blades, and then grouped according to the following stages: FG but immature (~0.65 mm, Stage III), MV (~0.50 mm, Stage III), EV (~0.40 mm, Stage III), PV (~0.30 mm, Stage II or cortical alveolus stage), and PG (~0.1 mm, Stage I) stage (Wang & Ge 2004). The process of dissection and isolation normally took about 4–6 h at room temperature.

Ovarian fragment incubation

After anesthetization and decapitation, the ovaries from four to six fishes were carefully removed and placed in a dish containing ice-cold Cortland's solution. The ovaries were briefly dispersed into small fragments, which were then distributed randomly into the wells of a 24-well plate for drug treatment. For the time course experiment, all groups including the control were incubated in Cortland's solution for 24 h, and the drugs were added at different times towards the end; different groups therefore had the same incubation time but different durations of exposure to drug treatment. The ovarian fragments were incubated at 28 °C.

Preparation of spontaneously matured follicles

To obtain FG follicles that had undergone spontaneous maturation (Stage IV), the immature FG follicles were isolated according to our previous report (Pang & Ge 1999) and incubated at 28 °C for 6 h. The mature follicles, which appeared translucent due to spontaneous GVBD, were isolated.

RNA isolation and RT

Total RNA was isolated from the tissues, ovarian follicles, and ovarian fragments with Tri-Reagent (Molecular Research Center, Cincinnati, OH, USA) according to the protocol

of the manufacturer and our previous report (Pang & Ge 2002b). RT was performed at 37 °C for 2 h in a total volume of 10 µl containing 2–3 µg total RNA, 1×MMLV buffer, 0.5 mM each dNTP, 0.5 µg oligo-dT and 100 U MMLV reverse transcriptase (Invitrogen). The RT reaction mix was 1:20 diluted with water (200 µl in total) before PCR amplification.

Real-time and semi-quantitative qPCR quantification of expression

RT-PCR analysis was performed using specific primers designed according to the sequences from GenBank (Table 1). Most quantitative measurements of expression were carried out by real-time qPCR. The standards for *inha* and glyceraldehyde-3-phosphate dehydrogenase (*gapdh*, housekeeping gene) were prepared by RT-PCR amplification of cDNA fragments with specific primers. The amplicons were resolved by agarose electrophoresis, purified, and quantitated by electrophoresis along with the Mass Ruler DNA marker (MBI Fermentas, Hanover, MD, USA). These amplified amplicons were used to construct standard curves in all real-time qPCR assays.

Real-time qPCR was performed on the iCycler iQ Real-time PCR Detection System (Bio-Rad) in a volume of 30 µl containing 10 µl 1:20 diluted RT reaction mix, 1×PCR buffer, 0.2 mM each dNTP, 2.5 mM MgCl₂, 0.2 µM each primer, 0.75 U Taq polymerase, SYBR Green (1:35 000, Molecular Probes, Leiden, The Netherlands), and 20 nM fluorescein (Bio-Rad). The reaction profile consisted of 38 cycles of 94 °C for 30 s, 56 °C for 30 s, 72 °C for 1 min, and 80 °C for 7 s for signal detection. A melt-curve analysis was performed at the end of the reaction consisting of 180 cycles of 7 s with temperature increased at 0.2 °C/cycle to demonstrate the specificity of the reaction, which was also confirmed by agarose gel electrophoresis and sequencing.

To localize the expression of *inha* in different compartments of the follicle and to confirm the results produced by real-time qPCR, semi-quantitative qPCR was also frequently used. For semi-quantitative qPCR analysis of *inha* expression, the cycle numbers used were optimized according to our previous report (Wang & Ge 2003). Briefly, PCR was carried out in a volume of 30 µl consisting of 1×PCR buffer, 0.2 mM each dNTP, 2.5 mM MgCl₂, 0.2 µM each primer, and 0.6 U Taq polymerase for various cycles: 26 for *inha*, 29 for *gdf9*, 35 for *lhcr*, and 27 for *gapdh*.

Table 1 Primers used in RT-PCR.

Gene	Primer			Application	bp	Accession no.
	Name	Sequence	Strand			
<i>inha</i>	INH520	AGCCTCTCTGCCAGTGTTG	S	Semi-quantitative and real-time qPCR		XM_693951
	INH521	ATGTTGATGGAAGCGATGGTCTC	AS	Semi-quantitative qPCR	301	
	INH604	AGCATCAGAAGAGTGGTCAGGTATA	AS	Real-time qPCR	188	
<i>gdf9</i>	GDF206	GAGTCTGTTGAACCCGACG	S	Semi-quantitative qRT-PCR	544	AY833104
	GDF207	GCAGGTGGATGTCCTTCTTA	AS			
<i>lhcr</i>	LHR103	AAGGACGAGTCGCTGAAAC	S	Semi-quantitative qPCR	663	AY424302
	LHR104	GATTCATTGTGGCGTATTC	AS			
<i>gapdh</i>	GAPDH47	AATGAAGGGAATTCTGGGA	S	Semi-quantitative and real-time qPCR		NM_213094
	GAPDH48	AACAACCTACAGCAATGCCTG	AS	Semi-quantitative qPCR	398	
	GAPDH404	GCGTCTCGTAGGTGGGAACAG	AS	Real-time qPCR	149	

Data analysis

The expression level of zebrafish *inha* in each sample was first calculated as the ratio to that of the internal control *gapdh*, and then expressed as fold change versus the reference group for statistical analysis. All values were expressed as the mean $\pm$ S.E.M., and the data were analyzed by one-way ANOVA followed by Newman–Keuls test (for comparing all pairs of groups) or unpaired *t*-test using Prism 5.0 for Macintosh OS X (GraphPad Software, San Diego, CA, USA). $P < 0.05$ was considered statistically significant.

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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