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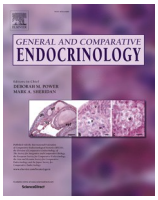

Research paper

Elevated expression of *inhibin α* gene in sterile allotriploid crucian carp

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ABSTRACT

Inhibin and Activin, belong to the transforming growth factor β superfamily (TGF-β), which associate with the regulation of the reproductive process by the modulation of the hypothalamic-pituitary-gonad (HPG) axis. In this study, we reported the molecular cloning and tissue expression of *inhibin α* in allotriploid crucian carp and its parent- diploid red crucian carp. The full-length cDNA of *inhibin α* were respectively 1632 bp and 1642 bp in allotriploids and diploids, which both consisted of a 1044 bp open reading frame (ORF) encoding 347 amino acids. Real-time quantitative PCR (RT-qPCR) showed that allotriploids and diploids had significant expression of *inhibin α* in testis and ovary, and the expression of *inhibin α* in the gonads of allotriploids was higher than that of diploids. The immunohistochemistry indicated that the ovarian development of allotriploids was abnormal, and the expression of Inhibin α in the ovary of allotriploids was higher than that of diploids. Results of co-immunoprecitation (co-IP) demonstrated that the Inhibin α and Activin β_A, Inhibin α and Activin β_B can form dimers. These findings suggested that the elevated expression of *inhibin α* and the competitive binding of Inhibin α subunit with Activin β subunits in allotriploids may be releted to the sterility of allotriploids. Furthermore, these results will facilitate the investigation of reproduction characteristics in allotriploids and provide theoretical basis for the study of polyploid breeding in the future.

1. Introduction

Hybridization is a crucial process for speciation in animals and plants (Mallet, 2007). Distant hybridization can combine two distantly related genomes together, and the genotype and phenotype of the hybrid offspring are different (Liu, 2010). In previous study, our laboratory successfully bred the allotetraploid population of F_3-F_{30} (4*n*AT, $4n = 200$), a bisexual fertile population, which was successfully produced by mating red crucian carp (*Carassius auratus* red var., $2n = 100$, ♀) with common carp (*Cyprinus carpio*, $2n = 100$, ♂) (Liu et al., 2001a; Liu et al., 2001b). Allotriploid crucian carps ($3n = 150$) with sterility, faster growth rate, good flesh quality, and higher anti-disease ability, which were obtained by crossing the males of allotetraploids with the females of red crucian carp. The allotriploids had three types of gonads including testis, ovary, and fat type, which all cannot produce normal gametes (Liu et al., 2000). Studies on chromosome spreading showed that there were abnormal pairings between homologous chromosomes during meiosis in the testis of allotriploids (Zhang et al., 2005). In addition, expression of the meiotic gene *dmc1* in the ovaries of allotriploids was lower than that of allotetraploids (Tao et al., 2008). Abnormal expressions of HPG axis genes (*gnrh*, *gthβ*, *gthr*, *gnih*, and *gnihr*) were related to sterility of allotriploids (Long et al., 2006; Long et al., 2009).

Fish reproduction is mainly regulated by the HPG axis. Gonadotropin-releasing hormone (GnRH), gonadotropin hormone (GTH) and gonadotropin hormone receptor (GTHR) are vital signal molecules in the HPG axis. GnRH secreted from the hypothalamus to stimulate the synthesis of GTH, including follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Charlton, 2008; Long et al., 2009). FSH involved in gonadal growth, and LH induced final maturation and ovulation (Prat et al., 1996; Richards et al., 1994; Swanson et al., 1991). Inhibin and Activin belong to TGF-β, which play an important role in regulating the development and maturation of gonads. Researchers found that Inhibin mainly was expressed in the ovary and testis and could inhibit the secretion of FSH, but Activin could stimulate the synthesis and secretion of FSH (Meunier et al., 1988; Ge et al., 1997; Lu et al., 2020).

Inhibin was first discovered in an aqueous extract solution of testis by McCullagh in 1932 (McCullagh, 1932) and consists of two types: Inhibin

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A (α-β_A) and Inhibin B (α-β_B). Inhibin A (α-β_A) is composed of α subunit and β_A subunit, and Inhibin B (α-β_B) is constituted of α subunit and β_B subunit. Activin consists of two β subunits (Tao et al. 2019), and studies showed that Inhibin and Activin share common β subunits (either β_A or β_B) (Hotten et al., 1995; Fang et al., 1996; Oda et al., 1995; Yamashita et al., 1995, Lu et al., 2020). In rats, excessive expression of Inhibin α subunit will reduce the FSH level and ovulation rate (Cho et al., 2001), and autocrine action of Inhibin can induce the production of estradiol in rat granulosa cells (Ying et al., 1986). And Adu-Gyamf et al. reported that dysregulated expression of Inhibin was involved in pregnancy complications and disturb reproduction (Adu-Gyamfi et al., 2020). Ge et al. found that Inhibin can influence the secretion of type-II gonadotrophin (GTH-II) in goldfish (Ge et al., 1993). Inhibin can cause the changes of ovarian functions, such as changes in basal and gonadotropin-induced steroids (LaPolt and Hsueh, 1991). Tsafriri et al. indicated that Inhibin and Activin can influence the follicular growth in ovary, production and development of spermatogonia (Tsafriri et al., 1989; Mather et al., 1990). Inhibin-deficient mice showed symptoms of infertility (Matzuk et al., 1992; Matzuk et al., 1994). These studies have shown that Inhibin is involved in gonadal development and reproductive regulation.

The gonadal developmental mechanism is very complicated that includes plenty of genes and networks acting synergistically or antagonistically. The studies of *inhibin α* gene related to gonadal development have been reported in many species (Woodruff et al., 1987; Onagbesan et al., 2004; Akhtar et al., 2019; Wheaton et al., 2003; Fallahiam et al., 2009), but few studies reported in polyploid fish. The sterile allotriploid crucian carp provides a good experimental material for studying the function and mechanism of fertility. In this study, we obtained the full length of *inhibin α* gene in allotriploid crucian carp and its parent-diploid red crucian carp and investigated the tissue distribution and location of *inhibin α* gene. In addition, we studied the association of Inhibin α subunit and Activin β subunits in allotriploid crucian carp.

2. Materials and methods

2.1. Experimental fish and materials

Allotriploid crucian carp and diploid red crucian carp were obtained from Engineering Research Center of Polyploid Fish Reproduction and Breeding of the Ministry of Education at Hunan Normal University during breeding season and non-breeding season periods (April and October). All of the fish were anesthetized before decapitation, and the tissues (brains, pituitaries, testes, ovaries, muscles, hearts, livers, spleens and kidneys) were excised and stored at −80℃. All experiments followed guidelines of the Administration of Affairs Concerning Experimental Animals of China.

HEK293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA), 2 mM L-glutamine (Gibco, USA), 100 Units/ml penicillin (Gibco, USA) and 100 ug/ml streptomycin (Gibco, USA) at 37 °C with 5% CO_2.

N-terminal c-HA-tagged Inhibin α, N-terminal c-Myc-tagged Activin β_A, N-terminal c-Myc-tagged Activin β_B were amplified and subcloned into pcDNA3.1 vector by Sangon (Shanghai, China) to obtain the expression plasmids.

2.2. RNA isolation and generation of first-strand cDNA

Total RNA was purified using Trizol™ Reagen (Invitrogen, Carlsbad, CA, USA). The integrity and the concentration of RNA were detected with the agarose gel electrophoresis and the NanoDrop (Thermo Fisher Scientific, Wilmington, DE, USA), respectively. PrimeScript RT reagent kit with gDNA Eraser (TaKaRa, Tokyo, Japan) was used to synthesize first-strand cDNA. Then the products of reverse transcription were stored at −20℃ for other experiments.

2.3. Cloning of partial inhibin α cDNA fragment and RACE

Primer premier 5.0 was used to design gene specific primers (Table 1) of *inhibin α* according to the cDNA sequence of *inhibin α* gene in the goldfish (XM_026262782), which was obtained from National Center for Biotechnology Information (NCBI). The partial cDNA fragments of the *inhibin α* coding region in the two types of fish were amplified by PCR. The cDNA fragments of *inhibin α* untranslated region (UTR) in these fish were obtained by 5′ - and 3′-rapid amplification of cDNA ends (RACE). The specific 3′-RACE forward primers (3R1 and 3R2) and the 5′-RACE reverse primers (5R1 and 5R2) in table 1 were designed according to the known partial cDNA sequences of *inhibin α* gene in the two types of fish. For 3′-RACE, the first-strand template was synthesized by a reverse transcription reaction with Oligo dT-3 sites Adapter Primer using 3′-Full RACE Core Set (Takara, Tokyo, Japan). Then the 3R1 and 3′-sites Adaptor Primer were used with the first PCR reaction and the 3R2 and 3′-sites Adaptor Primer were used with the nested PCR reaction. For 5′-RACE, the 5′ ends of *inhibin α* cDNAs of the two types of fish were synthesized with a SMART™ RACE cDNA Amplifcation Kit (Clontech, San Francisco, CA, USA). SMART II A Oligonucleotide, 5′-RACE CDS Primer A (Table 1), and PowerScript™ Reverse Transcriptase were used to synthesize the first-strand cDNA by a reverse transcription reaction. Universal Primer A Mix (UPM) and 5R1 were used with the first PCR reaction and Nested Universal Primer A (NUP) and 5R2 were used with nested PCR reaction.

Productions of these PCR reactions were analyzed with 1.2% agarose gels, purified by a Gel Extraction Kit (Sangon, Shanghai, China), then all the purifications were connected with pMD18-T vector (Takara, Dalian, China) for night at 4℃, recombinant plasmids were transformed into Escherichia coli DH5α (Sangon, Shanghai, China). Finally, they were sequenced by Sangon.

2.4. Real-time quantitative PCR (RT-qPCR)

To analyze the distribution of *inhibin α* gene in the brains, testes, ovaries, muscles, hearts, livers, spleens, kidneys and pituitaries, these tissues were taken from allotriploid crucian carp and diploid red crucian carp (three males and three females, respectively), and the RT-qPCR was performed by a Prism 7500 Sequence Detection System (ABI, Foster City, CA, USA) according to the manufacturer's instructions. The specific primers (*inhibin α*-qF and *inhibin α*-qR) were designed by AlleleID 6, and *actin*-qF *and actin*-qR were used as the internal control (Table 1). The volume of 10 μL mixture consisted of 5 μL SYBR green PCR Master Mix,

Table 1
Primers used in this study.

Primer name	Primer sequence (from 5′- 3′)	Usage
inhibin α-F	ACAAGGGAGACAAGAGTGGAAA	PCR
inhibin α-R	GGTGGTGGTGAAACGGAGAGAC	PCR
3′ sites Adaptor Primer	CTGATCTAGAGGTACCGGATCC	3′ RACE
3R1	GCTATGATTCAGAAGATAAAAC	3′ RACE
3R2	CCTGCTTCTGATGACACTGATT	3′ RACE
SMART II™ A oligo	AAGCAGTGGTATCAACGCAGAGTACATGGG	5′ RACE
5′- CDS Primer A	(T)25VN (V = A/G/C; N = A/C/G/T)	5′ RACE
UPM (Mix)	CTAATACGACTCACTATAGGGCAAGCAGT GGTATCAACGCAGAGT (long) CTAATACGACTCACTATAGGGC (short)	5′ RACE
NUP	AAGCAGTGGT AACAACGCAGAGT	5′ RACE
5R1	ACTGGACTTACAGATGCTTGGA	5′ RACE
5R2	GAAAGTAGTAAGTAAAGTAACC	5′ RACE
inhibin α-qF	AGGGAGACAAGAGTGGAAAGA	RT-qPCR
inhibin α-qR	TGATGATGGAGTCCTGGCTGT	RT-qPCR
actin-qF	TCCCTTGCTCCTTCCACCA	RT-qPCR
actin-qR	GGAAGGGCCAGACTCATCGTA	RT-qPCR

3 μL water, 1 μL cDNA sample, 0.5 μL *inhibin α*-qF and 0.5 μL *inhibin α*-qR. The following conditions were used: 50℃ for 2 min, 95℃ for 10 min, followed by 40 cycles at 95℃ for 15 sec and 61℃ for 45 sec. To ensure the accuracy of the PCR results, experimental samples were added to a 96-well plate repeated thrice. The $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) was used to analyze the relative expression of the *inhibin α* gene. Finally, the dissociation curve examined whether PCR products were specific or not.

2.5. Immunohistochemistry

The pv-9000 polymer detection system (ZSGB-Bio, Beijing, China) was used to examine the expression and cellular localization of Inhibin α in ovary of allotriploids and diploids during the different phases. Firstly, ovary samples were fixed in the Bouin's solution for 24 h, and then dehydrated in a series of ethanol. Secondly, tissues were embedded in paraffin wax blocks and sectioned at 6 μm. Thirdly, paraffin sections were deparaffinized in xylene, rehydrated through a serial ethanol gradient and incubated in 3% H_2O_2 solution in methanol for 10 min to block endogenous peroxidase activity. Next, the citrate buffer method was used for antigen retrieval and then the sections were incubated with anti-Inhibin α antibody overnight, which anti-Inhibin α antibody was synthesized by Genscript (NJ, USA, Order ID:C742YFC030-1, Lot: C7825FC030-1, Immunogen: Peptide-KLH conjugate, Host Strain: New Zealand Rabbit) followed by processing with the pv-9000 Polymer Detection System according to the manufacturer's instructions. In the gap between each step, sections were washed with PBS. Finally, the 3, 3-diaminobenzidine tetrahydrochloride (DAB) (ZSGB-Bio, Beijing, China) and hematoxylin were used for staining and counterstaining respectively until the positive signals were detected. Negative control was incubated with PBS instead of the primary antibody.

2.6. Co-immunoprecipitation (co-IP)

The recombinant expression vector pcDNA3.1-HA-Inhibin α was constructed by inserting the ORF of *inhibin α* in allotriploids into pcDNA3.1, and the HA tag was fused at the N-terminus of *inhibin α*. The recombinant plasmids pcDNA3.1-Myc-Activin β_A and pcDNA3.1-Myc-Activin β_B were constructed by cloning ORF of *activin* β_A and *activin* β_B in allotriploids into pcDNA3.1, and the Myc tag was fused at the N-terminus of *activin* β_A and *activin* β_B, respectively.

To verify the direct interactions between Inhibin α subunit and Activin β_A subunits, Inhibin α subunit and Activin β_B subunits in allotriploids, pcDNA3.1-HA-Inhibin α and pcDNA3.1-Myc-Activin β_A, pcDNA3.1-HA-Inhibin α and pcDNA3.1-Myc, pcDNA3.1-HA-Inhibin α and pcDNA3.1-Myc-Activin β_B were co-transfected into HEK293T cells respectively by using Lipomax (SUDGEN, China). After these transfected cells were incubated for 36–48 h, these whole cell lysates were collected for co-IP (Li et al., 2018). Ultrasonic cell crusher (SONICS, USA) were used to crush the whole cell lysates, which were incubated with protein A/G agarose at 4℃ for 1 h, then the supernatants were collected and Myc-conjugated protein A/G agarose beads were added to the supernatants for night at 4℃. Finally, after Myc-conjugated protein A/G agarose beads were washed with 1% NP-40 for 6–7 times, boiled with 6 × sample buffer for 20 min, and then the proteins were used for immunoblot (IB) (Zhou et al., 2015).

Procedures of IB as follows: firstly, the proteins were separated by 10% SDS-PAGE and transferred to PVDF membrane, next the 5% skimmed milk was used to block the transferred membrane for 45 min, and the transferred membrane was probed with mouse monoclonal anti-HA antibody (1:2000, Sigma, USA, H3663-100UL, Lot#038M4810V) or mouse monoclonal anti-Myc antibody (1:2500, Sigma, USA, M4439-100UL, Lot#029M4849V) at 4℃ overnight, then were incubated with goat-anti mouse IgG (1:1500, Auragene Bioscience, China). Finally, BCIP/NBT Alkaline Phosphatase Color Development kit (Sigma, USA) was used to visualize the target protein.

2.7. Statistics analysis

Multiple sequence alignment was analyzed using DNAMAN version 5.0 (Lynnon Biosoft, Quebec, Canada). Phylogenetic tree was constructed with MEGA 5 software (Philadelphia, Pennsylvania). Significant differences were determined by SPSS 19.0 software (IBM, Chicago, IL, USA). The value of $P < 0.05$ was considered as statistically significant.

3. Results

3.1. Nucleotide and deduced amino acid sequences of Inhibin α

The cloned full-length cDNAs of *inhibin α* in allotriploid crucian carp (MW149267) and diploid red crucian carp (MW149266) were 1632 bp and 1642 bp, respectively. And allotriploids and diploids *inhibin α* had 1044 bp ORF that encoded a putative protein of 326 amino acids. Analysis of multiple sequence alignment showed that Inhibin α had high homology of 99.7% between allotriploids and diploids, and also had higher homology among the cyprinid fish. However, Inhibin α had lower homology in other fish and mammals (Fig. 1). Phylogenetic tree analysis indicated that Inhibin α in allotriploids and diploids both were localized in a clade with other teleosts, and nested with *Cyprinus carpio* and *Danio rerio* (Fig. 2).

3.2. Tissue expression of inhibin α in allotriploids and diploids

RT-qPCR was used to determine the relative mRNA expression of *inhibin α* in allotriploids and diploids (Fig. 3). The dissociation curve confirmed the specificity of PCR products. The results reported that *inhibin α* gene was highly expressed in the gonads. In female allotriploids, *inhibin α* gene was present in the brain, heart, liver, ovary and pituitary, but the highest expression was in the ovary (Fig. 3a). In male allotriploids, expression of *inhibin α* gene was higher in the testis and pituitary, and was also partially expressed in the brain, heart and liver (Fig. 3b). In female diploids, *inhibin α* gene was highly expressed in the ovary, and moderately expressed in the liver and spleen, but expressed at low levels in the brain and pituitary or even not expressed in other peripheral tissues (heart, kidney and muscle) (Fig. 3c). In male diploids, *inhibin α* gene was mainly expressed in the testis, and had a lower expression in the brain, muscle, pituitary (Fig. 3d).

The differential expression of *inhibin α* in ovaries of different ploidy fish during breeding season and non-breeding season periods was analyzed by RT-qPCR. Dissociation curve confirmed specificity of PCR products. The results showed that the expression of *inhibin α* in allotriploids ovaries was higher than diploids in both the breeding and non-breeding periods (Fig. 4).

3.3. The localization of Inhibin α in ovaries

The study explored the localization and expression of Inhibin α protein in the ovaries of allotriploids and diploids at different phases by immunohistochemistry. The ovaries of allotriploids consisted of some small oogonium-like cells and a few oocytes of stage III in the breeding season, but only some small oogonium-like cells were observed in the non-breeding season (Fig. 5a, b, e, and f). However, three oocyte phases (phases II, III and IV) were observed in diploids, and we found that the main phases of oocytes were slightly different between non-breeding season and breeding season. Oocytes were mainly in phases II and III during non-breeding season (Fig. 5c, and g), but oocytes were mainly in phases III, IV and a few in phases II during the breeding season (Fig. 5d, and h). Inhibin α was highly expressed in the ovaries of both types of fish (Fig. 5). In allotriploids, Inhibin α was strongly expressed in the small oogonium-like cells (Fig. 5a, and b). In addition, Inhibin α was present in the zona radiate, nucleoplasm and cytoplasm of the oocytes in stages III in allotriploids during breeding season (Fig. 5b). In diploids, Inhibin α

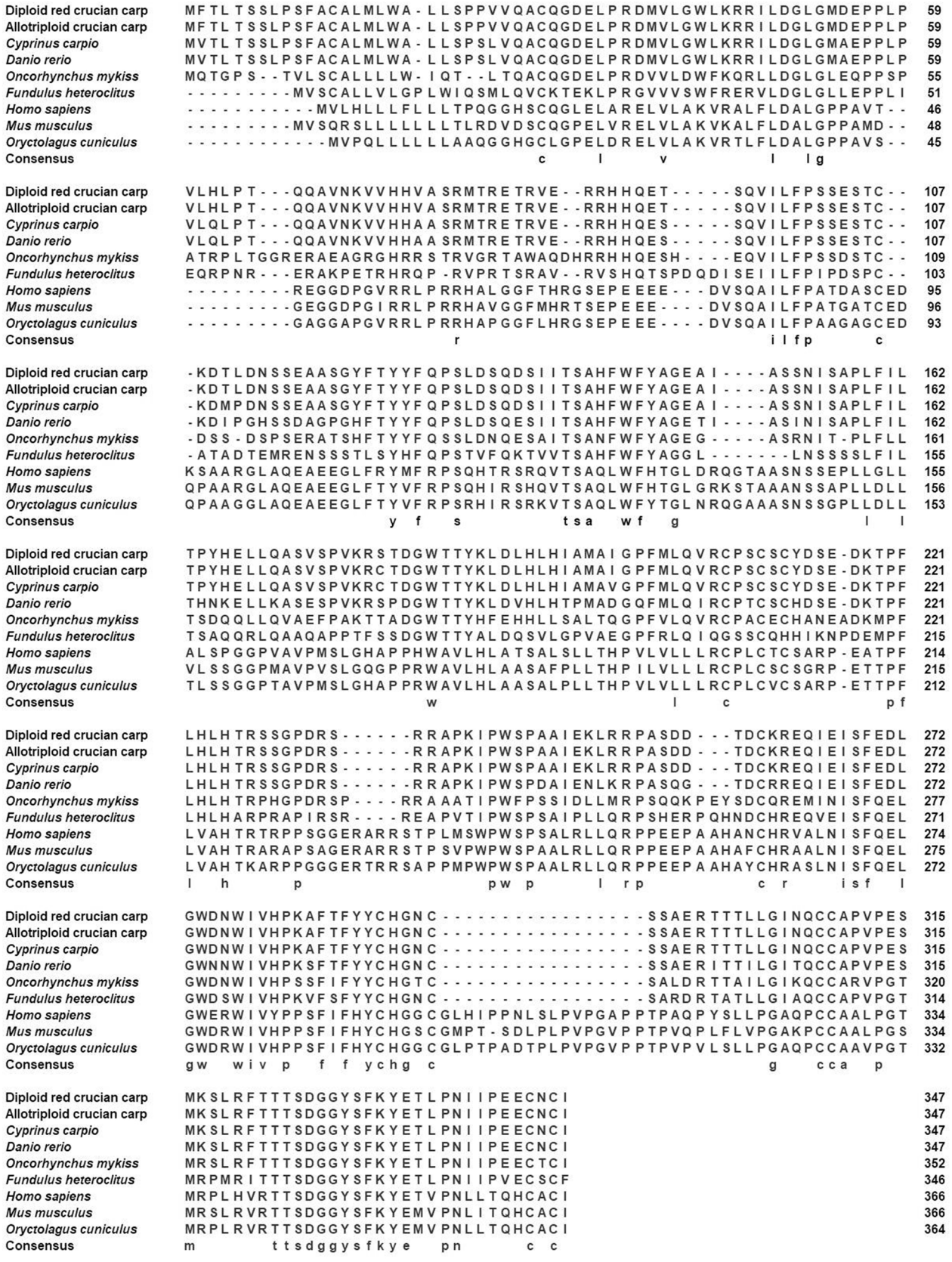

Fig. 1. Homology analysis of Inhibin α protein sequences. Identical and similar amino acid residues are indicated with lowercases below each amino acid. *Cyprinus carpio* (Common carp, XP_018966331); *Danio rerio* (Zebrafish, NP_001038669); *Oncorhynchus mykiss* (Rainbow Trout, NP_001117672); *Fundulus heteroclitus* (Supercooled killifish, NP_001296890); *Mus musculus* (Mouse, NP_034694); *Oryctolagus cuniculus* (European rabbit, NP_001315998); *Homo sapiens* (Human, NP_002182).

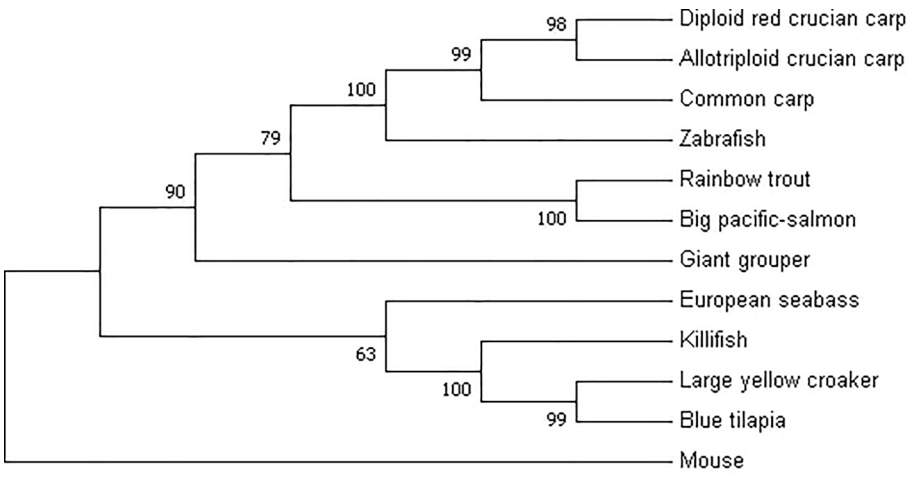

Fig. 2. The phylogenetic tree based on Inhibin α in different species. The tree was constructed by the NJ method. Numbers at nodes indicate the bootstrap value, as percentages, obtained for 1,000 replicates. *Cyprinus carpio* (Common carp, XP_018966331); *Danio rerio* (Zebrafish, NP_001038669); *Oncorhynchus mykiss* (Rainbow Trout, NP_001117672); *Fundulus heteroclitus* (killifish, NP_001296890); *Mus musculus* (Mouse, NP_034694); *Dicentrarchus labrax* (European seabass, CCJ67607); *Larimichthys crocea* (Large yellow croaker, TMS11537); *Oreochromis aureus* (Blue tilapia, XP_031608986); *Oncorhynchus keta* (Big pacific-salmon, XP_035611138), *Epinephelus lanceolatus* (Giant grouper, XP_033471549).

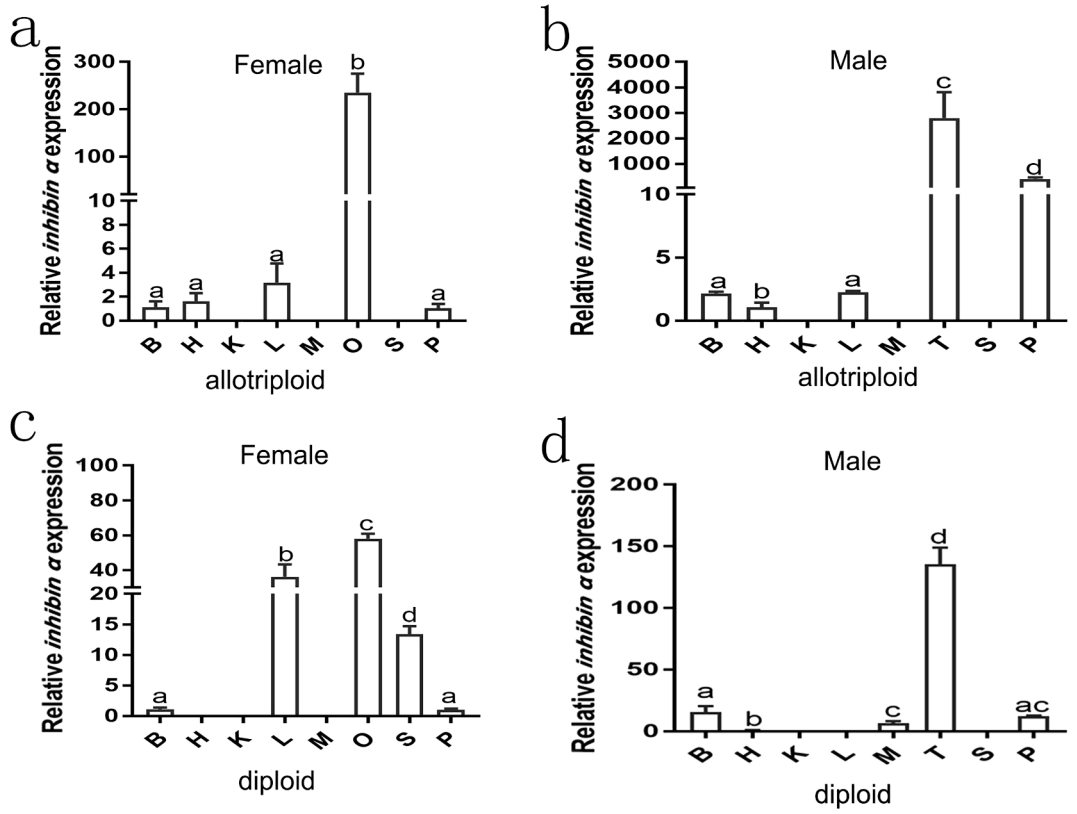

Fig. 3. Expression of *inhibin α* gene in tissues of allotriploid crucian carp and diploid red crucian carp. Expression of the *inhibin α* gene in the tissues of female allotriploid crucian carp (a), male allotriploid crucian carp (b), female diploid red crucian carp (c) and male diploid red crucian carp (d). B: brain; H: heart; K: kidney; L: liver; M: muscle; O: ovary; S: spleen; T: testis; P: pituitary. The different lowercase letters on each bar indicated a significant difference ($p < 0.05$).

was observed in zona radiate, nucleoplasm of oocytes in phases II, III, IV and cytoplasm of oocytes in phases III, IV, but it was not observed in the cytoplasm of oocytes in phases II (Fig. 5c, and d).

3.4. Associations between Inhibin α subunit and Activin β subunits

Inhibin and Activin share common β subunits (either β_A or β_B), which suggests that Inhibin α subunit and Activin β subunits (β_A and β_B) may form dimers. To further verify interactions between Inhibin α subunit and Activin β subunits (β_A and β_B) in allotriploids, we carried out a co-IP assay in HEK293T cells. Whole cell lysates of HEK293T cells co-transfected with pcDNA3.1-HA-Inhibin α and pcDNA3.1-Myc-Activin β_A or pcDNA3.1-HA-Inhibin α and pcDNA3.1-Myc-Activin β_B, which were precipitated with anti-Myc antibody and the precipitates were analyzed by IB with anti-HA antibody. The results showed that pcDNA3.1-HA-Inhibin α can be precipitated with anti-Myc antibody in the presence of pcDNA3.1-Myc-Activin β_A or pcDNA3.1-Myc-Activin β_B. To ensure the reliability of the results, pcDNA3.1-HA-Inhibin α and empty vector were co-transfected into HEK293T cells as control. As well as, Whole cell lysates of HEK293T cells co-transfected with pcDNA3.1-HA-Inhibin α and empty vector were precipitated with anti-Myc antibody and the precipitates were analyzed by IB with anti-HA antibody, but the results of IB showed no bands (Fig. 6). The results of co-IP assays showed that pcDNA3.1-HA-Inhibin α can be precipitated with anti-Myc

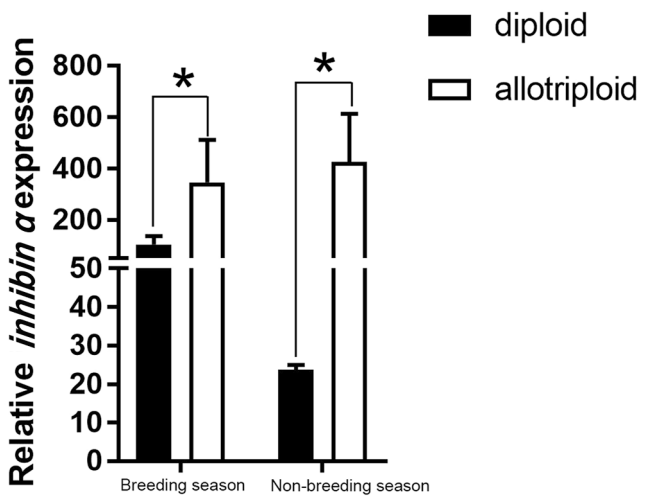

Fig. 4. Expression of *inhibin α* gene in ovary of allotriploid crucian carp and diploid red crucian carp in both the breeding and non-breeding periods. Asterisk (*) indicated a significant difference ($p < 0.05$).

antibody only in the presence of pcDNA3.1-Myc-Activin β_A and pcDNA3.1-Myc-Activin β_B, indicating the interactions between Inhibin α subunit and Activin β subunits (β_A and β_B).

4. Discussion

In this study, we cloned the full-length cDNA of *inhibin α* gene in allotriploid crucian carp and diploid red crucian carp were 1632 bp and 1642 bp, respectively. And allotriploids and diploids *inhibin α* gene had 1044 bp ORF that encoded a putative protein of 326 amino acids. Analysis of multiple alignment of amino acid sequence and phylogenetic tree revealed that Inhibin α was remarkable conservation in allotriploid crucian carp and diploid red crucian carp. Furthermore, the conservative domains were mainly distributed in C-terminus but the N-terminal sequence was less conservative with other species (Fig. 1 and Fig. 2).

The expression of *inhibin α* gene in allotriploids and diploids was analyzed by RT-qPCR. The results showed that *inhibin α* gene was highly expressed in the gonads (Fig. 3) coincided with the previous reports (Woodruff et al., 1996; Lewis et al., 2000; Meunier et al., 1988; Yamashita et al., 1995), which indicated that *inhibin α* gene may play a certain role in the processes of gonadal development, reproduction and gametogenesis in allotriploids and diploids. We observed that *inhibin α* gene was highly expressed in the gonads. Moreover, *inhibin α* gene was also observed in the brains, pituitaries, livers, hearts, spleens and muscles (Fig. 3). These data indicated that *inhibin α* gene might play an important role in regulating multiple physiological functions such as reproduction, growth and embryonic development.

Researchers found that Inhibin can regulate the release of FSH through their feedback mechanism in female fish (Ahmad et al., 2018; Lu et al., 2020). To further explore the role of the Inhibin α in reproductive development of fish, we studied the expression and location of *inhibin α* in the ovaries of different ploidy cyprinid fish. The results of RT-qPCR indicated that the expression of *inhibin α* gene in allotriploid crucian carp was significantly higher than that of diploid red crucian carp during both the breeding and non-breeding periods (Fig. 4). Previous studies showed that the expressions of many genes (such as: *dmc1*, *gnrh2*, *gthβ*, *gthr*, *and vasa*, etc.) were abnormal in allotriploid crucian carp (Zhang et al., 2005; Tao et al., 2008; Long et al., 2009; Yu et al., 2015). In addition, the results of immunohistochemistry showed that the expression of Inhibin α in the ovaries of allotriploid crucian carp was higher than that in diploid red crucian carp (Fig. 5), which in accordance with the results of RT-qPCR. It was well documented that the main function of Inhibin was to selectively inhibit the synthesis and secretion of the FSH, and to regulate the growth and development of follicles, the release of gonadotropins, the maturation of oocytes, and ovulation through autocrine and paracrine effects (Setchell and Jacks 1974; De Jong and Sharpe 1976; Nishimori and Matzuk, 1996). Hence, we speculated that the higher expression of Inhibin α in allotriploids may be related to its sterility through inhibiting the synthesis and secretion of FSH.

The importance of Inhibin during ovarian development and stages of oogenesis had been demonstrated in several vertebrate species (Ahmad et al., 2020; Kohli et al., 2003). The studies of Inhibin in zebrafish (Wu et al., 2000), rainbow trout (Tada et al., 2002) and pigtail (Mousa et al., 2003) also verified the function of Inhibin in the oogenesis, and study showed that Inhibin was involved in disruption of folliculogenesis and female infertility in fish (Lu et al., 2020). Results of immunohistochemistry showed that the ovarian development of allotriploid crucian carp was abnormal in both the breeding and non-breeding periods, and signals of Inhibin α were detected in ovarian zona radiata, cytoplasm of oocytes, nucleoplasm of oocytes and oogonium-like cells (Fig. 5). In addition, Inhibin α were detected in oocytes of all stages (Fig. 5), which similar to recent studies in Chinese tongue sole (Zhang et al., 2020). Co-IP assay was used to verify whether Inhibin α subunit and Activin β subunits (β_A and β_B) can form dimers, the results were indeed the case

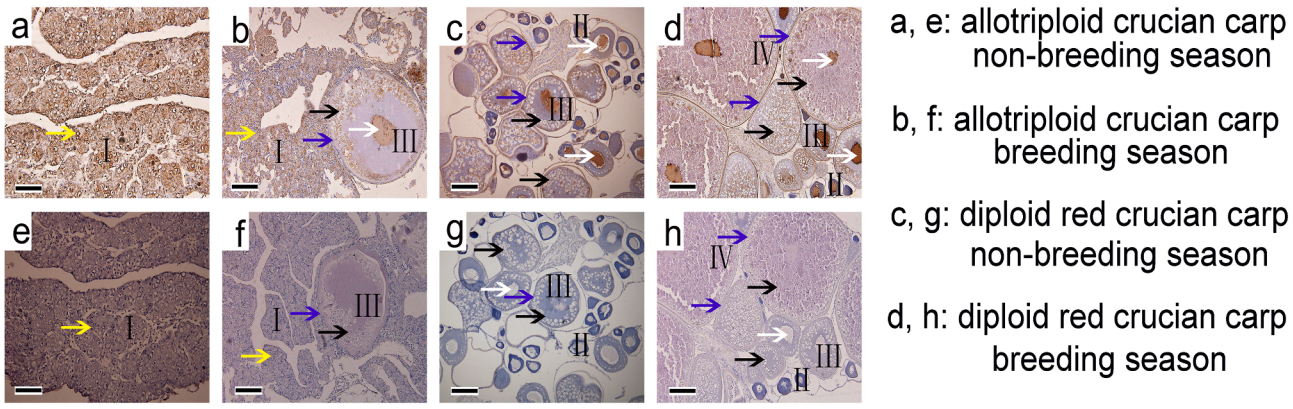

Fig. 5. Distribution of Inhibin α in the ovaries of allotriploid crucian carp and diploid red crucian carp. (a) Inhibin α of allotriploid crucian carp in the non-breeding season. (b) Inhibin α of allotriploid crucian carp in the breeding season. (c) Inhibin α of diploid red crucian carp in the non-breeding season. (d) Inhibin α of diploid red crucian carp in the breeding season. (e) Negative control of allotriploid crucian carp in the non-breeding season. (f) Negative control of allotriploid crucian carp in the breeding season. (g) Negative control of diploid red crucian carp in the non-breeding season. (h) Negative control of diploid red crucian carp in the breeding season. Blue arrows indicate zona radiata; white arrows indicate nucleoplasm of oocytes; black arrows indicate cytoplasm of oocytes; yellow arrows indicate oogonium-like cells. I, II, III and IV indicate oocytes in phases I, II, III and IV, respectively. Scale bars: a and e, 20 μm; b, c, d, f, g and h, 40 μm.

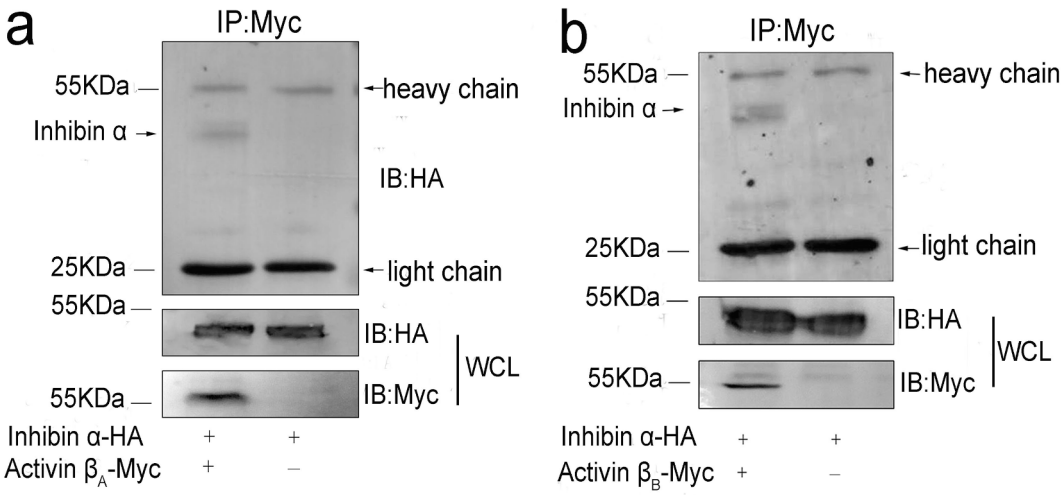

Fig. 6. Associations between Inhibin α-subunit and Activinβ_A- /β_B-subunits. (a) HEK293T cells were transfected with plasmids including Inhibin α-HA and Activin β_A-Myc. Proteins precipitated with Myc-conjugated protein A/G agarose beads were resolved by SDS-PAGE and analyzed by immunoblot (IB) with anti-HA antibody. Whole cell lysates (WCLs) were analyzed by IB with anti-HA and anti-Myc antibodies. (b) HEK293T cells were transfected with plasmids including Inhibin α-HA and Activin β_B-Myc. Proteins precipitated with Myc-conjugated protein A/G agarose beads were resolved by SDS-PAGE and analyzed by IB with anti-HA antibody. WCLs were analyzed by IB with anti-HA and anti-Myc antibodies. IP: immunoprecipitation; Inhibin α-HA: pcDNA3.1-HA-Inhibin α; Activin β_A-Myc: pcDNA3.1-Myc-Activin β_A; Activin β_B-Myc: pcDNA3.1-Myc-Activin β_B. The heavy chain and light chain were indicated with black arrow.

(Fig. 6). Previous studies have shown that Inhibin and Activin share common β subunits (β_A and β_B) and Inhibin can exert its antagonistic effect by competitively binding to Act IIR receptor of Activin (Yamashita et al., 1995). Inhibin can form a high-affinity complex with the Activin receptor type II, which will block the intracellular signaling cascade of Activin molecules (Ahmad et al., 2020). As well as, previous studies showed that there was a potentially negative feedback between the pituitary and the gonad in zebrafish, which the Inhibin produced by the gonad can control the production of FSH from the pituitary (Poon et al. 2009). Therefore, we speculated as follows: on the one hand, Inhibin α subunit competitively combined with Activin β_A/β_B subunits to form Inhibin A/Inhibin B so that Activin was reduced, which might be one of the reasons for sterility of allotriploid crucian carp. On the other hand, the higher expression of Inhibin α in allotriploids might inhibit the secretion and synthesis of pituitary FSH in a dose-dependent manner through a negative feedback mechanism, the lower production of FSH might lead to abnormal gonadal development and gametogenesis, which might cause the sterility of allotriploids. In summary, these findings suggested that Inhibin α might participate in the regulation of gonad development of allotriploid crucian carp and laid the foundation for the molecular mechanisms of allotriploids sterility.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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