Molecular and Cellular Endocrinology 383 (2014) 1–9

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

Elevated expression of Piwi and piRNAs in ovaries of triploid crucian carp

Yi Zhou [1], Huan Zhong [1], Shaojun Liu *, Fan Yu, Jie Hu, Chun Zhang, Min Tao, Yun Liu

Key Laboratory of Protein Chemistry and Developmental Biology of State Education Ministry of China, College of Life Sciences, Hunan Normal University, Changsha 410081, China

ARTICLE INFO

Article history:
Received 20 July 2013
Received in revised form 19 October 2013
Accepted 28 November 2013
Available online 4 December 2013

Keywords:
Piwi
piRNAs
Triploid
Tetraploid
HPG axis

ABSTRACT

Increasing piRNAs provide RNA-interference pathways to regulate transposons and gene expression in germline cells. We demonstrate that *Piwi* transcripts are exclusively expressed in adult testes and ovaries in teleosts, with triploids showing the highest Piwi expression in the ovaries. Studies *in vivo* and *in vitro* showed that hCG and E2 treatment suppressed Piwi expression. We further cloned 200 small RNAs in the three kinds of fish. Seven piRNAs were obtained from all the three different ploidy fishes. During ovulation, five piRNAs showed significantly higher expression in the ovaries of sterile triploids than fertile diploids and tetraploids. Furthermore, E2 suppressed the expression of the six piRNAs at different levels *in vivo* and *in vitro*. The present study bridges the gap between the HPG axis and Piwi–piRNA pathway by suggesting that a dysfunctional HPG axis abrogated the piRNA suppression in triploid fish.

1. Introduction

Piwi proteins belong to the Argonaute family, which is characterized by the presence of PAZ and Piwi domains (Filipowicz, 2005). The Argonaute family is divided into two subclasses: Ago and Piwi families. Based on their interaction with microRNA (miRNA) and small interfering RNA, Ago proteins are well studied. In contrast, Piwi proteins were not fully understood until Piwi-interacting RNA (piRNA) was elucidated recently (Megosh et al., 2006; Seto et al., 2007; Höck and Meister, 2008). *Piwi* was first found in *Drosopila* as a crucial factor in germline stem cell maintenance, with *Piwi* mutations resulting in failure of germline stem cell division (Lin and Spradling, 1997; Cox et al., 1998). By contrast, *Piwi* mutations are limited to the male germline in mammals. In humans (*Homo sapiens*), four homologs of Piwis have been identified, including Hiwi, Hiwi2, Hiwi3 and Hili (Qiao et al., 2002). The mouse (*Mus musculus*) Piwi subfamily contains three homologs (Miwi1, Miwi2, and Mili) (Kuramochi-Miyagawa et al., 2001; Carmell et al., 2007). In lower vertebrates, such as *Xenopus* (Wilczynska et al., 2009) and zebrafish (*Danio rerio*) (Tan et al., 2002; Houwing et al., 2007, 2008), Piwis are expressed in both male and female gonads. Houwing et al. reported that Ziwi (*Piwi-like 1*, *Piwil-1*) and Zili (*Piwi-like 2*, *Piwil-2*) were exclusively expressed in the germline of zebrafish (Houwing et al., 2007, 2008). In addition, a Ziwi mutation resulted in germ cell apoptosis and a Zili mutation caused the failure of germ cell differentiation.

Similar to Ago proteins, Piwi proteins have been implicated in both transcriptional and posttranscriptional gene silencing. However, unlike the well-studied miRNA, piRNAs were not unraveled until 2006 (Girard et al., 2006; Lau et al., 2006). Several studies confirmed the role of piRNA in repressing transcription of the transposable elements (Houwing et al., 2007; Klattenhoff and Theurkauf, 2008; Grentzinger et al., 2012; Kawaoka et al., 2012). In addition, Robine et al. reported that some piRNAs were generated from 3′ untranslated regions (3′UTR) in *Drosophila* ovaries, murine testes, and *Xenopus* eggs (Robine et al., 2009). This study revealed that piRNA might regulate transcripts via partially complementary sequences, which is similar to miRNA. Meanwhile, a series of other studies have demonstrated that Piwi–piRNA complexes are essential in gene silencing and transposon regulation during germ cell differentiation and gonadal development in animals (Klattenhoff and Theurkauf, 2008; Grentzinger et al., 2012; Kawaoka et al., 2012).

In vertebrates, reproductive activity is regulated by the hypothalamic-pituitary-gonad (HPG) axis. Gonadotropin-releasing hormones secreted by the hypothalamus act on the pituitary gland to stimulate the synthesis of gonadotropins (GTH), including follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Charlton, 2008). By interacting with their receptors (GTH receptor, GTHr), particularly the LH receptor (LHr), gonads are stimulated to produce steroids that induce final maturation and ovulation. In a previous study, we reported that human chorionic gonadotropin (hCG) suppressed *Piwi* expression in common carp (*Cyprinus carpio*) (Zhou et al., 2012). However, the HPG axis regulation of Piwi–piRNA pathway has not been documented.

* Corresponding author. Tel.: +86 731 88873010; fax: +86 731 88873074.
E-mail address: lsj@hunnu.edu.cn (S. Liu).
[1] These authors contributed equally to this work.

0303-7207/$ - see front matter © 2013 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.mce.2013.11.019

In previous study, we obtained an allotetraploid population of F_3–F_{21} by crossing red crucian carp (*Carassius auratus* red var.) with common carp (Liu et al., 2001). We also produced triploids by crossing the males of allotetraploids with the females of Japanese crucian carp (*Carassius auratus langsdorfii*). The sterile and fast-growing triploids are commercially valuable, with the sterile offspring serving as a model for comparative studies in fish reproduction and aquatic application (Long et al., 2006). Previously, Long et al. reported that triploids showed disordered expression of HPG axis genes (Long et al., 2009). *In situ* hybridization results suggested that the gonadal GTHr expression in triploids was lower compared to diploids and tetraploids, which weakened the combination of GTHr with pituitary GTH, resulting in sterile triploids. However, the expression profile and the impact of the disordered HPG axis on the Piwi–piRNA pathway in sterile triploids, is still unavailable. Herein, we cloned *Piwi* and piRNAs and present their expression patterns in the ovaries of different ploidy fishes. In addition, using *in vivo* and *in vitro* approaches we examined the action of HPG axis on *Piwi* and piRNAs expression in ovary. Finally, we explored the differential expression levels of *LHr* among different ploidy fishes.

2. Materials and methods

2.1. Experimental fish

Diploid, triploid, and allotetraploid were provided by the Engineering Center of Polyploidy Fish Breeding of the National Education Ministry, Hunan Normal University. All the fish were anesthetized with 2-phenoxyenthanol before being euthanized. The fish were treated humanely, in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals for the Science and Technology Bureau of China. The tissues were frozen in liquid nitrogen and immediately stored at −80 °C, until further analysis.

2.2. Piwi cloning, phylogenetic analysis and tissue distribution

The total RNAs were extracted with TRIzol® Reagent (Invitrogen) and then processed with DNase I (Fermentas) to eliminate DNA contamination. First-strand cDNA was synthesized with RevertAid™ First Strand cDNA Synthesis Kit (Fermentas). The degenerate primers (Supplemental data 1) were designed, based on conserved sequences of other teleosts and polymerase chain reaction (PCR) was carried out to obtain core partial cDNA sequences of *Piwil-1* and *Piwil-2*. Subsequently, rapid Amplification of cDNA ends (RACE) was performed using SMART™ RACE cDNA Amplification Kit (Clontech). Specific nested primers were designed, based on the core partial sequences (Supplemental data 1).

Phylogenetic analysis was conducted to compare the sequences with other vertebrates using the MEGA 4.1 software (neighbor-joining method with 1000 bootstrap replicates).

Tissue distributions of *Piwi* transcripts were performed by reverse transcription-PCR (RT-PCR). The specific primers (Supplemental data 1) were designed with Primer Express® 3.0 (Applied Biosystems). We ensured that primer pairs for the *Piwis* did not cross-react, by sequencing the products.

2.3. Real-time RT-PCR

The primers were designed according to the cloned sequences (Supplemental data 1). Each test was repeated three times to improve the accuracy of the results in a Prism 7500 Sequence Detection System (Applied Biosystems). β-Actin was used as the internal control. The conditions were: 50 °C for 5 min and 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s and 60 °C for 45 s. Finally, melting curve analysis was performed to validate the specific generation of the expected product. Negative RT control and negative NTC control were employed to rule out DNA and/or dimer contamination.

2.4. Western blot analysis

Frozen ovarian samples were homogenized in RIPA buffer (Pierce) according to the manufacturer's instructions. Samples were resolved on 15% polyacrylamide gels and then transferred onto a PVDF membrane. The membranes were blocked and incubated with different antibodies (Piwil-1, SAB1300682, sigma) (Piwil-2 antibody, sc-68932, Santa Cruz Biotechnology), followed by incubation with horseradish peroxidase (HRP)-linked second antibody. The specificity of the primary antibodies was described in Supplementary data (Supplemental data 1). The signals were detected using ECL Reagent (Bio-Rad).

2.5. Immunohistochemistry

Using the 3,3-diaminobenzidine tetrahydrochloride (DAB) immunohistochemistry, we localized Piwi proteins in ovarian tissues. After deparaffinization in xylene, tissue sections (8 μm) were rehydrated in a series of ethanol and water solutions. Antigen retrieval was performed in citrate buffer (0.01 M, pH = 6), followed by washing in 3% hydrogen peroxidase in methanol to block endogenous peroxidase activity. After blocking with normal rabbit serum, the sections were incubated with either Piwil-1 or Piwil-2 antibody overnight. The sections were then incubated in anti-rabbit biotinylated secondary antibody followed by streptavidin-HRP. Finally, the signals were detected by DAB until positive staining was obtained. A negative control for the immunohistochemistry was included with rabbit immunoglobin G (IgG) (Santa Cruz Biotechnology).

2.6. Small RNA cloning

The protocol was based on Cloning Small RNAs for Sequencing with 454 Technology methods (Simon et al., 2009). Using 15% PAA urea gel, the total RNAs were separated, and the RNAs of desired size were excised. The small RNAs were isolated by 0.4 M NaCl and frozen in dry ice after spinning the gel slices through a microcolumn (Nanosep® 100 filter). After two rounds of ligation, the small RNAs were cloned and sequenced.

2.7. piRNA quantification by real-time quantitative RT-PCR

Real-time quantitative RT-PCR was performed using miScript SYBR Green PCR kit (Qiagen) according to the manufacturer's instructions. Specific primers for piRNAs were designed, based on the cloned piRNAs (Supplemental data 1). The amplification conditions were as follows: 50 °C for 5 min and 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s and 60 °C for 45 s. β-Actin was used as internal control.

2.8. In vivo experiments

Ovarian *Piwi* mRNA levels were analyzed during the pre-ovulation [2.5-years-old, November, Body weight (BW) = 358.53 ± 31.34 g, gonadosomatic index (GSI) = 0.047 ± 0.009, $n = 4$] and ovulation period (2-years-old, April, BW = 403.13 ± 21.09 g, GSI = 0.091 ± 0.011, $n = 4$). After RNA isolation and cDNA synthesis, *Piwi* expression levels were assayed.

We injected 8 adult (2.5-years-old, BW = 375.83 ± 43.94 g, GSI = 0.040 ± 0.007) red crucian carp females with hCG (Sigma).