Video Article

Dissection of Larval Zebrafish Gonadal Tissue

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Abstract

Although wild zebrafish possess a ZZ/ZW sex-determination system, domesticated zebrafish have lost the sex chromosome. They utilize a polygenic sex determination system, where several genes distributed throughout the genome collectively determine the sex identities of individual fish. Currently, the genes involved in regulating gonad development and how they work remain elusive. Normally, isolating gonadal tissue is the first step to examine the sex developmental processes. Here, we present a procedure to isolate gonadal tissue from 17 dpf (days post fertilization) and 25 dpf zebrafish larvae. The isolated gonadal tissue may be subsequently examined by morphology and gene expression profiling.

Video Link

The video component of this article can be found at https://www.jove.com/video/55294/

Introduction

The major female sex determinant in wild zebrafish chromosome 4 is lost or modified in the domesticated zebrafish (*i.e.* common lab strains)[1]. Instead, they have a polygenic sex determination system accompanied by environmental factors such as temperature, hypoxia, food availability and population density. The detailed mechanisms of zebrafish sex development are not fully understood. Fundamental questions such as when zebrafish sex determination occurs, what the primary sex determination signal(s) is/are, and which genes regulate the first step of gonad transformation remain to be answered[2,3].

In the process of zebrafish sex development, several important stages have been recognized. In the early stage of development, starting from 4 hpf (hours post fertilization) primordial germ-cells (PGCs) undergo specification, migration to genital ridge and proliferation. PGC numbers and reciprocal interactions between germ cells and somatic cells are important for gonad differentiation[4]. At 13 dpf (days post fertilization), the gonads are in the undifferentiated stage. By 17 dpf, the gonads develop into bi-potential ovaries in both future females and males. The apoptosis-dependent transition from ovary to testis begin at 21 to 25 dpf and may continue for several weeks. By 35 dpf, the sex of the gonad has been determined and sex-specific gamete production is underway in both ovaries and testes[5,6,7].

To date, diverse candidate genes and mechanisms of sex determination have been proposed. Proteomics and transcriptomic analysis have isolated many genes with sexually dimorphic expression and these genes have been utilized to study sex differentiation in zebrafish[8,9,10]. For example, in larval zebrafish, the *cyp19a1a* gene is specifically expressed in the ovary but not in the testis[11,12]. In addition, *amh* gene is weakly expressed in the ovarian follicle granulosa cells, but strongly in testis Sertoli cells[13]. In contrast, *vasa* gene is continuously expressed in the germ cells of both female and male zebrafish, making it a suitable gonad marker[14,15].

Investigating gonadal gene expression levels is critical to understand the molecular mechanism of sex determination and differentiation especially in the bi-potential ovary stage[3,9]. However, the small size of larval zebrafish and correspondingly small gonads complicate the isolation of gonadal tissue for further molecular analysis. Previous studies used dissected whole trunk region between the opercula and anal pore[16]. This preparation, although containing gonads, consists of multiple tissues and organs. Alternatively, transgenic animals with gonad-specific GFP expression such as vasa: EGFP were used for gonadal tissue isolation *via* fluorescence activated cell sorting (FACS) and laser capture micro-dissection[17,18]. But their widespread application is limited. Here, we describe a simple procedure to isolate gonadal tissue from larval zebrafish at 17 dpf and 25 dpf. We demonstrate the position of the gonads with respect to other organs and isolate the morphologically intact gonads from the surrounding tissues. We further show the gonad-specific genes such as vasa and *cyp19a1a* are highly expressed in the isolated gonads compared with the trunk tissue through quantitative PCR (qPCR) analysis. The present protocol allows identification, isolation, RNA purification and amplification of gonadal specific genes from larval zebrafish, thereby enabling subsequent molecular analysis of gonadal tissue[19].

Protocol

Zebrafish experiments were approved by the Fudan University Institutional Animal Care and Use Committee. Zebrafish were raised and bred according to standard procedures[20].

1. Preparations

1. Cultivate 17 dpf and 25 dpf larval zebrafish
    1. Transfer 2 male and 2 female adult zebrafish (healthy, 3 to 6 months old, laboratory AB strain) to a crossing tank in the late afternoon before the crossing day. Separate the males and females with a barrier. The next morning, refresh the tank water and remove the barrier to allow them to mate. Collect eggs in 100 mm Petri dishes 1 to 2 h after fertilization.
    2. Keep about 40 embryos in a 100 mm plate with 40 mL embryo medium (EM) at 28.5 °C during the early development period (4 days) and refresh the EM twice a day. Transfer larvae to 1 L tanks (40 larvae to 300 mL system water) and feed with live rotifers (full supply, twice a day) after 5 dpf. Feed live brine shrimp instead of rotifer diet after 10 dpf. Transfer the fish into re-circulating water system at 14 dpf[21].
    3. Raise larval zebrafish in the re-circulating water system till 17 dpf or 25 dpf. Ensure the light/dark cycle is 14/10 h and pH value of system water is about 7.2. Measure the body length of 17 dpf (5.5 to 6.8 mm) and 25 dpf (8 to 11 mm) larvae[22].

2. Prepare 2% agar plates for the dissection.
    1. Add 4 g agar to 200 mL sterile water. Heat the mixture in a microwave oven until it turns transparent.
    2. Cool the agar solution for about 15 min, then pour it into 60 mm diameter Petri dishes (approximately 1/3 volume of the plate). Store the solidified agar plates at 4 °C.

2. Protocol 1: Dissect the Gonadal Tissue of 17 and 25 dpf Larvae

1. Add crushed ice into the tank to anesthetize 17 dpf larvae. Transfer the anesthetized larvae to a 100 mm chilled Petri dish with 30 mL cold Ringer's solution. Keep the fish incubated in the chilled Ringer's solution for at least 15 min to fully anesthetize the larvae.
2. Transfer the larvae to a precooled agar plate with a small plastic spoon. Submerge the entire fish body in 10 mL chilled Ringer's solution and gently lay it on its side.
3. Do the following operations under the vision field of 25X stereo microscope. Clamp the fish trunk with tweezers for stabilization. Rip the abdomen longitudinally from the anus to the heart with another pair of tweezers. Gently remove the skin and muscles on one side of the body to expose the internal organs.
4. Remove the mass of organs ventral to the swim bladder carefully. Avoid damaging the gonad attached to the swim bladder.
5. Cut off the connection between the swim bladder and anterior body. Pull out the entire swim bladder and gonadal tissue gently.
   NOTE: In most cases, the gonadal tissue at 17 dpf contains surrounding epithelial tissue and protonephridium. They are not easily separated from each other, but at 25 dpf it can be easily separated. The gonad is also connected to the anus. A junction with left and right gonad connected to each other at the anus will be clearly visible.
6. Use tweezers to carefully separate the gonadal tissue from the swim bladder and clean up the surrounding adipose tissues.
7. Immediately transfer the isolated gonadal tissue to a prechilled 1.5 mL centrifuge tube containing 200 µL Ringer's solution. Keep the tube on ice until all gonadal tissues are separated from the larvae.
8. Dissect the gonads of 25 dpf larvae as described in steps 2.1 - 2.7.

3. Protocol 2: Analyze Gene Expression of the Isolated Gonadal Tissues

1. Extract total RNA from the larval gonadal tissues.
    1. Transfer the isolated gonadal tissues to a new RNase-free 1.5 mL tube and remove Ringer's solution. Add 100 µL lysis solution. Vortex until the tissues are completely lysed.
    2. Perform the total RNA extraction procedure according to the manufacturer's instructions.
    3. Add 1/10 volume of 10x DNase I buffer and 1 µL of DNase I to the RNA solution and mix gently. Incubate for 20 min at 37 °C.
    4. Add 1/10 volume DNase inactivation reagent to the RNA solution. Incubate for 10 min at 70 °C. Measure the concentration of the total RNA using a spectrophotometer. Store at -20 °C.

2. Perform first-strand cDNA synthesis
    1. Use an oligo(dT)-linker primer to perform first-strand cDNA synthesis following the manufacture's protocol. Add 1 to 2 µg of RNA to a total reaction volume to 20 µL.
    2. Incubate the reaction solution for 90 min at 45 °C. Terminate the reaction by heating at 70 °C for 5 min.
    3. Add 1 µL RNase H to the cDNA solution to remove the residual RNA. Mix gently and centrifuge for 10 s at ~ 13,000 x g. Incubate for 20 min at 37 °C. Add 20 µl nuclease free water. Store at -70 °C.

3. Perform fluorescent quantitative PCR
    1. Perform qPCR on a cycler system with a fluorescent dye. Use the following PCR cycling conditions: 35 cycles at 95 °C for 15 s, Tm-5 °C for 15 s, 68 °C for 30 s. Use primer sequences as follows: *amh* (fwd: GTGGATGGCAGCAGTACGAC; rev: GCGGAGAGGTGGAAGAGAGAATG), *cyp19a1a* (fwd: GTCCTGTTGTCTCCTACTGTCG; rev: CATTTGAGTTGAATATGATGCCCTG), *nanos3* (fwd: GCTCGGTGTACGCCAAATCAACAT; rev: CCAAGTGAAAACACAACACCAGTGC), *vasa* (fwd:

ATCGCATAGGAAGAACTGGACGCT; rev: CCAAGTGAAAACACAACACCAGTGC) and *β-actin* (fwd: AGTGCGACGTGGACATCCGTA; rev: GCACTTCCTGTGGACGATGGA)[19].

Representative Results

Dissections of the gonads were performed on AB strain larval zebrafish. **Figure 1** shows typical gonadal tissue of larval zebrafish at 17 dpf and 25 dpf. Firstly, the skin and muscles of one side of the abdomen is cut to expose the internal organs. After removing the mass of internal organs, the swim bladder together with the gonad remain in the trunk. The gonad was attached to the ventral side of swim bladder (arrow in **Figure 1B'**). At 17 dpf, the gonad was in the bi-potential ovary stage. The isolated gonad contained the left and the right gonad and was translucent (**Figure 1C**). In most cases, it was surrounded by epithelial tissue and protonephridium (arrow in **Figure 1C**). At 25 dpf the gonad was often wrapped by adipose tissues (**Figure 1D**). At this development stage, either big or small gonad may be observed. The immature ovary is big as the development of the secondary oocytes, and follicle cells increase the gonad volume (**Figure 1E**). The immature testis is small because of degeneration and apoptosis of the larval ovary (**Figure 1F**).

To analyze the molecular properties of the isolated gonadal tissues, we first examined gene expression levels of *amh*, *cyp19a1a*, *nanos3* and *vasa*, four gonad markers in zebrafish, by qPCR. Total RNA was extracted from larval gonadal tissues (n = 35) using a RNA isolation kit. In addition, we removed the head and tail of 17 dpf larva, and used the trunk tissue (between the structure of the heart and anal pore) to extract RNA as the control group (n = 15). The trunk tissue included skin, muscle, bone (vertebrae and ribs), swim bladder, kidney and gonad. Oligo dT-primed cDNA was used for qPCR. The qPCR result showed increases in *amh*, *cyp19a1a*, *nanos3* and *vasa* expression levels in 17 dpf isolated gonadal tissue by approximately 397, 342, 45 and 170-fold, respectively (**Figure 2**).

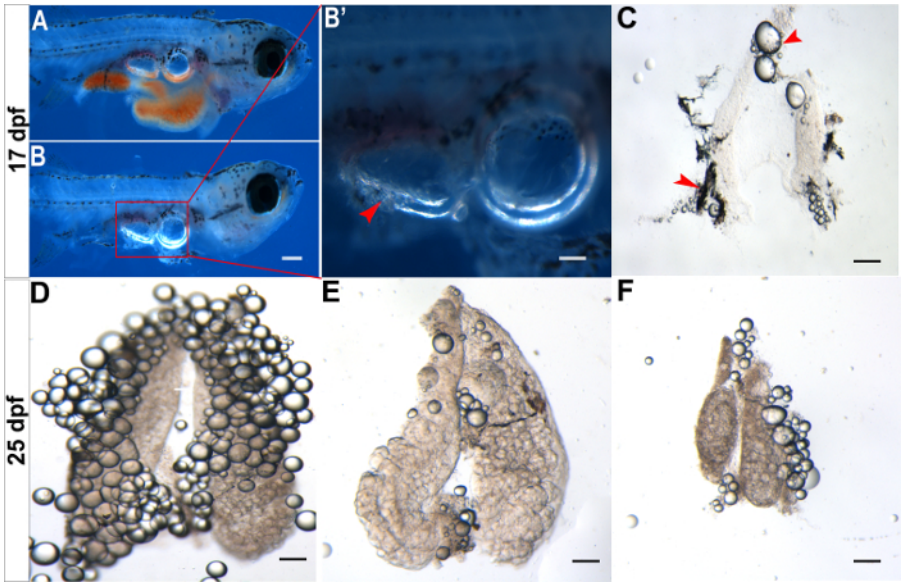

Figure 1. Microphotographs of Typical Gonadal Tissues in Larval Zebrafish at 17 dpf and 25 dpf. (A) Rip the skin and muscles of one side of the abdomen to expose the internal organs under a 25X stereo microscope. **(B)** After removing the mass of internal organs, the swim bladder and the gonad remain attached to the trunk. **(B')** Amplified view of the red box in panel B to show the relative position of the swim bladder and the gonad. The gonad is indicated by the arrow. **(C)** Isolated gonadal tissue at 17 dpf. The black tissues in the picture (arrows) are the endothelial tissue and protonephridium attached to gonad. **(D-E)** A big gonad before and after adipose tissue removal at 25 dpf. **(F)** A small gonad at 25 dpf. Scale bars: 200 μm. Please click here to view a larger version of this figure.

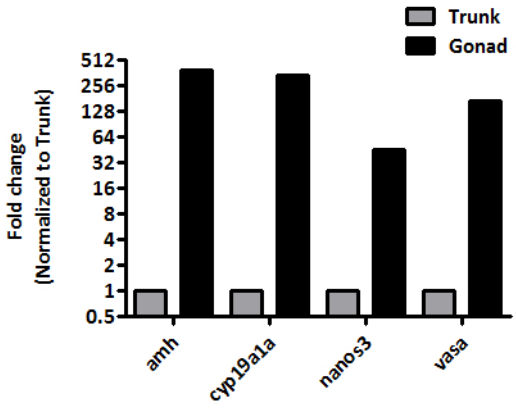

Figure 2. Normalized Gene Expression Levels of *amh, cyp19ala, nanos3* and *vasa* in the Trunks and Isolated Gonads at 17 dpf. Numbers of animals used: control group, n = 15; gonad group, n = 35. The trunk tissues in the control group are without the head and the tail structures. Gonad group refers to the isolated gonadal tissues. Please click here to view a larger version of this figure.

Discussion

The zebrafish has become a powerful model and is extensively used in development and disease-related research. The methods for isolation of organs in adult zebrafish such as brain, heart, gonad, and kidney, have been well documented[23,24,25]. Due to the small size and dynamic remodeling of the gonadal tissues in the larval zebrafish, isolation of gonadal tissue is a challenging task. Previous studies used whole dissected trunk tissues or transgenic vasa: EGFP based cell sorting and laser microdissection to examine the larval gonads[26]. The modifications of chromosome 4 during domestication makes the sex determination a mystery in the domesticated zebrafish. Our method described here can provide relatively clean and early gonad preparations for further morphological and molecular examinations, which can be helpful to explore the sex-determining mechanisms.

It is not easy to separate the developing gonad from other structures at early the development stages. Our method describes how to perform the dissection. To successfully perform this protocol, some critical steps need to be noted. First, the growth condition of larval zebrafish is critical to yield expected results. The gonadal development of larval zebrafish is a highly dynamic process. The size and appearance of gonadal tissues are determined by the development stages of the animals[27]. It is important to maintain different batches of zebrafish in standardized condition. The factors that may influence the growth of larvae include population density, duration of light and dark cycles, food availability and feeding schedules. The standard length measurement of larval fish can be used as a guide to determine the growth status of the animals[22]. A second critical factor for successful gonadal tissue isolation is a good understanding of the relative position and morphological differences between the gonads and the surrounding tissues. Because the gonad is located to the ventral side of the swim bladder, it is convenient to initially isolate the gonads together with the swim bladder. In addition, the distal end of the gonads is tightly attached to the distal end of gut. So one has to be careful when removing the gut from the gonad at the distal ends. For subsequent gene expression analysis, it is essential to use pre-chilled media and agar plate, and perform the whole procedure quickly.

A similar method has successfully been utilized by R.F. Ketting *et al.*[28]. They applied the gonad isolation method for investigating the function of piRNAs and the PIWI pathway in 3-week-old larvae. Here, we dissected gonadal tissue from zebrafish larvae as early as 17 dpf to explore the molecular identity and gene expression profile of the developing zebrafish gonad. Future molecular analyses of the earlier isolated gonadal tissue may be performed to determine the transcriptome, methylome and histone acetylation patterns to elucidate the mechanisms underlying sex development in the zebrafish.

Disclosures

The authors declare that they have no competing financial interests.

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