RESEARCH ARTICLE

Germ cell sex prior to meiosis in the rainbow trout

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ABSTRACT

Germ cells make two major decisions when they move from an indeterminate state to their final stage of gamete production. One decision is sexual commitment for sperm or egg production, and the other is to maintain mitotic division or entry into meiosis. It is unclear whether the two decisions are made as a single event or separate events, because there has been no evidence for the presence of germ cell sex prior to meiosis. Here we report direct evidence in the fish rainbow trout that gonias have distinct sexuality. We show that *dazl* expression occurs in both male and female gonias but exhibits differential intracellular distribution. More strikingly, we show that *boule* is highly expressed in male gonias but absent in female gonias. Therefore, mitotic gonias possess sex, sperm/egg decision and mitosis/meiosis decision are two independent events, and sperm/egg decision precedes mitosis/meiosis decision in rainbow trout, making this organism a unique vertebrate model for mechanistic understanding of germ cell sex differentiation and relationship between the two decisions.

KEYWORDS *boule*, *dazl*, germ cell sex, gonias, meiosis, trout

INTRODUCTION

A multicellular organism comprises two major cell lineages, the soma and germline. The germline founder in diverse animals is specified during early embryogenesis as primordial germ cells (PGCs), which migrate into the developing gonad

for the production of gametes (Wylie, 1999). Once in the gonad, germ cells make a first major decision for sexual commitment for sperm or egg production. At the stage of gamete production, germ cells make a second decision, namely the maintenance of mitotic division or entry into meiosis (Kimble and Page, 2007). Meiosis is a specialized cell division that occurs exclusively in germ cells. During embryonic development, germ cells increase their number by using the standard mitotic division that occurs also in somatic cells. They undergo mitosis also in adults for stem cell maintenance. Upon maturation, germ cells commence meiosis, which reduces the chromosome number from the typical two sets (diploidy) to a single set (haploidy) in sperm and eggs. A diploid state is restored at fertilization when a sperm and an egg are fused to form the zygote, the start point of a new life of the next generation. How germ cells make the two decisions have remained longstanding questions. It is even unclear whether the two decisions are made as a single event or separate events (Kimble and Page, 2007). There are two models. In one model, sperm/egg and mitosis/meiosis decisions are one and the same event, and a sex-specific regulator induces sex-specific entry into meiosis and commits the germ cell to differentiation as sperm or egg. In the other model, the two decisions are controlled by distinct regulators, and a sex-neutral mechanism induces meiotic entry but sex-specific regulators operate in parallel to generate sex-specific aspects of meiosis and differentiation as sperm or egg (Kimble and Page, 2007). The identification of a molecular sex marker for premeiotic germ cells will be critical to distinguish between the two models.

Sex is a mystery since long, because it is the basis of sexual reproduction in multicellular organisms including

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human. Although germ cells are the final decision maker and ultimate player to produce the male gametes sperm or the female gametes eggs, most studies on sex differentiation have so far focused on the sex of gonadal somatic cells (Kocer et al., 2009). Formation of testis-triggering Sertoli cells or ovary-triggering granulosa cells determines the gonadal sex, which in turn determines the organismal sex and germ cell sexuality (Kocer et al., 2009; Uhlenhaut et al., 2009). In the fish medaka, the gonadal male sex is determined by *dmy/dmrt1y* (Matsuda et al., 2002; Volff et al., 2007). Paradoxically, with germ cells, animals usually develop into two opposite sexes, male and female; without germ cells, they produce only one sex, male, pointing to a conserved interplay between germ cells and gonadal sex (Kurokawa et al., 2007). Gonads within a testis (spermatogonia) or an ovary (oogonia) contain germ stem cells capable of self-renewal and differentiation even in culture (Hong et al., 2004). Transplantation data in fish support that gonads are sexually indeterminate or plastic (Okutsu et al., 2006; Okutsu et al., 2007; Yoshizaki et al., 2010). However, there is no evidence for the absence or presence of gonadal sex (Kocer et al., 2009). Here we show in the fish trout that male gonads are *dazl*⁺/*boule*⁺, whereas female gonads are *dazl*⁺/*boule*⁻, providing direct evidence for germ cell sexuality prior to meiosis.

RESULTS AND DISCUSSION

We chose trout as a test-organism to study the sexuality of germ cells prior to meiosis, because in this organism, spermatogonia and oogonia have been demonstrated to take the male or female pathway according to the sex of a host gonad but irrespective of the sex of their origin (Okutsu et al., 2006; Yoshizaki et al., 2010). Recently, we have established that *boule* and *dazl* encoding RNA binding proteins of the DAZ family are germ cell markers in rainbow trout (Li et al., 2011). By using them and *vasa* as highly conserved germ cell marker also in rainbow trout (Xu et al., 2010), we performed *in situ* hybridization (ISH) to examine differential expression between male and female germ cells before and during meiosis.

In the adult testis, chromogenic ISH revealed that both *boule* and *dazl* were highly expressed in mitotic spermatogonia but undetectable in surrounding somatic cells (Fig. 1A and B), conforming to our previous observation and demonstrating the specificity of the ISH procedure. We then performed fluorescence ISH and examined RNA expression patterns before and during meiosis. Meiosis represents the earliest stage so far in mammals that shows germ cell sexuality at the cellular level (Kocer et al., 2009). In the adult testis, male germ cells at premeiotic, meiotic and post-meiotic stages of development can be clearly seen on the basis of relative locations and morphology (Fig. 1C). The *boule* signal was intense in gonads (mitosis), spermatocytes (meiosis)

and barely detectable in spermatids (post-meiosis), and distributed evenly in the cytoplasm of these cells (Fig. 1D). The expression of *dazl* was similar to *boule* in terms of developmental stages. However, we observed two salient features (Fig. 1E). One is the concentration of the *dazl* signal in several speckles in contrast to uniform *boule* distribution. The other is a relatively high level of expression in spermatids, in which *dazl* was found in the so-called chromatoid body (CB). Differences in stages and intracellular distribution became more evident upon merge (Fig. 1F). Thus, *boule* and *dazl* exhibit mitotic and meiotic germ cells expression, but possess different intracellular distribution in male.

In the young ovary, *boule* expression was absent in oogonia and became detectable in stage-I early oocytes, whereas *dazl* was easily detectable in both oogonia and oocytes and exhibited uniform cytoplasmic distribution (Fig. 2A), indicating the absence of *boule* expression in oogonia of immature female. In the adult ovary, absence of *boule* expression was also observed in oogonia, which displayed strong *dazl* expression (Fig. 2C–D). Interestingly, the *boule* signal was more abundant in the perinuclear region, whereas *dazl* distributed widely in the cytoplasm including the peripheral cytoplasm (Fig. 2D). These data suggest that *boule* expression is absent in oogonia of immature and mature females.

In rainbow trout, efforts have made toward the identification of several sex markers such as *dmrt1* and *foxl2*, which were found to show dimorphic expression at critical stages of gonadal development and sex differentiation (Baron et al., 2005; Vizziano et al., 2007). In these studies, sex-dimorphic expression of these genes was observed in the gonadal somatic cells. In the current study, we have revealed the differential expression of *boule* in male gonads and differential intracellular distribution of *dazl* transcript between male and female gonads in rainbow trout. Briefly, in rainbow trout, male gonads are *dazl*⁺/*boule*⁺, whereas female gonads are *dazl*⁺/*boule*⁻ instead. Furthermore, male gonads are *dazl*^s (speckle *dazl*), whereas female gonads are *dazl*^d (disperse *dazl*). In conclusion, rainbow trout male gonads are *dazl*⁺/*boule*⁺, whereas female gonads are *dazl*⁺/*boule*⁻ (Fig. 3). In fish, the expression of *boule*, *dazl* and *vasa* has also been examined in medaka (Xu et al., 2009), a laboratory fish model for germ cell biology (Herpin et al., 2007; Li et al., 2009) and stem cell biology (Hong et al., 1996, 1998; Yi et al., 2009). Notably, the medaka *dazl* expression also exhibits differential intracellular distribution between male and female gonads (Fig. 3), indicating that gonadal sexuality may not be limited to rainbow trout. Our data provide direct evidence in a fish that germ cells prior to meiosis possess an established sexuality as male or female gonads, instead of being sexually indeterminate. Our data also raise an interesting question as to whether *boule* and *dazl* are merely cellular markers or even important regulators of gonadal sexuality in rainbow trout.

Figure 1. Expression of *boule* and *dazl* transcripts in the adult trout testis. (A and B) Chromogenic ISH with antisense probe *boule* (A) and *dazl* (B). (C–F) Fluorescence ISH with antisense *boule* (green) and *dazl* (red) probes. Spermatogonia are *dazl*⁺/*boule*⁺. sg, spermatogonia; sc1, primary spermatocytes; sc2, secondary spermatocytes; st, spermatids; CB, chromatoid body; asterisk, somatic cells, ISH, *in situ* hybridization.

Figure 2. Expression of *boule* and *dazl* transcripts in the ovary. (A) One-year-old young ovary after fluorescence ISH with *boule* (green) and *dazl* (red) probes. (B–D) Three-year-old adult ovary after fluorescence ISH with *boule* (green) and *vasa* (red) probes. Oogonia (og) are *dazl*⁺/*boule*⁻. ISH, *in situ* hybridization. Scale bars, 50 μm.

Figure 3. Gonial sex. In rainbow trout and medaka, *vasa* expression and homogeneous distribution are seen in both male and female gonias, *dazl* expression is seen also in both male and female gonias but shows different distribution. Strikingly, *boule* shows expression and uniform distribution in both male and female gonias of medaka, but exhibits sex-specific expression only in male gonias of rainbow trout. In both rainbow trout and medaka, *vasa* expression in gonias does not show sex dimorphism. Data for rainbow trout are from the present study and (Li et al., 2011 In press), and data for medaka are according to (Xu et al., 2009).

Together with transplantation data (Okutsu et al., 2006; Yoshizaki et al., 2010), our finding suggests the plasticity of gonial sexuality for reprogramming into both sexes under the influence of the gonadal sex. Our findings suggest that sperm/egg decision and mitosis/meiosis decision are two independent events, and that sperm/egg decision appears to precede mitosis/meiosis decision.

We have shown that *boule* lacks expression in oogonia of both immature and mature ovaries. It will be interesting to determine in the future whether *boule* expression occurs in sexually indeterminate PGCs, or whether its sex-specific expression commences concurrently with gonadal/organismal sex differentiation. Although our study cannot reveal the role of *boule* and *dazl* in germ cell sex establishment, the ability to identify the germ cell sexuality prior to meiosis opens new avenues for studying sex differentiation directly in germ cells themselves.

In many organisms, *boule* expression has been reported to be meiotic in one sex (Xu et al., 2009; Li et al., 2011). One exception is medaka, where *boule* expression occurs in PGCs, mitotic and meiotic germ cells of both sexes (Xu et al., 2009). Therefore, the male-specific gonial expression of *boule* is not an evolutionarily conserved premeiotic germ cell marker. This situation appears to parallel the enormous diversity of somatic sex determination. For example, *dmy/dmrt1y* acts as a male determining gene in medaka (Matsuda et al., 2002; Nanda et al., 2002), which is, however, absent in other species of the genus *Oryzias* to which medaka belongs (Kondo et al., 2003); and *sox9* is a male-sex-determining gene in mammals but does not show sex-dimorphic expression in medaka (Nakamoto et al., 2005). The diversity of regulators governing sperm/egg decision and male/female decision provides the complexity to identify premeiotic germ cell sex in different organisms. Among germ cell markers examined so far, *boule* exhibits exceptional variation in its expression, ranging from male/female meiotic expression to bisexual mitotic and meiotic expression (Xu et al., 2009; Li et al., 2011). In this regard, it is not surprising that *boule* becomes a gonial sex marker in one fish but not in another. Whether *boule* and *dazl* show sex-specific expression or intracellular distribution in other fish species remains unknown. Our data make rainbow trout a prototype organism to study mechanisms underlying germ cell sexuality in a vertebrate.

CONCLUSION

We revealed differential expression of *boule* and differential intracellular distribution of *dazl* in premeiotic germ cells between rainbow trout male and female, providing convincing evidence for gonial sex in rainbow trout. This finding demonstrates sperm/egg and mitosis/meiosis decisions as two independent events, and puts germ cell sex ahead of mitosis/meiosis switch.

MATERIALS AND METHODS

Fish and chemicals

This study was carried out in strict accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals of the National Advisory Committee for Laboratory Animal Research in Singapore* and approved by this committee (Permit Number: 27/09). Rainbow trout females were purchased from a fish farmer in Würzburg, Germany, and adult testis samples were kindly provided by Dr. Bo Ma (the Heilongjiang Fisheries Research Institute, Ha'erbing City, China). Chemicals and enzymes were from Sigma and Promega, respectively, unless otherwise indicated.

Sections

The trout testis and ovary were fixed overnight or longer at 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) at 4°C. They were dehydrated by passing through an increasing series of ethanol (70%, 80%, 90% and 100%) for 1 h each. After an additional treatment in 100% ethanol, the samples were treated for 30 min each in a mix of Histoclear:ethanol (1:1) and 100% Histoclear. After a 30-min incubation in paraffin at 60°C, the samples were treated overnight in paraffin at 60°C and embedded in paraffin. The testis was cut at 5 µm and ovary at 8 µm on the Leica RM2135 Microtomes (Leica, Germany). Slides of sections were dewaxed by immersion in xylene with three changes, each for 10 min. After rehydration through a descending ethanol series (100%, 90%, 70%, 50% and 30%), the slides were subjected to treatments twice with PBS containing 0.1% Tween-20 (PBST) for 5 min each, once with 0.1 M HCl for 5 min and three washes in PBST for 5 min each. The samples were treated with proteinase K (10 µg/mL) for 30 min at 37°C, washed three times in PBST for 5 min each, and refixed in 4% for 15 min at room temperature. After three PBST rinses, the samples were subjected to *in situ* hybridization.

Chromogenic *in situ* hybridization

Chromogenic ISH by using BCIP/NBT as substrates was performed as described previously with minor modifications (Xu et al., 2005). Briefly, pOMBol, pOMdazl and pOMvasa were linearized with *Xho* I and *Sac* II and used for the synthesis of sense and anti-sense RNA probes from SP6 or T7 promoter by using the digoxigenin (DIG) Labeling Kit (Roche) for *dazl* and *vasa*, or the FITC RNA Labeling Kit (Roche) for *boule* (Li et al., 2011). The RNA probes were treated with RNase-free TURBO DNase (Ambion) and purified by using the LiCl method according to the supplier's instruction (cat #AM1340, Ambion). DIG probe and FITC probes were denatured and incubated at 90°C for 5 min followed by rapid cooling on ice. Chromogenic ISH was performed as described previously (Xu et al., 2005). Briefly, the probe was added at ~1 µg/mL to the hybridization buffer (50% formamide, 5 × SSC, 500 µg/mL yeast tRNA, 0.1% Tween-20 and 50 µg/mL heparin). The samples on each slide were covered with 100 µL of the hybridization buffer containing a probe and wrapped in parafilm. The slides were put into a Petri dish layered at bottom a Whatmann paper presoaked with 2 × SSC containing 0.1% Tween-20 (SSCT) and 50% formamide. The dish was sealed with parafilm and

incubated overnight at 65°C. The slides were washed twice in 2 × SSCT-50% formamide for 30 min each, twice in 2 × SSCT and twice in 0.2 × SSCT for 30 min each. After blocking for 1 h in PBST containing 10% goat serum at room temperature, the samples were incubated with either the alkaline phosphatase (AP) conjugated anti-DIG-antibody or AP-conjugated anti-FITC-antibody (Sigma) at 1:2000 in the blocking buffer (PBST containing 2% blocking reagent and 10% heat-inactivated fetal bovine serum) for 2 h at room temperature. Following six PBST washes and preincubation in the pre-staining buffer (NTMT; 100 mM NaCl, 50 mM MgCl₂, 100 mM Tris-Cl, pH 9.5, 0.1% Tween-20) for 30 min, the samples were incubated in the staining buffer (0.1 mg/mL NBT/BCIP in NTMT) in darkness at room temperature for 30–60 min or at 4°C overnight. Color development was microscopically monitored at regular intervals. Nuclear staining was done by using 4',6-Diamidino-2-phenylindole (DAPI; 1 µg/mL) and embedded in the Gold Antifade reagent (Invitrogen) for microscopy.

Fluorescence *in situ* hybridization

Two-color fluorescence *in situ* hybridization (FISH) (Campbell et al., 2007) was performed by using the tyramide signal amplification (TSATM) Plus Fluorescence Systems according to the manufacturer's instruction (NEL756, PerkinElmer Life Science)(Xu et al., 2009). Briefly, after hybridization and blocking as described above, the samples were incubated with the peroxidase (POD)-conjugated anti-FITC-antibody (Sigma) at a 12 000 dilution for 2 h at room temperature to detect the FITC-labeled probes. Following six PBST washes the samples were incubated for 30 min in the TSA-Fluorecein at a 1:100 dilution in the TSA Amplification Buffer (0.004% H₂O₂ in 0.1 M borate buffer, pH 8). The samples were then subjected to detection of the DIG-labeled probe: They were treated for 1 h in 1% H₂O₂ and incubated for two hours with the horseradish POD-conjugated anti-DIG antibody (Sigma) at a 1:2000 dilution, followed by a 30-min incubation in TSA-Cy3 for 30 min. The samples were finally stained for nuclei by using DAPI and embedded in the Gold Antifade reagent (Invitrogen) for microscopy. In this FISH procedure, two differently labeled antisense RNA probes are co-incubated with a sample in the hybridization step and subjected to sequential color development into green and red fluorescence, and nuclei are stained with DAPI for blue fluorescence. This procedure features multiple combinations between bright-field optics and three colors of fluorescent optics, allowing for precise comparisons of the relative signal intensity and distribution of different molecules. FISH in medaka provides detection sensitivity ~100 times that of the chromogenic ISH (Liu et al., 2009; Xu et al., 2009).

Microscopy and photography

Observation and photography under a Leica MZFIII stereo microscope, Zeiss Axiovertinvert and Axiovert upright microscopes with a Zeiss AxioCam M5Rc digital camera (Zeiss Corp) were as described previously (Xu et al., 2007).

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ABBREVIATIONS

AP, alkaline phosphatase; CB, chromatoid body; DAPI, 4',6-Diamidino-2-phenylindole; FISH, fluorescence *in situ* hybridization; ISH, *in situ* hybridization; PBS, phosphate-buffered saline; PFA, paraformaldehyde; POD, peroxidase

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