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In vitro germ cell differentiation during sex differentiation in a teleost fish

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ABSTRACT To clarify the sexually dimorphic mechanisms of gonadal sex differentiation, we established an *in vitro* culture system for gonadal sex differentiation using the teleost fish *Oreochromis niloticus*. *In vivo*, the entry of germ cells into meiosis occurs around 35 days after hatching (dah) in XX gonads, whereas in XY gonads, meiotic cells became differentiated around 85 dah. In our *in vitro* culture system using gonads from young fry at 23 dah, the meiotic cells in the XX gonads appeared after 21 days of culture. In contrast, in the XY gonads, no meiotic cells were detected after 21 days. These results indicate that germ cell differentiation in this culture system progresses in a manner similar to that *in vivo*. To identify the gene products that are involved in the entry of germ cells into meiosis or in the arrest of germ cells at the gonial stage of gonadal sex differentiation, we performed subtractive hybridization screening with this *in vitro* culture system. From the screening process, we identified the female-related gene, *FR-3,* which is a homolog of zebrafish *nanos-related gene (nos)*. The *nos* gene was expressed after gonadal formation around 35 dah in XX gonads, but not in XY gonads. *In situ* hybridization indicated that *nos* is expressed in oogenic meiotic cells, but not in spermatogenic meiotic cells. Further examination revealed that *nos* was expressed in oogenic meiotic cells after gonadal formation, specifically in teleost fish. Together, *nos* may be also involved in oogenic meiosis, with the exception of primordial germ cell migration.

KEY WORDS: *germ cell, meiosis, sex differentiation, teleost, in vitro*

Introduction

Gametogenesis consists of the following four events: 1) stem cell renewal; 2) proliferation; 3) meiosis; and 4) final maturation. In general, female gametogenesis in the form of oogenesis proceeds faster than male gametogenesis in the form of spermatogenesis. In particular, meiosis is the most important event in gametogenesis. Although many researchers have tried to clarify the mechanisms of meiosis, the details remain unclear. In meiosis, there are two crucial phenomena: the entry of embryonic germ cells into meiosis during gonadal sex differentiation (sex differentiation of germ cells and oogenesis), and the entry of spermatogenic cells into meiosis, i.e., the development of spermatocytes (spermatogenesis). For mammals, previous reports have suggested the possibility that meiosis-inducing and meiosis-preventing substances are present during gonadal sex differentiation (McLaren, 2003). Recent reports have indicated that in mice, loss of retinoic acid signaling plays an important role in the entry of embryonic germ cells into meiosis via CYP26B1 (Bowles *et al*., 2006; Koubova *et al*., 2006). However, it remains unclear whether this represents a conservative cascade for germ cell differentiation in non-mammalian vertebrates.

In lower vertebrates, especially teleost fish, many studies have been conducted on gonadal sex differentiation (Nakamura *et al*., 1998). Recent studies implicate *DMY* (a *DMRT1* homolog) as a male-determining gene in the teleost fish medaka, *Oryzias latipes* (Matsuda *et al*., 2002; Nanda *et al*., 2002), although *DMY* is present only in two species of the genus *Oryzias* (Matsuda *et al*., 2003). Nevertheless, the mechanisms of gonadal sex differ-

Abbreviations used in this paper: dah, days after hatching; DMC, dosage suppressor of mck1 homolog; FR, female-related gene; nos, nanos-related gene.

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entiation, which include sexual dimorphisms of germ and somatic cells, are not yet understood. In vertebrates, one of the conservative phenomena for gonadal sex differentiation is the entry of embryonic germ cells into meiosis during gonadal sex differentiation in females (McLaren and Southee, 1997). In some species of lower vertebrates, before entry of the embryonic germ cells into meiosis, germ cell proliferation occurs in the female gonads, whereas no proliferation is observed in the male gonads (Hardisty, 1967). However, to date, no information has emerged on the mechanisms underlying the entry of embryonic germ cells into meiosis during gonadal sex differentiation, especially in lower vertebrates, mainly due to a lack of information regarding the molecular events and adequate functional analysis, such as an *in vitro* system for analyzing meiosis during gonadal sex differentiation. To clarify the mechanisms of meiosis, tools that specifically recognize the meiotic stage are required. Dmc1 and Scp3 are specific gene products that are expressed in the leptotene to pachytene meiotic prophase (Wolgemuth, 2003). In yeast, the absence of Dmc1 prevents interhomolog interactions, which illustrates the role of Dmc1 in promoting interhomolog recombination during meiosis (Schwacha and Kleckner, 1997). Furthermore, the Dmc1-null mutation in mice causes meiotic arrest at the zygotene stage without homolog synapsis (Pittman *et al*., 1998) or with occasional synapsis between non-homologs (Yoshida *et al*., 1998). The role of Dmc1 in promoting interactions between homologous chromosomes appears to be conserved from yeast to mammals (Tarsounas *et al*., 1999). On the other hand, our recent study indicates that the Dmc1 protein is expressed in meiotic prophase specifically during spermatogenesis in the teleost fish *Anguilla japonica* (Kajiura-Kobayashi *et al*., 2005).

The teleost fish Nile tilapia *Oreochromis niloticus* is one of most intensely studied species with respect to gonadal sex differentiation (Nakamura *et al*., 1998; Kobayashi *et al*., 2000, 2002, 2003, 2008), although a sex-determining gene has not yet been identified. In tilapia, germ cell proliferation occurs and the cells enter into meiosis in genetic females, whereas in genetic males, the germ cells never enter into meiosis during gonadal sex differentiation. To clarify the mechanisms of sexual dimorphism of germ cell differentiation during gonadal sex differentiation, an *in vitro* culture system that enables functional analysis is required. Therefore, we attempted to establish an *in vitro* culture system for germ cell differentiation during gonadal sex differentiation in tilapia, with emphasis on the entry of germ cells into meiosis. In our *in vitro* system, germ cell differentiation and meiosis during gonadal sex differentiation progress in a manner similar to that observed *in vivo*. To identify the gene products that are involved in the entry of germ cells into meiosis or in germ cell arrest at the gonial stage of gonadal sex differentiation, we performed subtractive hybridization screening using this *in vitro* culture system.

Results

Germ cell differentiation in vivo

In tilapia, the gonadal primordium is formed at 3 dah. The first sexual dimorphism appears after 9 dah as a difference in germ cell numbers between the sexes, and the second sexual dimorphism appears at 20-25 dah as a difference in histogenesis between the sexes, e.g., the formation of the anlagen of the intratesticular efferent duct and ovarian cavity in XY and XX fry, respectively. Meiotic cells first appear after 35 dah in XX fry, but not in XY fry. In XY fry, the initiation of spermatogenesis is seen after 50 dah, and the first meiotic cells are observed around 85 dah. Our findings for the histogenesis are in agreement with those reported previously (Kobayashi *et al*., 2000; 2003; 2008) and are summarized in Fig. 1.

TABLE 1

EFFECTS OF 17A-METHYLTESTOSTERONE ON SEX DIFFERENTIATION IN TILAPIA*

	Male **	Female**
XX control	0/40	40/40
XX MT-treatment	45/45	0/45

* XX fry were treated with 17a-methytestosterone (MT) from 12 to 20 dah.
** Phenotypic sex examined at 50 dah.

Specific expression of Dmc1 in meiotic germ cells

The cloning of the tilapia Dmc1 and Rad51 cDNAs and their phylogenetic analysis have been published elsewhere (Kajiura-Kobayashi *et al*., 2005). In this study, we examined the localization of Dmc1 protein in the gonads using the anti-Dmc1 antibody. Immunoblot analysis for the Dmc1 protein was performed using testis preparations and recombinant tilapia Dmc1 and Rad51 protein derived from the rabbit reticulocyte lysate system. Dmc1 appeared as a 37-kDa band in the testicular protein preparation. When the anti-Dmc1 antibody was used in blots with recombinant Dmc1 and Rad51, only the recombinant Dmc1 protein was immunostained (Fig. 2A), which suggests that this antibody specifically recognizes tilapia Dmc1. Immunohistochemical examination of Dmc1 showed that in the testis, Dmc1 was localized

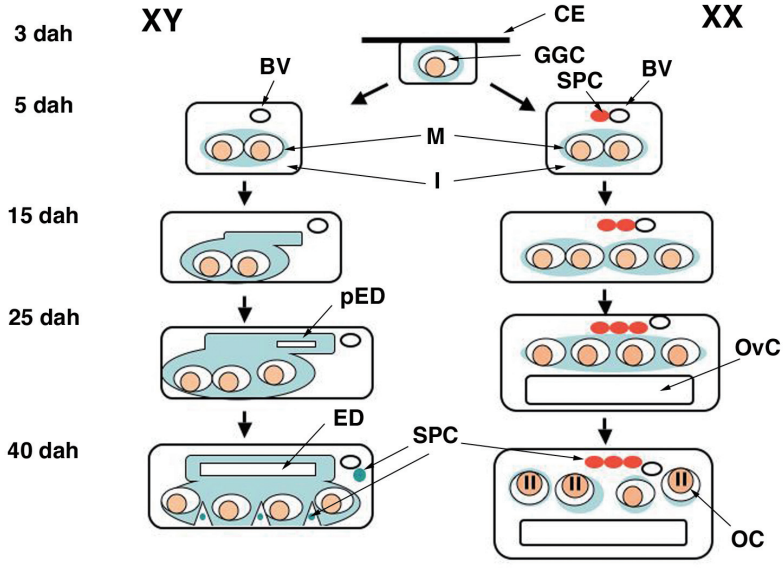

Fig. 1. Sexual dimorphism during gonadal differentiation in tilapia. *Schematic representation of gonadal histogenesis during gonadal differentiation. CE, coelomic epithelium. GGC, gonial germ cell. SPC, steroid producing cell. BV, blood vessel. OvC, ovarian cavity. pED, anlagen of intratesticular efferent duct. Oc, oocyte. ED, intratesticular efferent duct. M, medullary cell. I, interstitum. Dah, days after hatching.*

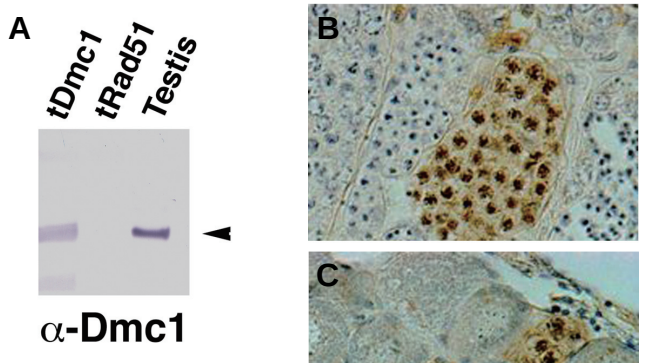

Fig. 2 (Left). Specificity of anti-Dmc1 antibody and localization of Dmc1 protein. (A) *Specificity of anti-Dmc1 antibody. Anti-Dmc1 antibody recognized recombinant Dmc1 protein, but not recombinant Rad51 protein. Anti-Dmc1 antibody recognized 37-kDa band in the testicular protein preparation.* **(B)** *Dmc1 was localized in spermatocytes, but not in spermatogonia, spermatids or spermatozoa. Further examination revealed that Dmc1 was localized in early primary spermatocytes but not in the late primary spermatocytes, which included the pachytene to diplotene primary spermatocytes.* **(C)** *In the ovary, Dmc1 was localized in early oocytes, particularly at the leptotene to zygotene stage, whereas no specific staining was detected in the oogonia or in oocytes after the pachytene stage.* **(D)** *This antibody also immunostained the medaka Dmc1 protein in meiotic cells that were similar to those of tilapia. Scale bar, 10 μm.*

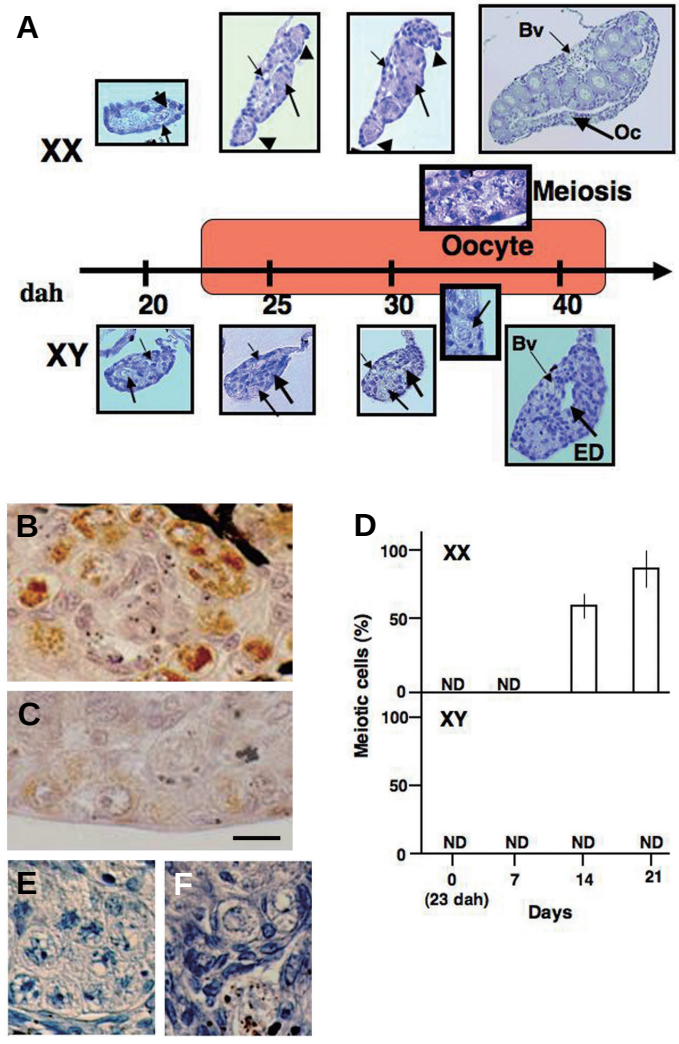

Fig. 3 (Right). Germ cell differentiation *in vitro* organ culture. (A) *Germ cell differentiation* in vivo. *Red region indicated the duration of* in vitro *organ culture of this study.* **(B,C)** *Immunohistochemical examination using the anti-Dmc1 antibody indicated that some of the germ cells were in meiosis. After 21 days of culture, the XY gonads contained a few germ cells but no meiotic cells* **(C)**, *while the XX gonads contained numerous germ cells and meiotic cells* **(B)**. **(D)** *Time schedule for the entry of germ cells into meiotic stage. When the gonads from XX fry at 23 dah were grown in the organ culture system, none of the germ cells in either sex had entered into meiosis at 7 days of culture, while only the XX gonads had entered into meiosis at 14 days of culture.* **(E,F)** *When XX fry were treated* in vivo *with 17α-methyltestosterone (MT) from 12 to 20 dah, complete sex reversal (female to male) was induced (Table 1). The XX gonads generated by MT treatment of the XX fry at 23 dah were applied to the* in vitro *culture system.* **(E)** *Control XX gonads.* **(F)** *MT treated XX gonads. After culturing for 21 days, the XX gonads did not contain any germ cells that had entered into meiosis, in similarity to XY gonads (C). Scale bar, 10 μm.*

in the spermatocytes but not in the spermatogonia, spermatids or spermatozoa. Further examination revealed that Dmc1 was localized in the early primary spermatocytes but not in the late primary spermatocytes, which included the pachytene to diplotene primary spermatocytes (Fig. 2B). In the ovary, Dmc1 was localized in early oocytes, particularly at the leptotene to zygotene stage, whereas no specific staining was detected in the oogonia or in oocytes after the pachytene stage (Fig. 2C). This specific staining pattern for Dmc1 was observed in the nuclei but not in the cytoplasm (Fig. 2B, C). This antibody also immunostained the medaka Dmc1 protein in meiotic cells, which were similar to those of tilapia (Fig. 2D). These findings indicate that Dmc1 expression occurs exclusively during the early meiotic stages in both sexes, and that Dmc1 protein represents an ideal molecular marker for identifying the entry of germ cells into meiosis in teleost fish, including tilapia.

In vitro *germ cell differentiation*

When the gonads from XX fry at 23 dah were grown in the organ culture system, none of the germ cells in either sex had entered into meiosis at 7 days of culture, while only the XX gonads had entered into meiosis at 14 days of culture (Fig. 3). Immunohistochemical examination using the anti-Dmc1 antibody demonstrated that some of the germ cells were in meiosis. After 21 days of culture, the XY gonads contained a few gonial germ cells but no meiotic cells (Fig. 3 C,D), while the XX gonads contained numerous gonial germ cells and meiotic cells (Fig. 3 B,D). These results indicate that our *in vitro* culture system simulates the genetic sex-

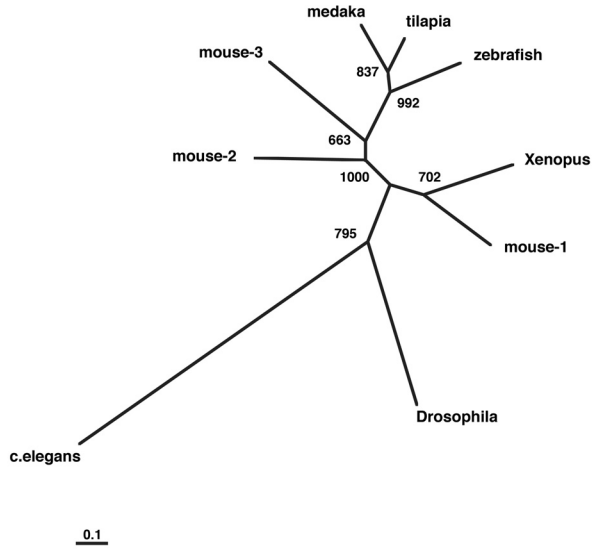

Fig. 4 (Left). Phylogenetic tree of the amino acid sequences of nanos family. *The tree was constructed with the neighbor-joining method. The numbers indicate bootstrap values from 1000 replicates. Horizontal lines indicate genetic distances. The GenBank accession numbers are: tilapia; AB453384, medaka; EU074259, zebrafish; NM131878,* Xenopus*; X72340, mouse (nos-1); NM178421, mouse (nos-2); NM194064, mouseu (nos-3); NM194059, Drosophila; M72421, C. elegans; NM063051.*

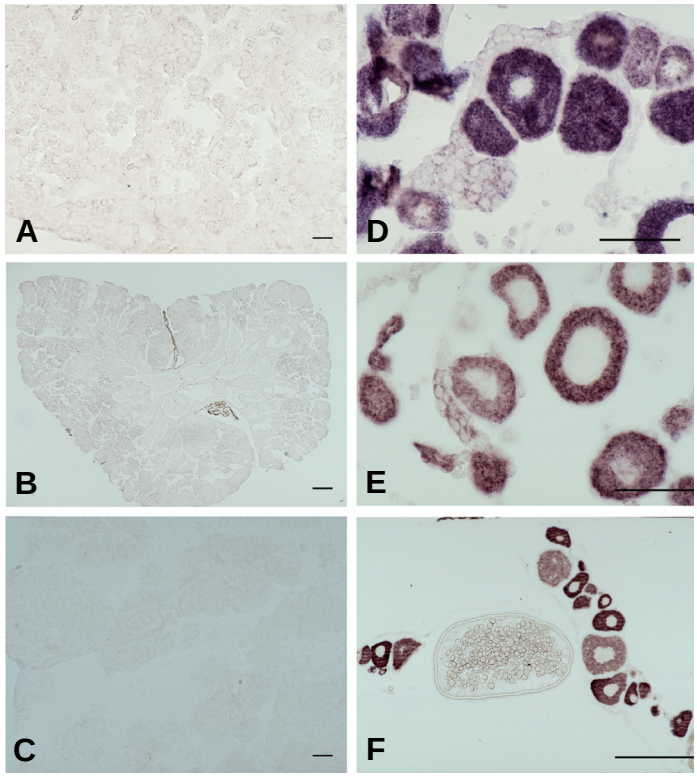

Fig. 5 (Right). Gene products involved in the entry of germ cells into meiosis. (A-C) *Testis;* **(D,E)** *Ovary.* **(A,D)** *Tilapia.* In situ *hybridization analysis for FR-3 (tNos-1) indicated that tNos-1 was expressed in the germ cells of the XX gonads, particularly in the oogenic meiotic cells* **(D)**, *but not in testicular cells.* **(B,E)** *Medaka.* **(C,F)** *Zebrafish. We also examined the expression profiles of nos-1 after gonadal formation in zebrafish and medaka. The zebrafish and medaka* nos-1 *genes were expressed in oogenic meiotic cells, specifically after gonadal formation, but not in testicular germ. Scale bar, 50 μm.*

dependent germ cell differentiation that occurs during gonadal sex differentiation *in vivo*. When XX fry were treated *in vivo* with 17α-methyltestosterone (MT) from 12 to 20 dah, complete sex reversal (female to male) was induced (Table 1). Therefore, we applied the XX gonads generated by MT treatment of the XX fry at 23 dah to the *in vitro* culture system. After culturing for 21 days, the XX gonads did not contain any germ cells that had entered into meiosis (Fig. 3F), in similarity to XY gonads described above. This suggests that XX gonads become differentiated as male gonads rather than as female gonads within 3 days after the induction of sex reversal by an androgen.

Screening of gene products involved in the entry of germ cells into meiosis

To identify gene products that are potentially involved in the entry of germ cells into meiosis or in the arrest of germ cells at the gonial stage of gonadal sex differentiation, we performed subtractive hybridization screening using the *in vitro* culture system. In all, 15 gene products were identified, 11 of which were expressed dominantly in XX gonads. One of the female-related genes, FR-3, was found to be similar to the zebrafish nanos-related gene 1 (nos) (Fig. 4). This gene was more similar to mouse nos-3 as compared to mouse nos-1 and nos-2. *In situ* hybridization analysis showed that FR-3 was expressed in the germ cells of the XX gonads, particularly in the oogenic meiotic cells (Fig. 5B). RT-PCR analysis for FR-3 indicated that FR-3 was expressed in the XX gonads around 35 dah, at which time-point the entry of germ cells into meiosis was observed. In contrast, FR-3 expression was not observed in the germ cells of the XY gonads (Fig. 6). To determine whether tilapia FR-3 is a nos homolog, we examined the expression profiles of nos after gonadal formation in zebrafish and medaka. The zebrafish and medaka nos genes were expressed in oogenic meiotic cells, specifically after gonadal formation (Fig. 5 C-F), but not in testicular germ cells and oogonia. In addition, nos was expressed in migrated primordial germ cells, and its expression decreased with gonadal formation in tilapia (data not shown), as seen for zebrafish nos-1 and medaka nos (Koprunner *et al.*, 2001; Kurokawa *et al.*, 2006). These results indicate that the tilapia FR-3 is a nanos-related gene homolog

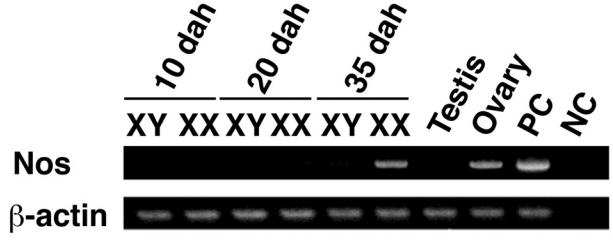

Fig. 6. RT-PCR analysis for *tNos-1* during gonadal differentiation in tilapia. *RT-PCR analysis indicated that* tNos-1 *was expressed in the XX gonads around 35 days after hatching, at which time-point the entry of germ cells into meiosis was observed (Kobayashi* et al.*, 2002; 2008). In contrast,* tNos-1 *expression was not observed in the XY gonads.*

(nos), and suggest that nos is expressed in oogenic meiotic cells, especially after gonadal formation, in teleost fish.

Discussion

In this study, we developed an *in vitro* culture system for sex differentiation, which simulates the entry of germ cells into meiosis in XX fry and the arrest of germ cells in the gonial stage in XY fry, in the teleost fish tilapia. Our experiments with this *in vitro* culture system demonstrate that germ cell differentiation (including meiosis) during gonadal sex differentiation progresses in a manner similar to that observed *in vivo*. To our knowledge, this is the first report that describes an *in vitro* culture system for studying the sexual dimorphism of germ cells during gonadal sex differentiation, with the exceptions of similar systems developed for the study of these phenomena in mammals.

Recent studies have elucidated some of the cellular and molecular events that occur during testicular somatic cell differentiation, such as testis cord formation (Tilmann and Capel, 2002). Nevertheless, many aspects of gonadal development, including the sex differentiation of embryonic germ cells, remain largely unclarified. In the mouse, sex differentiation of the somatic elements of the XY gonad is visually evident as the testis cord by E12.5. It is not until E13.5 that the first meiotic germ cells appear in the XX gonads; in contrast, the XY germ cells cease mitotic proliferation and arrest as prospermatogonia (McLaren, 1984, 2000). In studies using chimeric gonads, it has been revealed that XX embryonic germ cells in the testicular environment develop as male prospermatogonia, and that XY germ cells in the ovarian environment enter into meiosis, which suggests that the differentiation of germ cells as males or females is dependent upon the somatic environment rather than the sex chromosome content of the germ cell (mouse: McLaren and Monk, 1981; Taketo-Hosotani *et al.*, 1989; Palmer and Burgoyne, 1991; medaka: Shinomiya *et al.*, 2002). In the present study, when sex reversal of XX gonads was induced by androgen treatment from 12 to 20 dah, thereafter the XX germ cells never entered into meiosis during gonadal sex differentiation *in vivo* or *in vitro*, i.e., they resembled XY germ cells in this respect. In tilapia, sex differences in the histoarchitecture of the gonads are evident at 20 to 25 dah, particularly with respect to the ovarian cavity and the anlagen of the intratesticular efferent duct (Nakamura and Nagahama, 1985, 1989; Kobayashi *et al.*, 2000, 2008). A recent our report also indicated that androgen-induced testicular differentiation of XX fry caused the induction of DMRT1 expression in medullary cells including germ cell-surrounding cells (Kobayashi *et al.*, 2008). These results suggest that the development of teleost germ cells during gonadal sex differentiation is dependent upon the somatic milieu, as in the mouse.

In the present study, we attempted to screen for gene products involved in meiosis during gonadal sex differentiation using our established *in vitro* culture system. One of the identified gene products, FR-3, was expressed in oogenic meiotic cells, particularly after gonadal formation. Interestingly˘FR-3 is similar to the zebrafish nanos-related gene 1 (nos). It remains unclear as to whether the nanos homolog is expressed in oogenic cells after gonadal formation in non-mammalian vertebrates. For the mouse, three types of nanos homolog have been reported (Haraguchi *et al.*, 2003; Tsuda *et al.*, 2003), each of which has a specific expression profile and function, i.e., nanos-1 is expressed predominantly in the central nervous system, and nanos-2 and nanos-3 are expressed in germ cells in a cell-specific fashion. In particular, nanos-2 and nanos-3 are expressed in male PGCs and migrating PGCs, respectively. In fish, nos is expressed in migrating PGCs (Koprunner *et al.*, 2001; Kurokawa *et al.*, 2006). This suggests that the expression profile of nos in teleost fish after gonadal formation is similar to that in the mouse, and that the expression during PGC migration is similar to that of nanos-3 in the mouse. However, in contrast to nanos-2 and nanos-3 in the mouse, the fish nos is not expressed in germ cells after the PGC reach the gonadal anlagen in both sexes, and it is re-expressed in oogenic meiotic cells but not in oogonia or XY germ cells. This suggests that fish nos plays a role in oogenesis that is distinct from that of the homolog in the mouse. Although the role of nos in oogenesis has not been elucidated, to date, a recent study suggests that nos plays a role for maintain of oocytes in zebrafish (Draper *et al.*, 2007). Together with the expression profiles of nos in teleosts, the role of nos for oogenesis may be conserved in teleost fish including zebrafish, tilapia and medaka. Further studies are necessary for clarification of the function of nos during oogenesis in teleost fish.

In the present study, we established an *in vitro* culture system for germ cell differentiation during gonadal sex differentiation, including the meiotic transition in females and the arrest of germ cells in the gonial stage. Recent reports have suggested that the retinoic acid pathway and pstgD2/PGC-7 play important roles in meiotic transition during gonadal sex differentiation in the mouse (Adams and McLaren, 2002; Bowles *et al.*, 2006; Koubova *et al.*, 2006). However, it remains unclear whether these molecules are conserved in vertebrates. Recent findings suggested that 17α,20β-dihydroxy-4-pregnen-3-one (17,20β-DP) is involved in initiating meiosis in the teleost fishes, male eel and female Japanese huchen as well as common carp (Miura *et al.*, 2006; 2007). In addition, a recent report in tilapia showed that teleost specific Cyp17 isoform, *Cyp17a2*, lacking lyase activity synthesized 17,20β-DP efficiently and exhibited sexual dimorphic expression during gonadal differentiation by starting the expression at 11 and 75 dah in XX and XY gonads, respectively (Zhou *et al.*, 2007). In tilapia, the initiation of meiosis occurred around 35 and 85 dah (Kobayashi *et al.*, 2000; 2008). Although a time lag exists between *Cyp17a2* and the initiation of meiosis, 17,20β-DP might be involved in initiating meiosis during gonadal differentiation. Further study will be necessary to demonstrate this hypothesis. To address these questions, further analyses using the novel *in vitro* culture system established in this study will be available as a powerful tool.

Materials and Methods

Animals

Nile tilapia were kept in re-circulating fresh-water tanks with a capacity of 500 L at 26 ± 1°C until use. As described in our previous report (Kobayashi *et al.*, 2000), all the genetic females (XX) and males (XY) were obtained by artificial fertilization of eggs from a normal female (XX) with sperm from a sex-reversed male (XX) or from a supermale (YY), respectively. Fertilized eggs were cultured in re-circulating water at 26 ± 1°C. In our system, no hermaphroditic gonads were observed, as described previously (Kobayashi *et al.*, 2000, 2002, 2003).

Induction of sex reversal

The XX fry were fed commercial food (OTOHIME, Marubeni-Nisshin Feed Co. Ltd., Japan) containing 10 μg 17α-methyltestosterone (MT;

Sigma Co. Ltd., St. Louis) per gram of diet 12 to 20 days after hatching (dah). This food was given four times a day. The food was immersed in ethanol or ethanol containing MT for the control and the MT treatment, respectively. Then, the food was dried at 37°C overnight. MT containing food caused complete sex reversal to functional males (12 to 20 dah: 45/45 males and 0/45 females, see Table 1).

Production of the anti-Dmc1 antibody

To generate antibodies against Dmc1, we used as the immunizing antigen an oligopeptide that corresponds to the C-terminal amino acid sequence (tilapia Dmc1: AB18246, Kajiura-Kobayashi *et al.*, 2005) predicted from tilapia Dmc1 cDNA, with the addition of a cysteine at the N-terminus to facilitate linkage to the carrier protein, as described previously (Kobayashi *et al.*, 1996). Conjugation of the antigen to bovine serum albumin (BSA, fraction V; Sigma Chemical Co., St. Louis, MO) as the carrier protein was performed as described previously (Kobayashi *et al.*, 1998, 2002). Female Guinea pigs were immunized at 2-week intervals with four subcutaneous injections of peptide-linked protein (0.5 mg/Guinea pig/injection). Each antigen was emulsified in Freund's complete adjuvant for the first injection and in Freund's incomplete adjuvant for subsequent injections. One week after the last injection, blood samples were collected. Serum was separated and purified by affinity chromatography using the appropriate synthetic peptide. The specificity of the purified Dmc1 antibody was determined by assaying for reactivity with recombinant tilapia Dmc1 and Rad51 (Rad51: AB182647; Kajiura-Kobayashi *et al.*, 2005), which is a Rec A homolog and not a meiosis-specific gene product, generated using the rabbit reticulocyte lysate system (Promega, Madison, WI). In addition, proteins extracted from the ovary and testis were immunoblotted with the antibody, as described previously (Kobayashi *et al.*, 1996, 1998a,b). Anti-Dmc1 and anti-His antibodies were used at 1:1000 and 1:500 dilutions, respectively.

In vitro *organ culture*

The XX and XY gonads from fry 23 days after hatching (dah) were applied to an *in vitro* organ culture system, which comprised L-15 medium/10 mM Hepes (pH 7.4) that contained 0.5% BSA (fraction V), at 26°C. Briefly, gonads were placed onto the floats of 1.5% agarose block covered with a nitrocellulose membrane in 24-well plastic tissue culture dishes. The culture medium was exchanged once a week. After culture, the gonads were fixed in Bouin's fixative solution and embedded in paraffin. The gonads (5-μm-thick sections) were immunostained with 1:1000 dilutions of the anti-Vasa (Kobayashi *et al.*, 2002) and anti-Dmc1 antibodies. The sections were then stained with Carazzi's hematoxylin. Immunohistochemistry methods used have been described previously (Morrey *et al.*, 1998; Kobayashi *et al.*, 1998a, b; 2002).

Subtractive hybridization screening using the* in vitro *culture system

Subtractive hybridization screening was performed as described previously (Kobayashi *et al.*, 2003), using total RNA samples derived from XX and XY gonads that were cultured for 14 days (corresponding to 37 dah gonads) in the organ culture system described in the present study.

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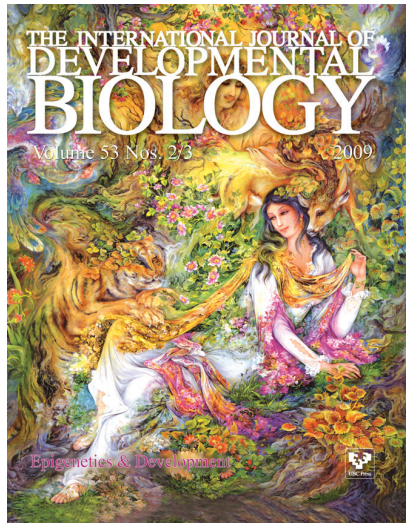

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