

# Sexual dimorphism of gonadal structure and gene expression in germ cell-deficient loach, a teleost fish

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Germ cell-deficient fish usually develop as phenotypic males. Thus, the presence of germ cells is generally considered to be essential for female gonadal differentiation or the maintenance of ovarian structure. However, little is known of the role of germ cells in the determination of the sexual fate of gonadal somatic cells. We have established an inducible germ cell deficiency system in the loach (*Misgurnus anguillicaudatus*, Cypriniformes: Cobitidae), a small freshwater fish, using knockdown of the *dead end* gene with a morpholino antisense oligonucleotide. Interestingly, loach lacking germ cells could develop as either phenotypic males or females, as characterized morphologically by the presence or absence of bony plates in the pectoral fins, respectively. The phenotypic males and females had testicular and ovarian structures, respectively, but lacked germ cells. Gene expression patterns in these male and female germ cell-deficient gonads were essentially the same as those in gonads of normal fish. Our observations indicate that sexually dimorphic gonads can develop in germ cell-deficient loach. In contrast to the situation in other model fish species, the gonadal somatic cells in phenotypic females autonomously differentiated into ovarian tissues and also played a role in the maintenance of gonadal structure. On the basis of our observations, we propose two possible models to explain the role of germ cells in sex determination in fish.

dead end | gonadal development | sex differentiation

Teleost species inhabit a wide range of aquatic environments, exhibit diverse life histories, and show an array of sex determination mechanisms (1). Although most fish species lack distinguishable heteromorphic sex chromosomes, they nevertheless have genetically based sex determination, based on single gene or polygenic systems (1). In some species, however, environmental conditions, such as temperature, population density, pH, or social status, influence sex determination rather than a genetic system as in most gonochoristic species (2). Moreover, phenotypic sex in some fish species can be modified by exogenous hormonal treatment before sex differentiation (1).

What factors determine gonadal sex? The model species zebrafish is categorized as an undifferentiated gonochorist. In this species, the gonads first differentiate into ovaries in all individuals after proliferation of germ cells, and then the process of sex differentiation occurs after a period of juvenile hermaphroditism (3). In medaka, the sexes are determined by the sex determining gene *DMY* (4) and embryos show sexual differentiation before hatching; this differentiation is the result of proliferation of germ cells (5). Furthermore, gonadal somatic cells of genetically XY male medaka can induce sex reversal in genetically XX females, suggesting that primary gonadal sex differentiation is susceptible to influence by gonadal somatic cells (6). However, germ cells themselves are important for the development of sexual dimorphism in medaka and zebrafish: embryos lacking germ cells develop into fish appearing phenotypically male and the gonads of germ cell-deficient fish differentiate into tube-like structures

that express male-specific genes (7–9). The results of germ cell depletion experiments in mammals also suggest that germ cells are necessary for the complete differentiation of an ovary, although the early stages of ovarian differentiation can occur in the absence of germ cells (10). Overall, the available data indicate that germ cells are essential for female gonad differentiation or the maintenance of ovarian structure, although little is known of the role of the germ cells in determining the sexual fate of gonadal somatic cells. However, this conclusion on the necessity of germ cells for complete ovarian differentiation is based on results from a small number of experimental species. Given the enormous biodiversity of sex differentiation systems among teleosts, it is unlikely that a single model for gonad differentiation will be universally applicable.

In this study, we used the loach *Misgurnus anguillicaudatus* (Cypriniformes: Cobitidae), a gonochoristic species, as the experimental material. Loaches are distributed across a wide area of the temperate zone of East Asia and live in rivers, lakes, ponds, and paddy fields. Sex determination in the loach was concluded to be a male-heterozygous XX–XY system because only female loaches were produced after induced gynogenesis in which a paternal contribution to the zygotes was eliminated (11). However, it has also been reported that sex ratio in the loach is influenced by high temperature during the sex differentiation period, and that female-to-male sex reversal frequently occurs (12). Male loaches take about 6 mo to achieve sexual maturity under our artificial rearing conditions whereas females take 1 y. The time taken to reach maturity in loach is higher than that in model species such as zebrafish and medaka. In the present study, we successfully induced germ cell deficiency in loach by knockdown of the *dead end* (*dnd*) gene using a morpholino antisense oligonucleotide (MO). This gene normally functions in primordial germ cell (PGC) migration and maintenance during embryogenesis (13). We then investigated gonadal development and gonadal structures in the resulting germ cell-deficient fish.

## Results

**Induction of Germ Cell-Deficient Loach.** To test the ability of *dnd*-specific morpholino antisense oligonucleotide (*dnd*-MO) to

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The authors declare no conflict of interest.

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Data deposition: The sequences reported in this paper have been deposited in the DDBJ database [accession nos. AB531493 (*vasa*), AB531494 (*dead end*), AB531495 (*dmt1*), AB531496 (*p450arom*), and AB531497 (*foxl2*)].

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knock down the target gene and to determine an optimum dosage of *dnd*-MO to induce germ cell deficiency in fish, we injected 5–10 nL of various concentrations of *dnd*-MO solution. We then carried out a histological analysis for the presence or absence of PGCs in each hatched larva that had been injected with *dnd*-MO (Table S1). In addition, we analyzed the morphological features of 13-mo-old fish grown from each experimental group (Table S2).

More than 90% of the intact control larvae and larvae injected with the standard control morpholino oligonucleotide (control MO) hatched normally. Histological analysis found a mean number of 48.5 and 50.5 PGCs in intact control larvae and control MO larvae, respectively. For larvae injected with *dnd*-MO, an increased concentration of *dnd*-MO was correlated with reduced numbers of PGCs in hatched larvae. Larvae injected with more than 1,000 pg *dnd*-MO showed a complete absence of PGCs at the genital ridge. The hatching rates of *dnd*-MO embryos were not significantly decreased.

Adult fish that developed from control larvae showed morphologically normal ovaries and testes. By contrast, two types of morphologically unusual gonads were observed in fish derived from *dnd*-MO-injected embryos (hereafter, *dnd* morphants). In one group, a testis developed on one side of the body, whereas on the other, an atypical gonad was present that resembled the germ cell-deficient gonads described in previous studies (8, 9). The proportion of *dnd* morphants with a normal gonad decreased with increase in *dnd*-MO dosage, whereas the rate with an atypical gonad increased. All *dnd* morphants injected with 2,000–4,000 pg *dnd*-MO had atypical gonads. These results indicate that germ cell-deficient fish could be reliably induced by injection of 2,000–4,000 pg *dnd*-MO, and in all subsequent analyses, we adopted this injection regime.

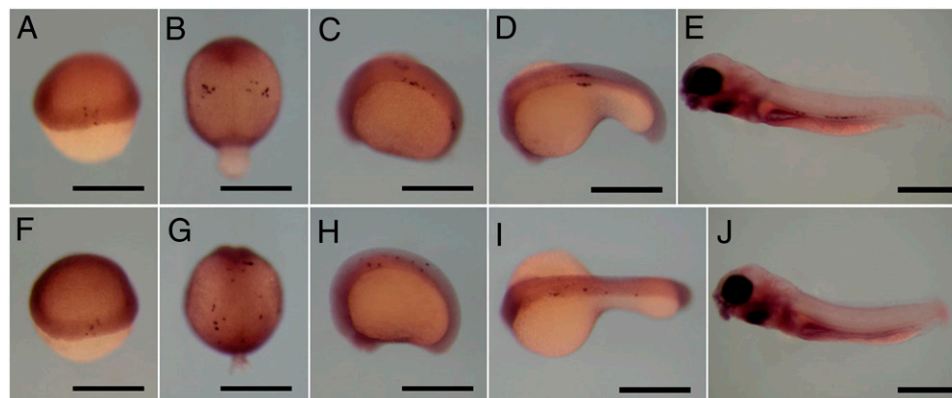
**Migration of PGCs During Embryogenesis in *dnd* Morphants.** The migration of primordial germ cells (PGCs) was investigated in wild-type and *dnd*-MO injected (2,000–4,000 pg/embryo) embryos from the embryonic shield stage to the larval stage. Whole mount in situ hybridization was used to determine the positions of PGCs stained for *vasa* gene expression. *vasa* is an useful molecular marker for tracing PGCs during embryogenesis in many organisms (14). At the embryonic shield stage, *vasa*-positive cells were located at the marginal region of the blastoderm in both wild-type embryos and *dnd* morphants (Fig. 1 *A* and *F*). At the 2- to 4-somite stage, *vasa*-positive cells formed clusters in the near-anterior somite of wild-type embryos, but were distributed along the body axis in *dnd* morphants (Fig. 1 *B* and *G*). At the 20-somite stage, many *vasa*-positive cells in wild-type embryos were clustered on both sides of the first to fifth somites; later, at the 30-somite stage, the clusters were located at

the base of the yolk extension region (Fig. 1 *C* and *D*). However, in *dnd* morphants at 20- and 30-somite stages, *vasa*-positive cells were found in the embryonic body at positions where PGCs are never located in wild-type embryos (Fig. 1 *H* and *I*). In 5-d postfertilization (dpf) wild-type larvae, *vasa*-positive cells were observed at the body cavity, whereas no *vasa*-positive cells were detected in *dnd* morphants (Fig. 1 *E* and *J*).

Embryos after whole mount in situ hybridization were categorized as “normal,” “normal and ectopic,” and “ectopic” on the basis of the distribution pattern of *vasa*-positive cells. *vasa*-positive cells in wild-type embryos and larvae were distributed normally except for four embryos at 30-somite stage (Table S3). In contrast, most *vasa*-positive cells in *dnd* morphants were ectopically positioned after the 2- to 4-somite stage and finally could not be detected at 5-dpf larvae (Table S3).

#### Sex Differentiation in Germ Cell-Deficient Loach During Gonadal Development.

Development of the gonads was examined to determine whether gonadal sex differentiation occurs in the same manner in *dnd* morphants as in wild-type fish. At 33 d post-hatching (dph), the germ cells of wild-type fish were surrounded by gonadal somatic cells and protruded into the coelomic cavity (Fig. 2*A*). At 55 dph, wild-type fish showed two gonadal types that could be distinguished with respect to the number of germ cells: in one type, germ cells had proliferated within a cyst (Fig. 2*C*); the second type had a few germ cells and no cyst (Fig. 2*D*). The distal edge of the gonads was attached to the coelomic wall at this stage. By contrast, the gonadal tissue was devoid of germ cells and protruded into the coelomic cavity at 33 dph in *dnd* morphants (Fig. 2*B*). As in the wild type, the distal edge of the gonads was attached to the coelomic wall at 55 dph in *dnd* morphants (Fig. 2*E*). At 70 dph, two gonadal types were seen in both groups. In the wild type, one of the gonadal types showed elongation from the dorsal to lateral coelomic wall and also proliferation of germ cells, which had entered meiosis, within a cyst (Fig. 2*F*). The second gonadal type had a club-like shape with only a few germ cells, and no meiotic cells were present (Fig. 2*G*). In the *dnd* morphants, one gonadal type showed elongation from the dorsal to lateral coelomic wall (Fig. 2*H*). The second was not elongated but exhibited a club-like shape and protruded from the dorsal coelomic wall (Fig. 2*I*). At 97–120 dph, female and male gonads in wild type could be discriminated clearly by their external appearances and by histological observation (Fig. 2*J* and *L*). Oocytes were apparent in the ovaries of wild-type fish, and an ovarian lumen was formed between the oocytes and the coelomic wall (Fig. 2*J*). In the testes, seminal lobules had begun to form, and a blood vessel and a sperm duct



**Fig. 1.** Migrating primordial germ cells (PGCs) were identified by *vasa* expression using whole mount in situ hybridization of wild-type and *dnd*-MO injected (2,000–4,000 pg/embryo) embryos at the embryonic shield to larval stages. (*A–E* and *F–J*) PGCs of wild-type and *dnd* morphants, respectively. (*A* and *F*) Embryonic shield stage. (*B* and *G*) Two- to four-somite stage. (*C* and *H*) Twenty-somite stage. (*D* and *I*) Thirty-somite stage. (*E* and *J*) Larvae at 5 d post-fertilization. (Scale bars, 500  $\mu$ m.)



**Table 1. Gonadal structures and morphological sexual characteristics of pectoral fins in 2-y-old wild-type fish and *dnd* morphants**

	Bony plates at pectoral fin	Wild-type		<i>dnd</i> morphant	
		Presence	Absence	Presence	Absence
Typical gonad	Testis	27	0	2	0
	Ovary	0	15	0	1
Atypical gonad	Tube	3	0	31	0
	Ribbon	1	2	1	22

males of *dnd* morphants consisted of presumptive testicular tubules that contained interstitial tissue but lacked germ cells, but otherwise resembled the testes of wild-type fish (Fig. 3 G and H). In the *dnd* morphants, a sheet of epithelial cells lined the inside of the tubules, and the epithelial cells were hypertrophied and ciliated (Fig. 3H). By contrast, the ribbon-like gonads of phenotypically female *dnd* morphants contained stromal tissue on the ventral side and epithelial cells lining the coelomic wall, similar to the ovaries of wild-type females (Fig. 3 I and J). Overall, the structure of the ribbon-like gonads resembled the normal ovary quite remarkably except for the lack of oocytes.

**Gene Expression in Germ Cell-Deficient Gonad.** RT-PCR analyses were used to determine the expression patterns of the following genes: *vasa*, *dmrt1*, *foxl2*, *p450 aromatase (p450arom)*, and  $\beta$ -*actin*. Two replicate RT-PCR experiments were performed using independent gonadal samples from 2-y-old adult loaches (Fig. 4).  $\beta$ -*actin* was used as the control. Analysis of gene expression patterns in germ cell-deficient gonads showed that *vasa* was not expressed in either phenotypically male or female *dnd* morphants (Fig. 4). For the other genes examined, the tube-like gonads showed a similar gene expression pattern to the wild-type male, except for *p450arom*, which showed a low level of expression in one wild-type testis (Fig. 4). Overall, the ribbon-like gonads of phenotypically female *dnd* morphants had the same expression pattern as wild-type ovaries (Fig. 4).

### Discussion

In wild-type fish, we identified sex-related variation in gonadal differentiation during development (Fig. 2 C and D). Two gonadal types were distinguished: one showed early proliferation of germ cells within a cyst and entry of the germ cells into meiosis (Fig. 2 C and F); the other had a small number of germ cells and no meiotic cells (Fig. 2 D and G). Sexual dimorphism in the proliferation of germ cells has been described previously in many species of teleosts (15). In general, presumptive ovaries have more germ cells than presumptive testes during early gonadal development, and the entry of germ cells into meiosis occurs earlier in ovaries than in testes (15). Therefore, we assume that the gonads that showed

greater proliferation of germ cells within a cyst, lateral elongation, and also evidence of meiosis were likely to develop into ovaries in the adult, whereas the gonads with only a few germ cells and with a club-like shape are presumed to develop into testes in the adult. In light of the sex differentiation process in the wild type, the elongated gonads and club-shaped gonads observed here in *dnd* morphants at 70 dph are presumed to develop into the ribbon-like and tube-like gonads, respectively, of the adult (Fig. 2 H and I). In addition to similar patterns of gonadal sex differentiation, we found that the sex ratio, based on gonadal structures, was similar between wild-type and *dnd* morphants. Therefore, we conclude that gonadal sex differentiation in germ cell-deficient loach occurs in the same manner as in the wild type.

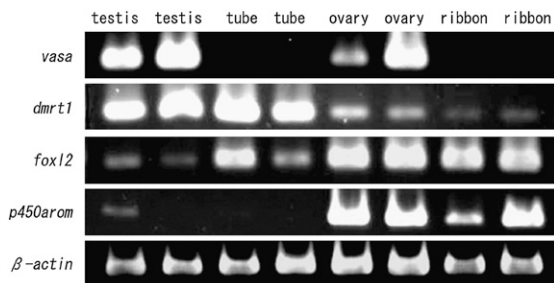
In adult *dnd* morphants, histological analysis showed that the tube-like gonads of phenotypic males had testicular structures, whereas the ribbon-like gonads of phenotypic females had ovarian structures (Fig. 3 H and J). The tube-like gonads of germ cell-deficient loach consisted of hypertrophied and ciliated cells, which lined the tubules. The gene expression patterns in these tube-like gonads were similar to those of testes of wild-type males. Overall, tubule structure and gene expression patterns were similar to those previously reported in the germ cell-deficient gonads of zebrafish and medaka (8, 9). We suggest that the somatic cells of the tube-like gonads of phenotypic males are responsible for the formation of the testicular structures typical of male sexual differentiation.

The most notable characteristic of the germ cell-deficient gonads of phenotypic females was the presence of ciliated and hypertrophied epithelial cells, which lined the coelomic walls of the ribbon-like gonads. It was previously reported that hypertrophic epithelial cells in the ovarian lumen were present in advance of ovarian maturation in medaka (16). Further, it was proposed that these cells played a role in both the maintenance of ovulated eggs and their transport through the oviduct for spawning. We suggest that the somatic cells of the ribbon-like gonads of phenotypic females differentiate into ovarian structures. In conclusion, germ cell-deficient gonads in loach can follow either the male or female differentiation pathway and can maintain the gonadal somatic structures of the testis or ovary.

We confirmed here that *dnd* morphants had complete ablation of germ cells using expression of the marker gene, *vasa*. Our RT-PCR analysis showed expression of *vasa* in testes and ovaries of wild type, but not in the tube-like gonads or ribbon-like gonads of the *dnd* morphants.

This study also showed sex-related variation in the expression of *dmrt1*, *foxl2*, and *p450arom* in the germ cell-deficient gonads. It was previously shown that *dmrt1* is expressed in testicular Sertoli cells before and during sex differentiation and is involved in the regulation of testicular differentiation (17). Here, we found a high level of *dmrt1* expression in wild-type testes and tube-like gonads of *dnd* morphants. Expression was also detected in ovaries and ribbon-like gonads, but the signal was weak when compared with that in the testes and tube-like gonads. These results indicate that a high level of *dmrt1* expression is necessary for testicular differentiation.

We examined the expression patterns of two marker genes for ovarian differentiation in vertebrates: *foxl2*, which belongs to the



**Fig. 4.** Patterns of expression of *vasa*, *dmrt1*, *foxl2*, *p450arom*, and  $\beta$ -*actin* in gonads of wild-type fish and *dnd* morphants. Two replicate RT-PCR analyses were performed using independent samples from each gonadal type.

forkhead gene family, and *p450arom*, which encodes an enzyme that converts testosterone to estradiol-17 $\beta$  (18). Because estradiol-17 $\beta$  is crucial for directing the initial steps of ovarian differentiation, regulation of *p450arom* expression has a key role in sex differentiation (17). A recent study in tilapia showed that *foxl2* likely binds directly to the promoter of *p450arom* and interacts with Ad4BP/SF-1 to form a heterodimer that also enhances transcription of *p450arom* (19). Here, we found that expression of *foxl2* was detectable not only in ovaries and ribbon-like gonads but also in testes and tube-like gonads. However, a high level of *p450arom* expression was detected only in ovaries and the ribbon-like gonads. Expression of *p450arom* was not detected in the germ cell-deficient gonads of zebrafish or medaka (8, 9). It is noteworthy that expression of both *foxl2* and *p450arom* was present in both ovaries and ribbon-like gonads of loach. This result strongly suggests that endogenous estrogen might give rise to ovarian differentiation in germ cell-deficient gonads. On the other hand, in zebrafish and medaka, germ cells are necessary for ovarian differentiation, although exogenous estrogen induced feminization in zebrafish lacking the germ cells (7). Thus, in these species, germ cells might control the expression of ovarian genes such as *foxl2* and *p450arom*. On the basis of our observations, we suggest that the dimorphic structures of germ cell-deficient gonads are maintained by expression of the same set of genes as normal testes or ovaries.

In conclusion, our study demonstrates that germ cell-deficient gonads in the loach can develop into testicular and ovarian structures and that gene expression patterns in these organs are similar to those of wild-type testes and ovaries. Therefore, it is no exaggeration to say that sexual dimorphism is evident in germ cell-deficient gonads in the loach. Thus, germ cells are not necessary for ovarian differentiation in the loach, indicating that germ cells do not control the expression of ovarian genes such as *foxl2* and *p450arom*. On the basis of this conclusion, two of the models that have been proposed for the role of germ cells in sex determination can be examined. In the first model, germ cells are indispensable for differentiation of gonadal somatic cells into ovarian tissues. The second model envisages that gonadal somatic cells autonomously differentiate into ovarian tissues and also play a role in their maintenance. Clearly, the available evidence indicates that gonadal differentiation in zebrafish, medaka, and mammals follows expectations of the first model, whereas, the present study shows that the loach is consistent with the second model. Comparative studies of the two models should help to elucidate the key factors controlling the differences in interactions between germ cells and somatic cells.

## Materials and Methods

Molecular cloning, PCR conditions, and primer sets (Table S4) appear in *SI Materials and Methods*.

**Ethics.** This study was carried out in accordance with the Guide for the Care and Use of Laboratory Animals at Hokkaido University.

**Experimental Fish and Embryos.** Wild-type loach *M. anguillicaudatus* was caught from a natural population in Iwamizawa, Hokkaido, Japan during the spawning season. The fish were reared as breeding stocks at Hokkaido

University, Faculty of Fisheries Sciences. The collection of gametes and fertilization of eggs were performed as previously described (20).

***dnd* Knockdown to Induce Germ Cell-Deficient Loach.** The *dnd*-specific morpholino antisense oligonucleotide (*dnd*-MO, 5'-GATCTGCTCCTTCATTGGC-TTTTC-3') including start codon was designed by and obtained from Gene Tools. Control MO was also obtained from Gene Tools. To determine optimum dosages of *dnd*-MO to induce germ cell-deficient fish, 250–500, 500–1,000, 1,000–2,000, and 2,000–4,000 pg of the *dnd*-MO diluted with 0.2 M KCl were injected at the central part of the blastodisc at one- to early two-cell-stage embryos. Control embryos were injected with 1,000–2,000 pg of control MO diluted with 0.2 M KCl.

After the injection treatment, germ cell deficiency was confirmed by a histological analysis to search for PGCs at the genital ridge in hatching fry and also by examination of the gonads of adult *dnd* morphants in each dosage group.

After hatching, fry were kept at 20–23 °C in 2-L containers at a density of 20–30 fish for 3 mo and, thereafter, were transferred to a 40-L tank. They were fed with brine shrimp and commercial food. Uninjected wild-type loaches were also kept under the same conditions.

**Sexing of Fish on the Basis of Secondary Sexual Characteristics and Gonadal Appearance.** The sex of individual fish was established by screening for the presence or absence of a pectoral fin ray and analyses of the gonads. In loach, the male can be distinguished by the formation of a bony plate at the base of the dorsal segment of the second pectoral fin ray (21). To make the bony plate visible, the pectoral fin was removed from the body and fixed with 10% formalin solution. The fin was then cleared by immersion in 2% KOH for 2–4 h, and the bones were subsequently stained with 0.1% Alizarin red S in 2% KOH for 2 h. After staining, the fin was washed with 1% KOH for 1–2 h and then dehydrated in 50% ethanol for 2 h. The specimen was cleared and stored by transfer to 100% glycerol plus 0.025% thymol.

**Histology.** Larvae at 5 dpf, young fish at 33–150 dph and undergoing sex differentiation, and 2-y-old adults were fixed overnight in Bouin's fixative. Fixed specimens were stored in 80% ethanol. The gonads of adult fish were dissected under a stereoscopic microscope and then the gonads and the stored young fish were dehydrated and infiltrated with and embedded in resin (Technovit 7100) or paraffin. Resin sections were cut at 2  $\mu$ m and stained with hematoxylin and alcoholic eosin. Paraffin sections were cut at 6  $\mu$ m and stained with hematoxylin and aqueous eosin.

**Whole Mount in Situ Hybridization.** The migration of PGCs during embryogenesis was examined in *dnd*-MO injected embryos by detection of *vasa* mRNA using whole mount in situ hybridization. Embryos, at the embryonic shield stage, 2–4 somite, 20 somite, 30 somite, and 5 dpf, were fixed with 4% paraformaldehyde in PBS for 30 h and stored in 100% methanol at –20 °C. *vasa* expression was detected with an antisense *vasa* RNA probe labeled with digoxigenin; the probes were designed using  $\approx$ 0.56-kb fragments from the 3'-UTR region of *vasa* cDNA in zebrafish (22). These probes were generated using an in vitro transcription kit according to the manufacturer's instructions (Roche). Hybridization, washing, and detection steps in whole mount in situ hybridization were carried out as previously described (23). Distribution pattern of *vasa*-positive cells at each stage was categorized as "normal," "normal and ectopic," and "ectopic." Normal position of *vasa*-positive cells was in accordance with our previous study (23).

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