The *hotei* mutation of medaka in the anti-Müllerian hormone receptor causes the dysregulation of germ cell and sexual development

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We previously performed mutant screens in the medaka for defects in gonadal development and identified a mutant of interest in this regard, which was designated as *hotei* (*hot*). This mutant manifests a number of remarkable phenotypic abnormalities including: (*i*) excessive proliferation of germ cells that initiates at around the hatching stage regardless of the genetic sex of the fish; (*ii*) initiation of premature meiosis in phenotypically male *hot* homozygotes; (*iii*) one-half of the *hot*-homozygous XY fish undergo sex reversal, which accompanies the expression of the female-characteristic *aromatase* gene in the somatic cells of the gonad; and (*iv*) in phenotypically female homozygotes, follicular development is arrested at an early stage. We have also performed genetic mapping, chromosome walking, and candidate gene sequencing analysis of *hot* and demonstrate that the underlying mutation occurs in the recently identified medaka anti-Müllerian hormone (Amh) receptor type II (*amhrII*) gene. Moreover, this gene was found to be responsible for each of the *hot* phenotypes, as an *amhrII* transgene rescues these abnormalities. In addition, the *amhrII* gene is expressed in the somatic cells of the gonads of both sexes. The phenotypes of the *hot* homozygotes indicate that there are multiple regulatory functions of the AMH/AMHRII signaling system in the development of the gonad, including the sex-dependent regulation of germ cell proliferation and follicular development. These presumably represent the basic roles of Amh, which precede Müllerian duct evolution during phylogeny.

gonad | sex differentiation | *hotei* mutant

Medaka, *Oryzias latipes*, is a small freshwater fish native to Japan and eastern Asia and has been cultivated in recent years as a genetically refined laboratory animal and model organism (1). An advantage of using the medaka in sexual differentiation studies is that the sex of this organism is primarily determined genetically, by the XX/XY chromosome system (2), which is analogous to mammals. In addition, the *DMY/dmrt1bY* gene on the Y chromosome of this fish species has been identified as the male-determining gene (3, 4), which further facilitates the investigation of gonadal and germ cell development in conjunction with sex differentiation. In both medaka and mammals, germ cells develop through various stages of mihotic proliferation and by entry into meiosis, reductive division, and also entry into a quiescent state awaiting specific fertilization signals that have different timings, depending on the sex. In females, germ cells begin to proliferate at around the hatching stage, and soon enter into meiosis and develop as oocytes. In males, germ cells remain in mihotic arrest for 2 weeks and initiate meiosis only 4 weeks after hatching. The sex of a normal medaka of a few weeks old can thus be judged by the abundance of germ cells in its gonads (5, 6).

In the developing gonad, multiple interactions occur between germ cells and somatic cells. Recent studies have also identified several genes that are essential for mammalian early gonadal development (7, 8). Anti-Müllerian hormone (Amh), also known as Müllerian inhibiting substance/factor (Mis/Mif), is one such gene and is a secreted intercellular signaling protein belonging to the TGF-β superfamily. Amh expression is restricted to the Sertoli cells in the testis and granulosa cells in the ovary (9–13). In amniotes, a hallmark function of Amh is to induce regression of the Müllerian ducts. Amh functions primarily through the anti-Müllerian hormone receptor type II (AmhrII) (14), a member of the type II receptors for the TGF-β -related protein family that possesses a single transmembrane domain and a serine/threonine kinase domain and functions in conjunction with a type I receptor of TGF-β superfamily (15–17). Bmpr1a (Alk3) has been shown to function as an AMH type I receptor (16). Interestingly, however, Amh is also expressed in lower vertebrates lacking a Müllerian duct (18–20), suggesting that there are evolutionally conserved functions of the Amh/Amhr signaling network, such as ovarian folliculogenesis and gonadal steroidogenesis.

Aromatase is an enzyme that is expressed in the follicular layer surrounding the oocytes in the ovary and catalyzes the conversion of androgen to estrogen (21). Although estrogen has been suggested to contribute to sexual differentiation of the gonads in lower vertebrates, the actual roles of aromatase and Amh in this process have not been fully clarified. The inhibition of the estrogen action results in the development of testicular structures that express *amh* (22), whereas Amh inhibits the aromatase-dependent estrogenic activity that is present in cultured fetal gonads (23, 24), suggesting that a reciprocal interaction occurs between these two pathways.

Recently, the medaka *amhrII* gene was identified, which represents the first characterization of this receptor in nonmammalian vertebrates (25). In fish, Wolffian duct is associated with the pronephros regardless of the sex, and sperm duct and oviduct are derived from the coelomic epithelium (26) analogous to amniote Müllerian duct. In medaka, *amh* and *amhrII* are ex-

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Abbreviations: Amh, anti-Müllerian hormone; Amhr, anti-Müllerian hormone receptor; dph, days post hatching.

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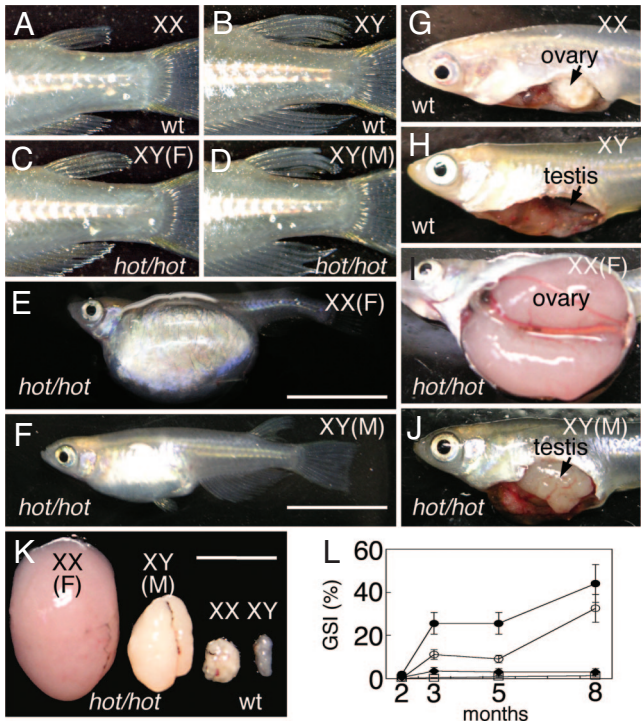

Fig. 1. Adult *hot*-homozygous mutant phenotypes. Primary sexual phenotypes of *hot* homozygotes are indicated by F (female) and M (male) in parentheses with the sex chromosome composition. (*A–D*) Sex-dependent fin morphologies. (*A*) Wild-type XX female. (*B*) Wild-type XY male. Male fins have rougher edges and are larger than female fins. (*C*) Phenotypically female XY *hot* homozygote. (*D*) Phenotypically male XY *hot* homozygote. (*E* and *F*) External appearance of the *hot*-homozygous fish at 6 months. (Scale bar, 10 mm.) (*E*) XX *hot* homozygote, showing an expanded abdomen. (*F*) Phenotypically male XY *hot* mutant, with a less pronounced abdominal expansion. (*G–J*) Exposed gonads in wild-type and *hot*-homozygous fish. (*G*) Wild-type ovary. (*H*) Testis. (*I*) The abdominal cavity of a female *hot* homozygote occupied by a hypertrophic ovary. (*J*) Enlarged testis in a phenotypically male homozygote. (*K*) Comparisons of the gonads isolated from wild-type and *hot* homozygotes fish at 6 months; from left to right, XX *hot* ovary, XY *hot* testis, wild-type ovary and wild-type testis. (Scale bar, 10 mm.) (*L*) Changes in the gonad-somatic index (GSI = gonad weight/body weight in percentage) were plotted for wild-type and *hot*-homozygous mutant fish at 2, 3, 5, and 8 months after hatching. The averages measurements for at least four fish are indicated. Filled diamond, wild-type XX; open square, wild-type XY; filled circle, ovary-containing *hot* homozygotes of either XX or XY; open circle, XY *hot* homozygotes with testes.

pressed in somatic cells of the developing gonads identically between the sexes during larval and juvenile stages. These results suggest that the medaka *amh* and *amhrII* genes are involved in gonadal development including the regulation of germ cells in both sexes.

To further elucidate the genes involved in gonadal development, we performed a screen of medaka mutants displaying abnormal gonad development (27, 28). As a result of this analysis, we successfully identified a number of mutants showing gonadal defects (27), among which a mutant of particular interest was *hotei* (*hot*), causing excessive germ cell proliferation and male-to-female sex reversal. Here, we show that the *hot* mutation resides in the *amhrII* gene and thus reveal important functions of Amh signaling that have not been characterized in amniotes.

Results

***hotei* Homozygous Mutants Develop Hypertrophic Gonads.** The *hot* mutation, which causes gonadal hypertrophy, behaves in an autosomal recessive manner [supporting information (SI) Fig. 7 and SI Table 2]. In wild-type fish, XX females and XY males are distinguishable by the shapes of their dorsal and anal fins (Fig. 1 *A* and *B*). Adult XX *hot*-homozygotes (*hot*/*hot*) show an enlarged abdomen (Fig. 1*E*). Adult XY *hot*-homozygous fish can be classified into two groups (Table 1), one-half of which have female characteristics (Fig. 1*C*) with an enlarged abdomen (as shown in Fig. 1*E*), and the remaining half of which show a male fin phenotype (Fig. 1*D*) with an abdominal enlargement (Fig. 1*F*), although this is less pronounced when compared with mutants of phenotypic females. The abdomens of the female *hot* homozygotes of either the XX or XY genotypes were occupied with hypertrophic ovaries (Fig. 1*I*) that had pushed the other internal organs against the abdominal wall. The *hot* homozygotes of XY males also display hypertrophic testis (Fig. 1*J*).

Table 1. Phenotypes of the *hot* homozygous fish at 6 months after hatching

Genotype	XX	XY	
Fin phenotypes	Female	Female	Male
Gonad development	Ovary	Ovary	Testis
The number of fish	35	14	13

Adult XY *hot*-homozygous fish are classified into two groups. One half of the XY *hot*-homozygotes display female-like fins (Fig. 1*C*) and hypertrophic ovaries (similar to Fig. 1*I*). The remaining XY *hot*-homozygotes have a male appearance (Fig. 1*D*) and enlarged testis (Fig. 1*J*).

At 3 months after hatching, wild-type medaka are sexually mature, and their gonads reach a maximum size. In contrast, the gonads of *hot* mutants were found to continue growing at 5 months after hatching (Fig. 1 *K* and *L*). Although most of the *hot*-homozygous mutants are sterile, a few female mutant fish were found to be fertile until 5 months of age, which contrasts with wild-type fish that show fertility for 1 year. Phenotypically, male *hot*-homozygous fish produced sperm, although the fertility of these fish is low. A fraction of the heterozygous males were also found to develop enlarged testis past 6 months after hatching. Very few of the *hot* homozygotes survive past one year, compared with the normal life span of around two years for wild-type medaka.

Wild-type mature ovaries during the spawning period of the medaka contain follicles at various developmental stages, from previtellogenic to fully grown (Fig. 2 *A* and *A′*). In contrast to wild-type, the hypertrophic ovaries of XX and XY female *hot* homozygotes are filled with small follicles (Fig. 2 *B* and *B′*). Thus, in *hot* homozygotes the germ cells continue to increase, whereas follicular development is arrested during early vitellogenesis, and these features can be observed even at 6 months after hatching. In wild-type testes, spermatogonia are located in the most peripheral regions of the lobule structures, and spermatogenesis proceeds synchronously within the cysts in these lobules (Fig. 2 *C* and *C′*). In the male-type XY *hot* mutants, hypertrophic testes develop that are filled with germ cells and sperm at various developmental stages (Fig. 2 *D* and *D′*). In a minor fraction of XY *hot* homozygotes, the gonads contained both testicular and ovarian components (see Fig. 6*E*).

In *hot* homozygotes, the germ cells had already increased their number at 20 days post hatching (dph), analogous to normal ovary, and regardless of ovarian or testicular development, hypertrophy of the gonad occur during the posthatching period. The increasing number of germ cells is illustrated by Fig. 3 *A–E*, which show gonads of *hot*-homozygous larvae at 20 dph that contain more abundant germ cells compared with wild type, regardless of the XX or XY genotypes. Even in the XY *hot* fish, the germ cells within the gonads rapidly increase their number and start meiosis by 20 dph regardless of the sex of the organs (Fig. 3 *D* and *E*). This contrasts markedly with wild-type testis,

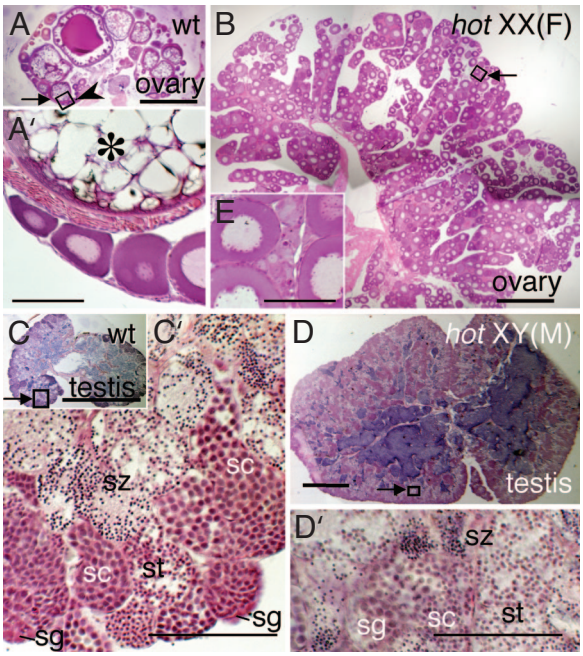

Fig. 2. Histological sections of gonads from wild-type and *hot*-homozygous fish at 6 months after hatching. The specimens are sections of the tissues shown in Fig. 1*K* stained with hematoxylin-eosin. (*A*) Wild-type ovary. (*B*) XX *hot*-homozygous ovary. The arrowhead in *A* (enlarged in *A′*) indicates previtellogenic follicles, with which the immature follicles abundant in *B* share a similar appearance (enlarged in *B′*). An oocyte at the yolk formation stage is indicated by an asterisk in *A′*. (*C*) Wild-type testis. (*C′*) Enlargement of the boxed area in *C* (arrow), where spermatogonia (sg), spermatocytes (sc), spermatids (st), and spermatozoa (sz) are labeled according to the morphological criteria (39). (*D′*) Enlargement of the boxed area in *D* (arrow), showing sp, sc, st and sz. [Scale bars, 0.5 mm (*A–D*); 0.1 mm (*A′–D′*).]

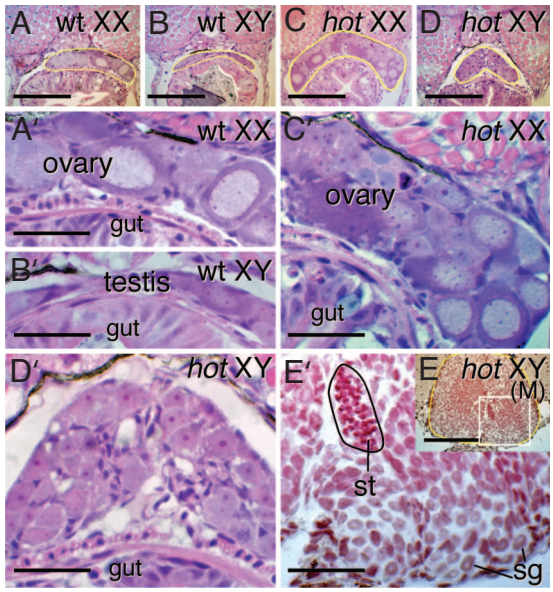

Fig. 3. Histology of wild-type and *hot*-homozygous mutant larvae. (*A–E*) Sections of 20-dph larvae. The gonads are encircled by a yellow line. (*A*) Wild-type XX. (*B*) Wild-type XY. (*C*) XX *hot/hot*. (*D*) An XX *hot/hot*, showing hyperplasic and undifferentiated gonad. (*E*) An XY *hot/hot* gonad showing exclusively testicular development. (Scale bars, 0.1 mm.) (*A′–E′*) Enlargement of the gonadal area in *A–E*. (Scale bars, 0.05 mm.) In XY *hot* testis (*E′*, showing enlargement of the boxed area in *E*), the germ cells have prematurely initiated meiosis at this larval stage. The area encircled by a black line indicates a cluster of spermatid-like cells. sg, spermatogonia-like cells. Hematoxylin-eosin staining is shown in *A–D*, and neutral red staining is shown in *E*.

where meiosis does not commence until ≈4 weeks after hatching. Furthermore, in the wild-type medaka, the germ cells are maintained in a thin layer in the gonads (Fig. 3 *A* and *B*). In contrast, the gonads of both XX and XY *hot* homozygotes were expanded in all dimensions, with more abundant and disorganized germ cells.

Identification of *hot* as an *amhrII* Gene Mutation by Positional Cloning. We performed positional cloning to characterize the specific gene that is mutated in the *hot* strains of medaka. This mutation was mapped to linkage group 7, based on its cosegregation with the Cab-line (southern strain)-associated polymorphic markers against Kaga (northern strain), within the 4 cM interval flanked by the EST markers Ola0109b and Olb2507h (Fig. 4*A*). Chromosomal walking was carried out by using these markers, and the position of the *hot* mutation was narrowed down to scaffold 3 [Medaka Whole-Genome Shotgun (WGS) sequencing project (http://medaka.utgenome.org), version 1.0]. Possible candidate genes for the *hot* mutation on this scaffold were then examined by sequencing, and this subsequently identified an A-to-G mutation in exon 9 of the recently identified *amhrII* gene (25) (Fig. 4 *B* and *C*). Cloning of the full-length *amhrII* cDNA confirmed that this gene consists of 11 exons and encodes a 492-aa-long Ser/Thr receptor kinase (Fig. 4*C*). The *hot* mutation causes a Cys substitution at the Tyr residue at position 390 in the kinase domain, which is a highly conserved amino acid among the type II receptors (Fig. 4 *D* and *E*).

To confirm that the Y390C mutation in exon 9 of the *amhrII* gene is responsible for the *hot* phenotypes, a 12-kb region derived from HNI (northern inbred line) genome (nucleotide positions 3172–3184 kb in scaffold 3; represented in Fig. 4*A* as Tg) was used as a transgene and injected into fertilized eggs of *hot* heterozygous parents. Among the progeny of the F_1 generation, two fish were identified as *hot*-homozygous females harboring this *amhrII* transgene. In both of these transgenic homozygotes, hypertrophy of the ovary was suppressed at 3 months after hatching (Fig. 4*F*). The GSI (gonad weight/body weight percentage) was calculated as 2.3% and 5.0% in these transgenic fish, whereas it was measured as 25.6% (20.3–30.0%) and 3.3% (0.6–6.1%) in a *hot*-homozygous and wild-type female, respectively (Fig. 1*L*). Furthermore, histological analysis indicated that the follicular development had been largely recovered in the transgenic fish (Fig. 4*F′*). These data further confirm that *hot* corresponds to a mutation in the *amhrII* gene.

Gonad-Restricted Expression of the *amhrII* Gene. To correlate the defect of AmhrII with the *hot*-homozygous phenotype, the tissue specificity of the expression of *amhrII* was investigated by RT-PCR and *in situ* hybridization. In adult fish, *amhrII* was found to be specifically expressed in the ovary and testis (Fig. 5*A*). In larvae, *amhrII* is also expressed in the gonads of both wild-type and *hot* homozygotes, regardless of their sex (Fig. 5*B*). *In situ* hybridization further indicated that *amhrII* is expressed in the somatic cells around the germ cells (Fig. 5 *C–E*). In addition, the *amh* gene, which encodes the ligand for AmhrII, has been reported to be expressed in the cells around the germ cells of wild-type fish (25, 29), which is also true for *hot* homozygotes, regardless of their sex genotype (data not shown).

Phenotypic Sex and Expression of Sex-Related Genes in *hot*-Homozygous Mutants. Because a significant fraction of the *hot*-homozygous XY fish examined in our experiments show reversal of their secondary sex characteristics, the expression of the estrogen-synthesizing *aromatase* gene was examined in these animals. In wild-type larvae, *aromatase* is highly expressed in the

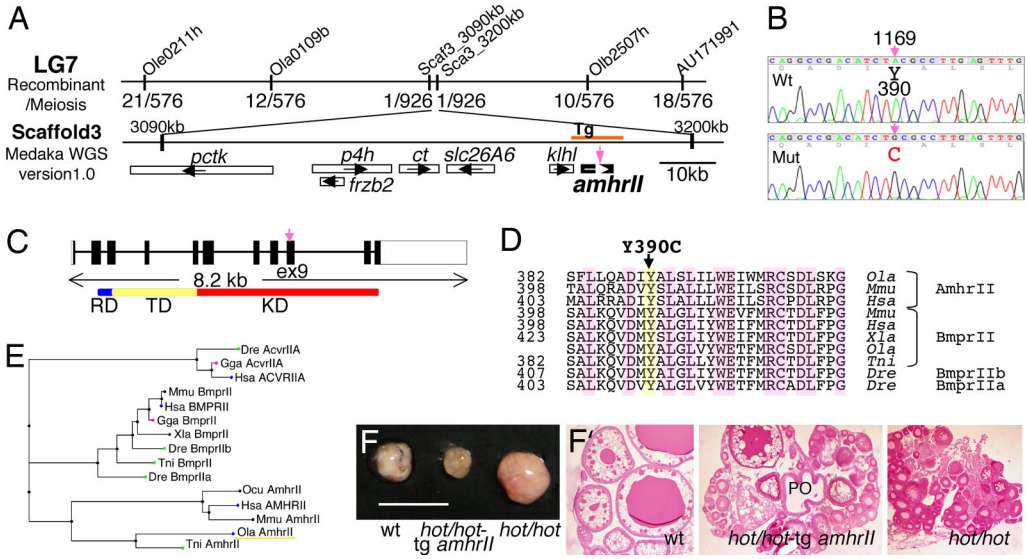

Fig. 4. Characterization of the *hot* mutation. (*A*) Genetic mapping of the *hot* mutation using polymorphic markers. Genetic map of medaka linkage group 7, indicating the recombination frequency of the *hot* mutation with markers that place this mutation in Scaffold 3 [Medaka Whole-Genome Shotgun (WGS) sequencing project, version 1.0]. Genes: pctk, PCTAIRE-motif protein kinase; p4h, prolyl-4-hydroxylase; ct, creatine transporter; slc26A6, solute carrier family 26, member 6; klhl, kelch-like protein. (*B*) A missense mutation (TAC to TGC) has occurred in exon 9 of the *amhrII* gene, causing an amino acid substitution (Tyr to Cys) in the intracellular kinase domain. (*C*) Schematic representation of the *amhrII* gene and the position of the *hot* mutation. RD, activin types I and II receptor domain; TD, transmembrane domain; KD, serine/threonine protein kinase domain. The *hot* mutation in *amhrII* gene exon 9 is indicated by a vertical arrow. (*D*) Comparisons of the deduced amino acid sequences of the medaka and mammalian AmhrII, and of the BmprII proteins of various vertebrate species. Note that the mutated Tyr residue in *hot* is conserved among the type II receptors and across the species shown. (*E*) Phylogenetic relationship between the medaka AmhrII with other type II receptors of the TGF-β superfamily. Dre, *Danio relio* (zebrafish); Gga, *Gallus gallus* (chicken); Hsa, *Homo sapiens* (human); Mmu, *Mus musculus* (mouse); Xla, *Xenopus laevis*; Tni, *Tetraodon nigroviridis* (pufferfish); Ocu, *Oryctolagus cuniculus* (rabbit); Ola, *O. latipes* (medaka). (*F*) Rescue of the *hot*-homozygous phenotype by expression of a wild-type *amhrII* transgene. The hyperproliferation of germ cells in the ovary was inhibited in the transgenic fish at 3 months of age. (*Left*) Wild-type ovary. (*Center*) *hot/hot* XX fish harboring the *amhrII* transgene. (*Right*) *hot/hot* XX fish. (Scale bar, 10 mm.) (*F'*) Sections of the ovaries shown in *F*. In the *hot/hot* XX fish carrying the *amhrII* transgene, the number of growing follicles is reduced compared with the *hot*-homozygous ovary, and postovulatory follicle is also observed (PO).

female XX ovary (Fig. 6 *A* and *A'*), but not in XY male testis (Fig. 6*D*) at 20 dph. In *hot*-homozygous larvae, the expression of *aromatase* is detectable in both XX and XY ovary-type gonads (Fig. 6 *B* and *B'*, and data not shown), whereas no signal can be observed in XY testis-type gonads by using whole mount specimens (Fig. 6*C*) or cross sections (data not shown). In the XY *hot*, gonads of either female-type development or female/male mixed development, *aromatase* was expressed only in the somatic cells around the oocytes (Fig. 6 *B* and *E*).

vitellogenin gene was also found to be expressed in the liver of phenotypically female adult fish, even those with XY chromosomes (Fig. 6*F*). Continuous incorporation of vitellogenin from the circulating serum results in the growth of large follicles in the normal ovary. However, in the adult *hot*-homozygous ovary, most follicles do not progress beyond the early stages of their development and do not accumulate vitellogenin, which indicates that the defects in Amh signaling cause multiple abnormalities associated with the functions of follicular cells in addition to the dysregulation of germ cell proliferation.

Discussion

hotei homozygous medaka mutants show remarkable phenotypes, including (*i*) excessive proliferation of germ cells that initiates soon after hatching regardless of the phenotypic sex of the fish; (*ii*) initiation of premature meiosis in phenotypically male *hot* homozygotes; (*iii*) sex reversal in 50% of the *hot*-homozygous XY fish, which accompanies the expression of *aromatase* in the somatic cells of the gonad; and (*iv*) growth arrest at an early stage of follicular development, despite *vitellogenin* expression in the liver. We also demonstrate in our current study that the *hot* mutation is a critical amino acid substitution (Y390C) in the *amhrII* gene, and that all of the phenotypes that arise in the corresponding mutant fish can be ascribed to the consequences of this defect on Amh/AmhrII signaling. Recessive Mendelian transmission of the mutation and the rescue of the mutant phenotype by the *amhrII* transgene suggest that the *hot* mutation caused loss of function of AmhrII.

The germ cells in normal female gonads initiate successive proliferative events to produce cystic clusters at the time of hatching, which results in the abundance of germ cells in females. Some populations of these clusters subsequently enter meiosis (D.S. and M.T., unpublished data). By contrast, in the normal wild-type male medaka, such successive proliferation does not occur until 4 weeks after hatching. It is possible, therefore, that Amh/AmhrII signaling regulates these successive proliferations and consequently that mutation of the *amhrII* gene causes unrestrained proliferation in males with a similar timing to that of the females. In this context, the phenotypically female *hot* homozygotes also show uncontrolled proliferation of germ cells and large increases in the germ cell number.

Of interest are the downstream effects of the disrupted Amh signaling in the *hot* homozygotes. The follicular cells in *hot* mutants express the gene for aromatase that catalyzes the synthesis of estrogen in the somatic cells around the developing oocytes, regardless of its genetic sex (Fig. 6). It had been demonstrated that estrogen promotes the hepatic synthesis of vitellogenin, and we subsequently confirmed *vitellogenin* expression in the liver of female *hot* homozygotes. In *hot* mutants, the growth of follicles is arrested, despite the occurrence of aromatase-dependent vitellogenin synthesis, suggesting that the vitellogenin uptake by the follicular cells (30) is affected.

Although the "anti-Müllerian duct" effects of Amh have been primarily emphasized in higher vertebrates, this protein is a phylogenetically conserved signaling molecule and has been

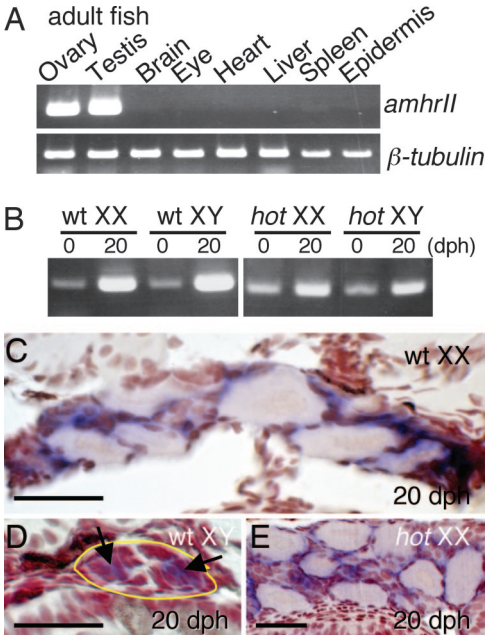

Fig. 5. Expression of *amhrII* in adult and developing larvae. (*A*) RT-PCR analysis of the tissue distribution of *amhrII* expression in adult medaka, using β-tubulin as control, showing restricted expression of *amhrII* in the ovary and testis. (*B*) Expression of *amhrII* in wild-type and *hot*-homozygous larvae, analyzed by RT-PCR. Regardless of the genetic sex, *amhrII* is expressed in both wild-type and *hot* homozygotes during the period between 0 and 20 dph. (*C–E*) *In situ* hybridization of the *amhrII* transcripts in cross sections of the gonads in 20-dph larvae. *AmhrII* expression is detectable in the somatic cells (arrows) surrounding the germ cells in the gonads of either phenotypic sex. The gonadal regions are enclosed with a yellow line in *D*. (*C*) Wild-type XX. (*D*) Wild-type XY. (*E*) XX *hot*/*hot*. (Scale bars, 0.1 mm.)

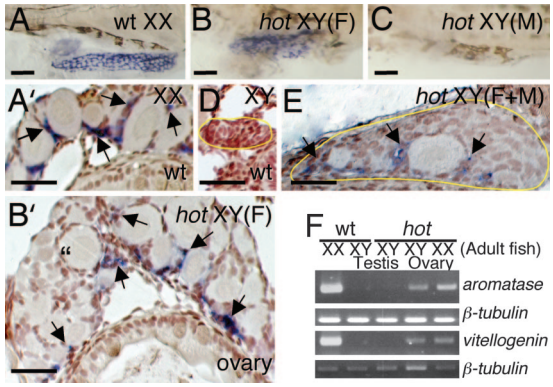

Fig. 6. Expression of *aromatase* transcripts in both wild-type and homozygous *hot* medaka. Wild-type (*A* and *D*) and *hot*-homozygous (*B*, *C*, and *E*) larvae at 20 dph were hybridized with an *aromatase* probe. *Aromatase* signals are detectable in the wild-type ovary (*A*) and in the ovarian-type gonads of the *hot*-homozygous mutant (*B*). (*A′* and *B′*) Shown are sections of the gonads in *A* and *B*, respectively, demonstrating expression of *aromatase* in the somatic cells near the ocytes (arrows) in both wild type (*A′*) and *hot* homozygotes (*B′*). *Aromatase* signals are not detected in the gonads of XY *hot* homozygotes with testicular development (*C*) or XY wild-type (*D*). In XY *hot*-homozygous gonads possessing both ovarian and testicular components (*E*), *aromatase* signals are detected only in the region of somatic cells that surround the oocytes (arrows). (*F*) RT-PCR analysis of *aromatase* in the gonads and *vitellogenin* in the liver in adult fish (6 months old). Both *vitellogenin* and *aromatase* are expressed in the phenotypically female fish (i.e., wild-type XX, XX *hot*-homozygotes, and XY *hot*-homozygotes, showing the development of ovaries), but not in phenotypically male fish. [Scale bars, 0.5 mm (*A–C*); 0.1 mm (*A′–C′*, *D*, and *E*).]

identified in many teleost fish species that lack a Müllerian duct (18–20, 25, 29). The phenotypes of the *hot* homozygotes indicate that there are conserved functions of Amh/AmhrII signaling that are distinct from its role in Müllerian duct regression. Indeed, Amh was initially reported as a spermatogenesis preventing substance (eSRS21), because it shows inhibitory activity toward the initiation of this process in cocultures of germ cells and somatic cells (18). A possibly related observation is that mammalian Amh also inhibits the proliferation of both Leydig cells (12, 13) and ovarian cancer cells (31). Amh has also been implicated in male sex differentiation, female follicular development, and also steroidogenesis in both sexes (9–13). In mammals, the lack of AMH signaling as manifested in *Amh* knockout mice and human patients causes precocious folliculogenesis (32, 33), in contrast to the defect of folliculogenesis in *hot* homozygotes described here. The mechanism underlying this difference is not clear at this point; however, the mouse model with the disrupted *amhrII* gene demonstrates male internal pseudohermaphroditism, having both male and female reproductive organs (34), again indicating analogy with the cases of *hot*-homozygous XY fish.

Because one half of the XY *hot* homozygotes develop sperm in their gonads, Amh/AmhrII signaling may not be a component of the major sex differentiation pathways in medaka. It is also possible that, in the *hot* mutants, signals that are derived from the somatic cells under the control of *DMY*/*dmrt1bY* and that masculinize the germ cells are not transmitted efficiently to the proliferating germ cells. In the gonad of female-dominant mixed phenotype XY *hot* homozygotes, *aromatase* is expressed only in the region of gonadal somatic cells where germ cells undergo folliculogenesis (Fig. 6*E*). The feminization of germ cells may then release the somatic cells from the repression of *aromatase* expression that is exerted by *DMY*/*dmrt1bY*. In zebrafish (35) and chicken (36), *amh* expression commences earlier than that of other genes, such as *sox9*, and its sexual dimorphic expression is not apparent during this early stage. Sexually dimorphic expression of the *amh* gene may therefore be a consequence of the sexual differentiation of the gonad that takes place first. How such species-dependent variations in the association of the Amh/AmhrII system with male development are coupled with sex-differentiation mechanisms is an interesting issue that is deserving of further study.

In conclusion, the multifaceted gonad- and sex-associated phenotypes of the *hotei* medaka mutant have further elucidated the central functions of the Amh/AmhrII system during gonadal development. This also underscores the value of focused mutant screening in vertebrates and demonstrates the advantage of the medaka as a model system in genetic studies.

Materials and Methods

Genetic Mapping and Gene Cloning. The *hotei* mutation was induced in the Cab-Kyoto line (called "*tot*" in refs. 27 and 28). The Kaga-Kyoto line (28) was used for polymorphic marker-based genetic mapping.

Genetic mapping and chromosome walking were performed essentially as described by Geisler (37), using restriction fragment length polymorphism markers [medaka linkage map database (ML Base), http://mbase.bioweb.ne.jp/~dclust/ml_base.html] and markers designed based on information from the Tetraodon Genome Browser (www.genoscope.cns.fr/externe/tetranew) and Medaka University of Tokyo Genome Browser (http://medaka.utgenome.org) databases. To identify the mutation, genomic DNA was amplified by PCR, using primers designed from the information in the University of Tokyo Genome Browser. This database was also used to predict introns and exons. The central fragment of the *amhrII* cDNA was isolated by RT-PCR from adult testes, and full-length cDNA was

obtained by 5′ RACE and 3′ RACE. Phylogenic distance was calculated by using BLAST Tree View Widget (National Center for Biotechnology Information).

Transgene Rescue of the *hot*-Homozygous Phenotype. The genomic fragment containing the entire *amhrII* gene plus *klhl* 3′ UTR was excised from BAC clone Mn0073N15 derived from the HNI line, and placed in the pCRII–TOPO vector (Invitrogen, Carlsbad, CA). The *klhl* sequence in the *hot* mutants contained no mutation. Fertilized eggs of *hot* heterozygous parents were injected with the DNA (6) and reared (28). Three *hot*-homozygous and two heterozygous fish carried the HNI-derived *amhrII* transgene. These G_0 fish were mated with *hot* heterozygotes, and two progeny fish identified as the transgene-carrying *hot* homozygotes were examined for the phenotype.

RT-PCR and In-Whole Mount *in Situ* Hybridization. RT-PCRwas performed by using the following primers: *amhrII* forward 5′-GTTGTGGGACAAGGACACTTTGCC-3′ and reverse 5′-CAGGATCTCAGGAGACATGTAGTGGG-3′; *Aromatase* forward 5′-CTGTGCTCATTGTTGCTTG-3′ and reverse 5′-CGTAAGACAAACTGCTCAGG-3′; *Vitellogenin* forward 5′-TCTGAGAGGTTCAACATCATACAG-3′ and reverse 5′-GCGTATCTCTGTACTTGCTCC-3′. The RT-PCR-derived sequences were also used as a probe for *in situ* hybridization (27, 38). The *amhrII* sequence, corresponding to to exons 6–9, is highly diversified from other TGFβ-family receptors (the closest being *bmprII* with 55% sequence identity), ruling out cross-hybridization with other type II receptor sequences.

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