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Hyperproliferation of mitotically active germ cells due to defective anti-Müllerian hormone signaling mediates sex reversal in medaka

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SUMMARY

The function of AMH (Anti-Müllerian hormone), a phylogenetically ancient member of the TGF β family of proteins, in lower vertebrates is largely unknown. Previously, we have shown that the gene encoding the type II anti-Müllerian hormone receptor, *amhrII*, is responsible for excessive germ cell proliferation and male-to-female sex reversal in the medaka *hotei* mutant. In this study, functional analyses in cultured cells and of other *amhrII* mutant alleles indicate that lack of AMH signaling causes the *hotei* phenotype. BrdU incorporation experiments identified the existence of both quiescent and mitotically active germ cells among the self-renewing, type I population of germ cells in the developing gonad. AMH signaling acts in supporting cells to promote the proliferation of mitotically active germ cells but does not trigger quiescent germ cells to proliferate in the developing gonad. Furthermore, we show that the male-to-female sex reversal phenotype in *hotei* mutants is not a direct consequence of AMH signaling in supporting cells, but is instead mediated by germ cells. Our data demonstrate that interfollicular AMH signaling regulates proliferation at a specific stage of germ cell development, and that this regulation is crucial for the proper manifestation of gonadal sex directed by sex determination genes.

KEY WORDS: AMH, Gonad, Medaka, Germ cells, Sex differentiation, Sex reversal

INTRODUCTION

Anti-Müllerian hormone (AMH) is a highly conserved member of the TGF β superfamily of proteins. In mammals, AMH is secreted from supporting cells of the testes (Sertoli cells) and binds to its receptor on the female reproductive primordium, the Müllerian duct. This results in regression of Müllerian duct-derived organs, the oviduct and upper vagina, in males (Behringer et al., 1994; De Santa Barbara et al., 1998; Arango et al., 1999). Although the role of AMH in early testis development is unclear, addition of recombinant AMH can induce mesonephric cell migration, which is crucial for mammalian testis formation and for development of male-specific steroidogenic cells, the Leydig cells (Ross et al., 2003). However, in lower vertebrates, such as teleost fish, the female reproductive primordium does not develop during embryogenesis and male-specific contribution of mesonephric cells to gonad formation is not apparent (Nakamura et al., 2006; Kinoshita et al., 2009). In addition, *amh* expression is detected in both the female and male developing gonads of fish (Rodriguez-Mari et al., 2005; Klüver et al., 2007). Recently, a medaka mutant with excess germ cells and male-to-female sex reversal was isolated (Morinaga et al., 2004). The gene underlying the *hotei*

mutant phenotype was identified as AMH type II receptor (*amhrII*) (Morinaga et al., 2007), but the cellular mechanism underlying the phenotype has not yet been elucidated. In this study, we report the cellular mechanism of germ cell and sex regulation by AMH signaling during gonadal development by analyzing of the *hotei* mutant phenotype.

MATERIALS AND METHODS

Strains and genotyping of medaka

The wild-type cab strain, *hotei* mutants (Morinaga et al., 2007) and *sox9b*-EGFP transgenic medaka (Nakamura et al., 2008) were used for this study. Isolation of tilling medaka was performed as previously described (Taniguchi et al., 2006).

For genotyping the *hotei* and *amhrII*^{K87X} allele, a Custom TaqMan SNP genotyping assay (ABI) was used. Primers for the *hotei* allele were: *hotei*_SNP F (5'-CCTGAACAACAGCTCCTTCCT-3'), *hotei*_SNP R (5'-AGCGCATCCATATTTCCACAAT-3'), *hotei*_VIC (5'-CCGACATC-TACGCCTTG-3') and *hotei*_FAM (5'-CGACATCTGCGCCTTG-3'). Primers for the *amhrII*^{K87X} allele were: K87X_F (5'-TGCGGTAAGTGGAAACCTTATGT-3'), K87X_R (5'-GCGATTTGAAAGCGTGGTT-3'), K87X_VIC (5'-CAACCTGCAAGGTAC-3') and K87X_FAM (5'-TGCAACCTGCTAGGTAC-3').

To genotype the *amhrII*^{SD} and *amhrII*^{Q89X} alleles, RFLP analysis was used. The genomic fragments were amplified by the following primers: *amhrII* tilling forward (5'-CACTCATCTGCTGGTAATGT-3') and *amhrII* tilling reverse (5'-CATAACAATTTTACACCGTT-3'). PCR products were digested with *RsaI* (NEB, Ipswich, MA, USA).

Constructs and reporter assays

WT-AMHRII del-AMHRII and hot-AMHRII (Y390C *hotei* mutation) constructs were prepared as previously described (Belville et al., 2009). P19 cells were co-transfected with Gal4-Smad1, Gal4-luc and different mutant cDNA expression vectors. AMH (10 μ g/ml) was added to the culture medium for 24 hours. Firefly and renilla luciferase activities were assessed according to the manufacturer's instructions (Dual Luciferase kit, Promega). Results were normalized to renilla luciferase activity.

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Immunohistochemistry, in situ hybridization and histology

In situ hybridization, immunohistochemistry and histology were performed as previously described (Nakamura et al., 2006). For immunohistochemistry, primary antibodies used were anti-OLVAS (1:100, rat or rabbit) (Aoki et al., 2008), anti-BrdU (1:100, mouse, Sigma) and anti-GFP (1:100, rat, Nacalai tesque, Kyoto, Japan).

BrdU incorporation analysis

BrdU incorporation analysis was performed as previously described (Nakamura et al., 2010). BrdU-positive single isolated germ cells were counted as self-renewing germ cells (type I), while clusters with more than two germ cells were counted as germ cells undergoing type II division. We counted the number of BrdU-positive type II clusters; for example, a two-cell cyst or 16-cell cyst was counted as one cluster.

Transplantation

Transplantation from *hotei* XY (donor) to wild-type XY (host) embryos and from wild-type XY (donor) to *hotei* XY (host) embryos was performed as previously described (Nakamura et al., 2012). Biotin-dextran was injected in the donor-derived cells and detected by Alexa-conjugated streptavidin (Molecular Probes).

Generation of germ cell-deficient gonads in *hotei* mutants

As described previously (Kurokawa et al., 2007), 2000 ng/μl *cxcr4b* morpholino with EGFP-*olvas* 3'UTR RNA was injected into the fertilized eggs of the *hotei* mutant. The germ cell-deficient embryos were screened by EGFP fluorescence and raised to adulthood.

RT-PCR

The isolation of total RNA and RT-PCR were performed previously (Kurokawa et al., 2007). For RT-PCR of *amhrII*, the following primers were used: *amhrII* f873 (5'-gaaacacacctcagc-3') and *amhrII* r1257 (5'-cttgataatcagagca-3').

RESULTS AND DISCUSSION

Characterization of *amhrII* mutations

In *hotei* mutants, the gene encoding the type II receptor for anti-Müllerian hormone (*amhrII*) contains a Tyr-to-Cys substitution at position 390 (Y390C) in the highly conserved kinase domain (Morinaga et al., 2007). To test the function of the *hotei* mutation, we conducted in vitro reporter assays using P19 cells, which possess all the AMH signaling components, except for a type II receptor (Belville et al., 2009). When a wild-type human *AMHRII* cDNA construct was co-transfected with the Gal4-luciferase reporter and Smad1-Gal4 fusion constructs into P19 cells, AMH was able to induce luciferase expression. By contrast, when human cDNA constructs encoding *hot-AMHRII*, which contains the Y to C point mutation, and *del-AMHRII*, in which the kinase domain is deleted, were co-transfected into P19 cells, no increase in luciferase activity was observed (Fig. 1A). *hot-AMHRII* and *del-AMHRII* mutant proteins were expressed at similar levels in the transfected cells, as assessed by western blotting (data not shown). These results indicate that the *hotei* mutant *AMHRII* receptor cannot mediate AMH signaling.

To further confirm that loss of *AMHRII* function underlies the *hotei* mutant phenotype, we have isolated three additional medaka *amhrII* tilling mutants. The mutant alleles lack the conserved kinase domain, the transmembrane domain and part of the receptor domain, and, in terms of the structure, are probably unable to mediate AMH signaling in cells (supplementary material Fig. S2A). Indeed, these mutants display *hotei*-like phenotypes, with excess germ cells and some XY mutants exhibiting complete sex reversal to female (supplementary material Table S1; Fig. S2B-E). Together with the cell culture experiments, these results indicate that the *hotei* mutant phenotype results from a lack of intracellular AMH signaling.

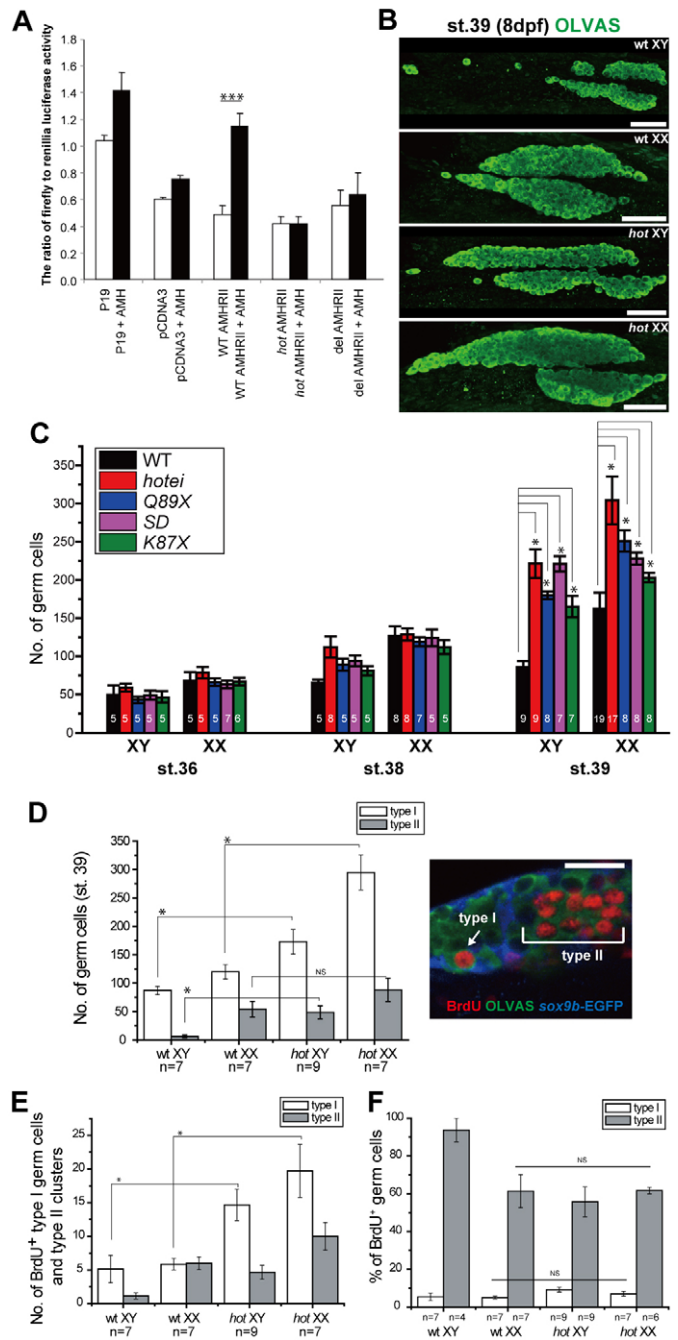


Fig. 1. Lack of AMH signaling causes excessive proliferation of germ cells during gonadal differentiation. (A) Luciferase reporter assays using P19 cells. (B) Ventral views of wild-type and *hotei* mutant gonads at stage 39. Germ cells are in green. (C) The total numbers of germ cells in wild-type and mutant embryos during gonadal differentiation. Differences between wild-type and mutant embryos of each sex are analysed for statistical significance. (D) The numbers of germ cells with type I or type II division in wild-type and *hotei* gonads at stage 39 (left). Representative images of germ cells undergoing type I or type II division (right). (E) The numbers of BrdU-positive type I germ cells and cystic type II germ cell clusters at stage 39. (F) The ratio of BrdU-positive type I or type II germ cells to the total number of type I or type II germ cells, respectively, at stage 39. Samples in D-F were immunostained after BrdU-pulse labeling for 30 minutes. Each value indicates the mean \pm s.e.m. *** $P < 0.0001$, * $P < 0.05$, Student's *t*-test. Scale bars: 50 μ m in B; 20 μ m in D.

AMH signaling is required to regulate germ cell proliferation during early gonad differentiation

Next, we examined the course of germ cell proliferation in both wild-type and mutant gonads. At stage 36, there is no significant difference in germ cell number between wild-type and mutant gonads (Fig. 1C). By stage 39 (8 days post-fertilization), the number of germ cells was increased in both XX and XY mutant gonads (Fig. 1B,C). The timing of germ cell expansion was consistent with the onset of *amh* expression (Nakamura et al., 2006). As this phenotype was observed in all four mutants, the *hotei* mutant was used hereafter for detailed analyses.

In developing medaka gonads, there are two types of germ cells: a self-renewing type that undergoes type I division and is important for germ cell maintenance, and a cystic type that undergoes type II division and is committed to gametogenesis. Female developing gonads have more germ cells due to the transition of germ cell division from type I to type II (supplementary material Fig. S1B) (Saito et al., 2007). At stage 39, the number of germ cells undergoing type I division was significantly increased in the gonads of both XX and XY *hotei* mutants compared with that in wild-type gonads (Fig. 1D). BrdU pulse labeling (Fig. 1E) revealed that the number of self-renewing germ cells in S phase at stage 39 is about threefold higher in *hotei* mutants (*hot* XX, 19.7 ± 3.9 , $n=7$; *hot* XY, 14.6 ± 2.3 , $n=9$) than that in wild type (wild-type XX, 5.8 ± 0.8 , $n=7$; wild-type XY, 5.1 ± 2.1 , $n=7$). In the *hotei* XY gonad, we found that the cystic type II division occurs precociously (Fig. 1D), an early indication of sex reversal of an XY gonad into an ovary. Interestingly, the number of the germ cell clusters with type II cystic division at S phase does not differ between *hotei* XY and wild-type XX gonads (approximately five type II germ cell clusters per gonad) (Fig. 1E). Furthermore, the ratio of type II germ cells at S phase to the total number of type II germ cells does not significantly differ among wild-type XX, *hotei* XY and *hotei* XX gonads at stage 39 (Fig. 1F). These results indicate that the type I division is principally affected in the *hotei* mutant. In other words, the excessive number of germ cells in both sexes of the

hotei mutant is primarily due to overproliferation of self-renewing germ cells. As there was no difference found in the number of cystic germ cell clusters at S phase between *hotei* and wild-type gonads (Fig. 1E), AMH signaling may not regulate the transition from type I to type II divisions.

Mitotically active self-renewing germ cells are regulated by AMH signaling

In the adult medaka ovary, germline stem cells are found in the Gs population of germ cells (Nakamura et al., 2010). The Gs population comprises two types of germ cells with different cell cycle length: fast-dividing (Gsf) and slow-dividing (Gss) germ cells. We hypothesized that self-renewing type I germ cells in the developing gonad also contain mitotically active and quiescent germ cells. To test this, the wild-type embryos at stage 36 (6 dpf) were incubated with BrdU for 48 or 72 hours. We found that, in each gonad, eight to ten self-renewing germ cells failed to incorporate BrdU in both sexes (Fig. 2A,C,E,F; data not shown), whereas cystic germ cells undergoing type II division in the XX gonad were all BrdU positive. Thus, self-renewing germ cells in the developing gonad includes a mitotically quiescent subpopulation that is clearly distinct from the mitotically active subpopulation. Surprisingly, the number of BrdU-negative germ cells does not differ significantly between wild-type and *hotei* mutant gonads (Fig. 2F). Together with our data on the number of germ cells and our cell cycle analysis (Fig. 1D,E), this suggests that AMH signaling in the developing gonad regulates the proliferation of mitotically active, self-renewing germ cells, but does not induce quiescent germ cells to enter mitosis.

AMH signaling acts in the *sox9b*-expressing supporting cells

To confirm previous *amh* and *amhrII* expression data (Nakamura et al., 2006; Klüver et al., 2007; Morinaga et al., 2007), we performed double in situ hybridization and showed that both *amh*

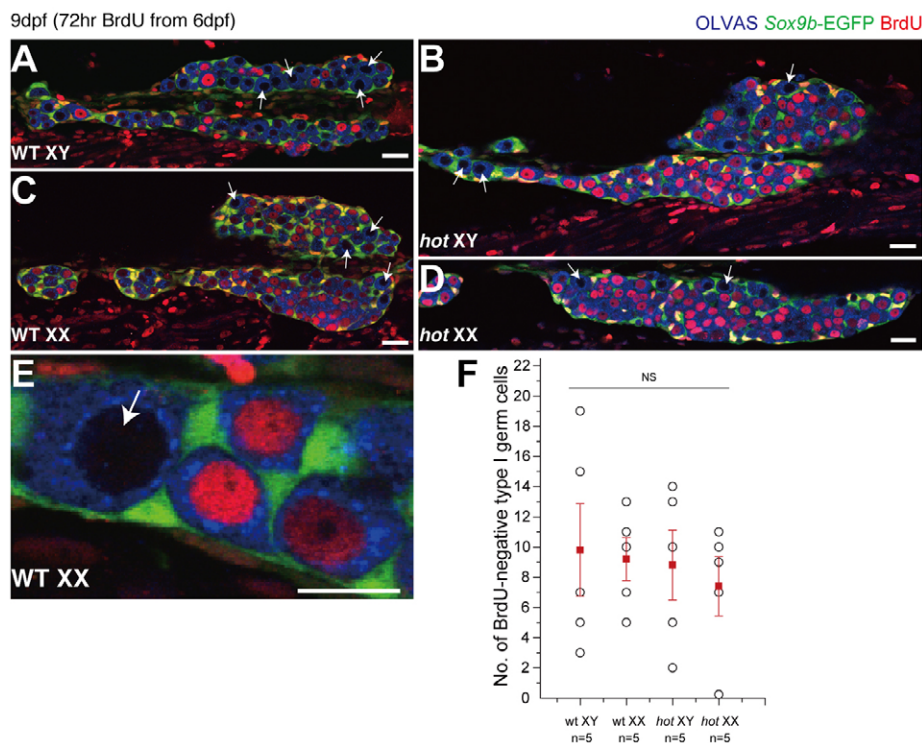


Fig. 2. Proliferation of mitotically active germ cells but not quiescent germ cells is accelerated in the *hotei* mutant.

(A-E) Ventral views of wild-type (A,C,E) and *hotei* (B,D) gonads at 9 dpf (stage 40), immunostained with OLVAS (blue), GFP (green) and BrdU (red) antibodies after 72 hours of BrdU exposure from 6 dpf (stage 36). GFP marks *sox9b*-expressing supporting cells. BrdU-negative germ cells (arrows) exist only among the self-renewing type I germ cells in both wild-type and *hotei* gonads, even after long-term BrdU exposure. A higher magnification view is shown in E. Scale bars: 20 μ m in A-D; 10 μ m in E. (F) The number of BrdU-negative germ cells after 72 hours of BrdU exposure from 6 dpf (stage 36). No significant difference was observed (one-way ANOVA). Open circles indicate each sample. Red squares and bars show the mean \pm s.e.m. Scale bars: 20 μ m in A-D; 10 μ m in E.

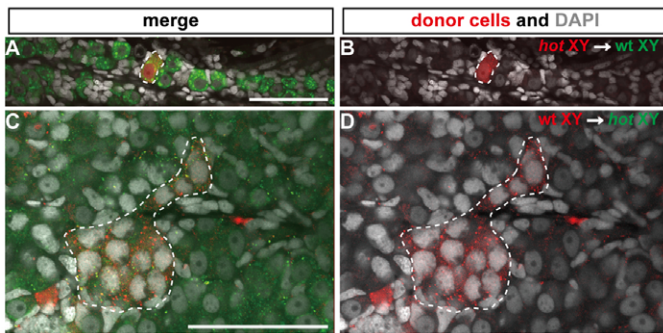


Fig. 3. AMH signaling does not act directly on germ cell proliferation. (A–D) The germline chimeric gonads at stage 39 stained by OLVAS antibody for germ cells (green) and streptavidin Alexa 568 (red) for biotin-dextran in donor-derived germ cells. When *hotei* homozygous XY donor germ cells (broken lines in A and B) were in the wild-type XY host gonad, the donor cells did not proliferate (A,B). By contrast, wild-type donor germ cells (broken lines in C and D) proliferate actively in the *hotei* XY host gonad (C,D). Scale bars: 50 μ m.

and *amhrII* are expressed in supporting cells, but not in germ cells, in both sexes during early gonad differentiation (supplementary material Fig. S3). This suggests that AMH signaling acts in *sox9b*-expressing supporting cells. To exclude the possibility that undetectable levels of *amh* and/or *amhrII* expression in germ cells affect germ cell proliferation, we generated germline chimeras. *hotei* mutant XY germ cells in host wild-type XY gonads failed to undergo cystic division characteristic of the XY *hotei* phenotype at stage 39 ($n=3$; Fig. 3A,B). However, when wild-type XY germ cells were transplanted into host *hotei* XY gonads, the donor-derived germ cells underwent type II cystic division, with some differentiating into meiotic oocytes, which are never observed in wild-type XY gonads at this stage ($n=3$; Fig. 3C,D; data not shown). These results demonstrate that AMH signaling does not directly act on germ cell proliferation. Rather, interfollicular AMH signaling controls germ cell proliferation via unknown signals.

Male-to-female sex reversal in *hotei* mutants is not primarily due to impaired *sox9b*-expressing supporting cells

Approximately half of the *hotei* XY mutants showed male-to-female sex reversal, as assessed by secondary sex characteristics (e.g. fins), gonad morphology and gene expression (Fig. 4A; supplementary material Table S2) (Morinaga et al., 2007). One possible explanation for this phenotype is that loss of AMH signaling results in a direct initiation of sex reversal events in other, non-germline, cell types. Another possibility is that sex reversal in *hotei* mutants is not a direct consequence of a supporting cell defect, but instead is mediated by the excessive number of germ cells. The latter model is supported by our previous work showing that germ cells are essential for differentiation of the undifferentiated gonad into an ovary in wild-type XX medaka. The absence of germ cells causes complete masculinization, with male-specific gene expression and male secondary sex characteristics (Kurokawa et al., 2007).

To determine which is accurate, we generated germ cell-deficient *hotei* mutants (Kurokawa et al., 2006; Kurokawa et al., 2007). None of the XY *hotei* mutants lacking germ cells exhibited female sex reversal, as judged by secondary sex characteristics and gene expression (Fig. 4A; supplementary material Table S2). These

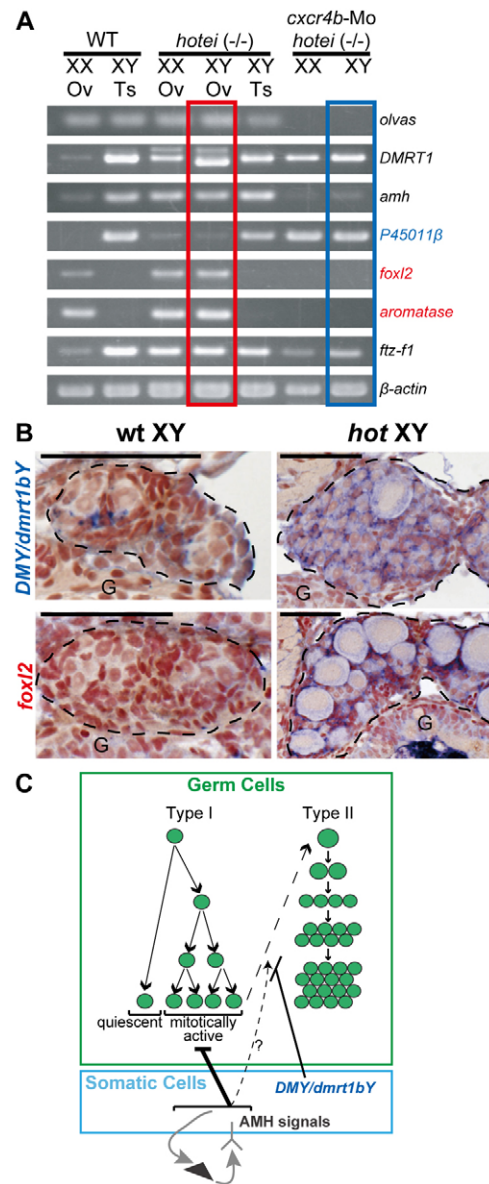


Fig. 4. Male-to-female sex reversal in *hotei* mutant depends on the presence of germ cells. (A) RT-PCR analysis of wild-type, *hotei* and germ cell-deficient *hotei* gonads. OV, ovary; Ts, testis. (B) *DMY/dmrt1bY* (upper) and *foxl2* (lower) expression in 20 dph wild-type XY (left) and *hotei* XY (right) gonads. G, gut. The gonad is outlined. Scale bars: 50 μ m. (C) Model of AMH signaling in germ cell proliferation.

results indicate that the male-to-female sex reversal in the XY *hotei* mutant is not a direct consequence of impaired AMH signaling in the *sox9b*-expressing supporting cells, but probably stems from precocious expansion of germ cells. Consistent with this, somatic cells expressed female makers, such as *foxl2* and a gene encoding an estrogen-producing enzyme, *aromatase* (*cyp19a1*), at 20 dph (days post hatching) in the *hotei* mutant (Fig. 4A,B) (Morinaga et al., 2007).

Interestingly, we noticed that several genes involved in masculinization of gonads, such as *DMY/dmrt1bY* and a male steroid hormone-producing genes, *P45011 β* , were also expressed in the *sox9b*-expressing supporting cells of the XY *hotei* feminized gonad (Fig. 4A,B). As the mutant XY developing gonad has

oocytes and is anticipated to form an ovary, germ cells are required not only for development and maintenance of the female gonad, but are also likely to have an antagonistic effect on male development, possibly by expressing a female-promoting or a male-repressing factor.

In this study, we found that AMH signaling functions in the somatic cells of both sexes and regulates the proliferation of mitotically active, self-renewing, type I germ cells (Fig. 4C). Loss of this signaling results in hyperproliferation. We also revealed the presence of a quiescent type of type I germ cells that are not affected by the AMH signaling. The intra- and intercellular signaling regulated by AMH signaling to control germ cell proliferation will be the next important target to further understand gonad development in medaka.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.076307/-DC1>

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Table S1. Phenotypic sex in *amhrII* tilling mutants

	<i>Q89X^{-/-}</i>		<i>SD^{-/-}</i>		<i>K87X^{-/-}</i>	
	Male	Female	Male	Female	Male	Female
XX	0	43	0	26	0	20
XY	24	3	13	10	19	2

The terms 'male' or 'female' are defined by secondary sex characteristics (e.g. male- or female-type fins) and adult gonad morphology (testis or ovary).

Table S2. Male-to-female sex reversal in *hot1* mutants depends on the presence of germ cells

	XX <i>hot1</i>^{-/-}	XY <i>hot1</i>^{-/-}		<i>cxcrb4</i> MO XY <i>hot1</i>^{-/-}	
Fin	Female	Female	Male	Femal e	Male
Morphology of gonad	Ovary	Ovary	Testis	-	-
Number of medaka	35	14	13	0	17