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RESEARCH ARTICLE

Genetic evidence for Amh modulation of gonadotropin actions to control gonadal homeostasis and gametogenesis in zebrafish and its noncanonical signaling through Bmpr2a receptor

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ABSTRACT

Anti-Müllerian hormone (Amh) plays an important role in gonadal function. Amh deficiency causes severe gonadal dysgenesis and dysfunction in zebrafish, with gonadal hypertrophy in both sexes. However, its mechanism of action remains unknown. Intriguingly, the Amh cognate type II receptor (Amhr2) is missing in the zebrafish genome, in sharp contrast to other species. Using a series of zebrafish mutants (*amh*, *fshb*, *fshr* and *lhcgr*), we provided unequivocal evidence for actions of Amh, via modulation of gonadotropin signaling, on both germ cell proliferation and differentiation. The gonadal hypertrophy in *amh* mutants was abolished in the absence of Fshr in females or Fshr/Lhcgr in males. Furthermore, we demonstrated that knockout of *bmpr2a*, but not *bmpr2b*, phenocopied all phenotypes of the *amh* mutant in both sexes, including gonadal hypertrophy, hyperproliferation of germ cells, retarded gametogenesis and reduced *fshb* expression. In summary, the present study provided comprehensive genetic evidence for an intimate interaction of gonadotropin and Amh pathways in gonadal homeostasis and gametogenesis and for Bmpr2a as the possible missing link for Amh signaling in zebrafish.

KEY WORDS: Anti-Müllerian hormone, Gonadal development, Folliculogenesis, Spermatogenesis, Zebrafish

INTRODUCTION

Anti-Müllerian hormone (AMH/Amh), also named Müllerian-inhibiting substance (MIS), is a distant member of the transforming growth factor-β (TGF-β) superfamily (Josso and di Clemente, 1999), and its primary function is to induce Müllerian duct regression in mammals during sexual differentiation (Behringer et al., 1994; Vigier et al., 1984). In addition, AMH also plays important roles in gonadal development and function (Durlinger et al., 2002; Rehman et al., 2017; Visser et al., 2007; Visser and Themmen, 2005). Given that AMH is produced primarily in early follicles, its serum concentration has been used as a major indicator for testing ovarian reserve in the clinical setting (Broer et al., 2014), and abnormal expression of AMH has been considered one of the factors associated with primary ovarian insufficiency or premature ovarian failure (Monniaux et al., 2014; Visser et al., 2012) and polycystic ovarian syndrome (Barbotin et al., 2019; Garg and Tal, 2016; Pigny et al., 2003). In mice, knockout of *Amh* led to increased ovarian weight and accelerated recruitment of primordial follicles (Durlinger et al., 1999); however, the number of preovulatory follicles remained relatively constant in *Amh* null mice, and this was attributed to an increase in atresia, which neutralized the increase in the number of preantral follicles recruited from the primordial follicle pool (Visser et al., 2007). In teleosts, recent studies by ourselves and others showed that disruption of *amh* in zebrafish (Lin et al., 2017; Yan et al., 2019; Zhang et al., 2020) and tilapia (Liu et al., 2019) resulted in gonadal hypertrophy owing to increased proliferation of early germ cells and decreased differentiation. However, the mechanisms by which Amh works remain largely unknown.

Like other TGF-β family members, AMH signals through a specific type II receptor, AMHR2 (Mishina et al., 1996). In humans, mutation of either *AMH* or *AMHR2* leads to persistence of the uterus and oviduct in males (Josso et al., 2005). In mice, knockout of *Amh* had no effect on gonadal differentiation; however, the *Amh*-deficient males developed reproductive tract systems of both sexes, including the uterus and oviduct (Behringer et al., 1994). The same phenotypes were also observed in *Amhr2* null male mice (Mishina et al., 1996). Amhr2 also exists in teleosts (Klüver et al., 2007), and an *amhr2* mutant (*hotei*) was identified by mutant screening and characterized in medaka (Morinaga et al., 2007). The *amhr2* mutant medaka showed significant phenotypic abnormalities, including excessive proliferation of germ cells in both sexes and arrest of folliculogenesis at the primary growth (PG) stage at 6 months after hatching (Morinaga et al., 2007). Further evidence showed that Amh primarily affected the germ cells undergoing mitotic self-renewal, but not the quiescent germ cells (Nakamura et al., 2012). The phenotypes of *amhr2* mutant medaka were similar to those of *amh* mutants in the zebrafish (Lin et al., 2017; Yan et al., 2019; Zhang et al., 2020). However, bioinformatics analysis has failed to identify any *amhr2* homologs in the zebrafish genome (Morinaga et al., 2007; Yan et al., 2019). How does Amh work in the zebrafish without its cognate receptor, Amhr2?

Using a CRISPR/Cas9 approach, we undertook this study to investigate the downstream mechanisms by which Amh works in zebrafish gonads. Our data support previous reports on roles of Amh in zebrafish and medaka gametogenesis. More importantly, in combination with the mutant lines for gonadotropin receptors (*fshr* and *lhcgr*), we provided strong evidence for interactions of gonadotrophic and Amh signaling pathways in both the ovary and the testis. Furthermore, we also provided strong evidence that Bmpr2a is likely to serve as the putative Amh type II receptor in zebrafish in the absence of its cognate receptor, Amhr2.

RESULTS

Gonadal dysgenesis of *amh* mutant involves gonadotropin signaling

Using the CRISPR/Cas9 method, we generated an indel mutation (+66, −4) in exon I of the *amh* gene, leading to a frameshift mutation

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of *amh* (Zhang et al., 2020). As reported recently in zebrafish (Lin et al., 2017; Yan et al., 2019; Zhang et al., 2020), disruption of *amh* resulted in severe gonadal hypertrophy and blockade of gametogenesis in both sexes, in particular in the testis, and both mutant ovaries and testes showed increased germ cell proliferation (PG follicles in the ovary and spermatogonia in the testis; $amh^{-/-};fshb^{+/-}$ versus $amh^{+/-};fshb^{+/-}$) but decreased germ cell differentiation, viz. meiosis in the testis and primary growth-previtellogenic (PG-PV) transition in the ovary (Fig. 1).

To investigate the mechanism by which Amh works, we focused our attention on pituitary gonadotropins, especially follicle-stimulating hormone (FSH), because FSH plays an important role in promoting ovarian and testicular growth in zebrafish (Zhang et al., 2015b). In mammalian models, AMH has been reported to stimulate pituitary FSH expression and secretion (Barbotin et al., 2019; Garrel et al., 2016; Kadokawa, 2020) but to suppress gonadal responsiveness to FSH (Durlinger et al., 2001; Visser and Themmen, 2014).

To address this issue, we first created an *amh* and *fshb* double mutant ($amh^{-/-};fshb^{-/-}$), with the aim of demonstrating how pituitary FSH was involved in the phenotypic abnormalities of the *amh* mutant. Surprisingly, we did not see any significant effect of FSH deficiency on phenotypic abnormalities in both females and males of the *amh* mutant at 4 months postfertilization (mpf) ($amh^{-/-};fshb^{-/-}$ versus $amh^{-/-};fshb^{+/-}$; Fig. 1). Given that the role of FSH in promoting gonadal growth could be taken up by luteinizing hormone (LH) after puberty in the *fshb* null mutant (Zhang et al., 2015b), we hypothesized that the lack of influence of FSH deficiency on the adult *amh* mutant could be attributable to the compensatory effect of LH for FSH, because both gonadotropins can activate follicle-stimulating hormone receptor (Fshr) in the zebrafish (So et al., 2005; Zhang et al., 2015b).

To test this hypothesis, we then created double and triple knockouts involving *amh* and the two gonadotropin receptors (*fshr* and *lhcgr*). Our recent study showed that the loss of *fshr* caused hypotrophy of the ovary and complete arrest of folliculogenesis at the early PG stage without a significant effect on spermatogenesis in mature adults, whereas double knockout of *fshr* and *lhcgr* led to complete arrest of spermatogenesis at an early stage (Zhang et al., 2015a). Based on these findings, we designed an experiment by creating an *amh* and *fshr* double knockout ($amh^{-/-};fshr^{-/-}$) to look at the ovary and a triple knockout ($amh^{-/-};fshr^{-/-};lhcgr^{-/-}$) to look at the testis. Analysis of these mutants with different gene

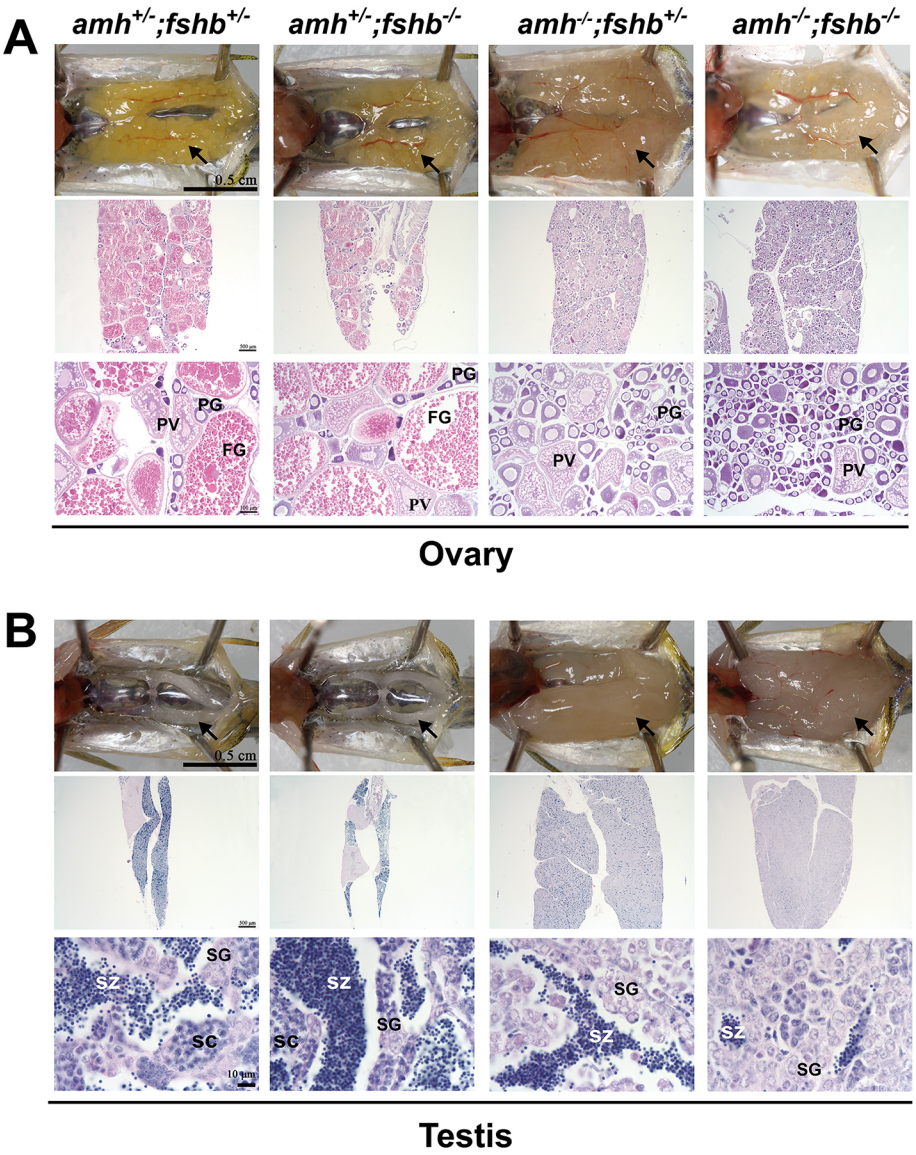

Fig. 1. Gonadal histology of *amh* mutant and double mutant with *fshb* mutation at 4 mpf. (A) Ovaries of four different genotypes: control ($amh^{+/-};fshb^{+/-}$), *fshb* single mutant ($amh^{+/-};fshb^{-/-}$), *amh* single mutant ($amh^{-/-};fshb^{+/-}$) and *amh* and *fshb* double mutant ($amh^{-/-};fshb^{-/-}$). (B) Testes of the four different genotypes. SC, spermatocytes; SG, spermatogonia; SZ, spermatozoa. Arrows indicate ovaries or testes.

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combinations provided crucial insights into the interactions of gonadotropin and Amh signaling pathways in controlling both germ cell proliferation (accumulation of PG follicles in the ovary and spermatogonia in the testis) and differentiation (PG-PV transition or follicle activation in the ovary and meiosis in the testis).

In females, the loss of Fshr in the *amh* and *fshr* double mutant ($amh^{-/-};fshr^{-/-}$) completely abolished the ovarian hypertrophy induced by *amh* deficiency with all follicles arrested at the PG stage as seen in *fshr* single mutant ($amh^{+/-};fshr^{-/-}$). Comparing *amh* mutants in the presence or absence of *fshr* ($amh^{-/-};fshr^{+/-}$ and $amh^{-/-};fshr^{-/-}$), we could see clearly that the ovarian hypertrophy occurred only in the presence of *fshr*. Interestingly, the accumulation of PG follicles in the hypertrophic $amh^{-/-};fshr^{+/-}$ ovary was accompanied by reduced exit of PG follicles to advanced stage or PG-PV transition. Full-scale folliculogenesis took place only when both *amh* and *fshr* were present, as seen in the control ($amh^{+/-};fshr^{+/-}$) (Fig. 2A).

Similar patterns were observed for testicular development. The lack of both gonadotropin receptors, Fshr and Lhcgr, abolished testicular hypertrophy induced by *amh* mutation ($amh^{-/-};fshr^{-/-};lhcgr^{-/-}$), resulting in extremely underdeveloped testes, with spermatogonia only, as seen in the *fshr/lhcgr* double mutant ($amh^{+/-};fshr^{-/-};lhcgr^{-/-}$). The testicular hypertrophy occurred only in the presence of *fshr/lhcgr* ($amh^{-/-};fshr^{+/-};lhcgr^{+/-}$) but with very limited meiotic entry. Like that in the ovary, normal testis size and full-scale spermatogenesis were achieved only in the presence of all three genes ($amh^{+/-};fshr^{+/-};lhcgr^{+/-}$) (Fig. 2B).

Loss of *bmpr2a* phenocopies *amh* mutant in controlling gametogenesis

One intriguing issue concerning the actions of Amh in zebrafish is that there is no Amh type II receptor (Amhr2/*amhr2*) in its genome, in sharp contrast to other species, including fish (Klüver et al., 2007; Liu et al., 2019; Morinaga et al., 2007). How, then, does Amh signal? Our hypothesis is that without its cognate type II receptor, Amh in zebrafish might signal through a related receptor of the TGF-β receptor family, and the most likely candidate would be Bmpr2a, according to its phylogenetic relationship with Amhr2 (Fig. S1A). To address this issue, we created a *bmpr2a* mutant line with a five-base deletion ($bmpr2a^{-5/-5}$) (Fig. S1B). Analysis of the phenotype showed that the female mutant ($bmpr2a^{-/-}$) was similar to the controls (+/+ and +/−) in gross morphology, including body weight and body length; however, the mutant showed an obviously expanded abdomen and significantly higher gonadosomatic index (GSI; gonad weight/body weight) at 5 mpf (Fig. 3A,C). Histological analysis of the ovaries revealed normal folliculogenesis in control females (+/+ and +/−), with all stages of follicles from primary growth (PG) to full-grown (FG) stage. By contrast, there was an enormous accumulation of follicles in the mutant ovaries (−/−), with most being at the PG stage and very few follicles beyond the PV stage (Fig. 3A), remarkably similar to the phenotype manifested by the age-matched *amh* mutant (Fig. 1A; Fig. 2A). With regard to males, the *bmpr2a* mutant (−/−) had a much larger belly, with enormous size of the testis, compared with the male controls (+/+ and +/−) (Fig. 3B,C), which was also identical to that of *amh* mutant males at the same stage (Fig. 1B; Fig. 2B). Histological analysis showed abundant spermatogonia in mutant testes, with limited meiosis (Fig. 3B). Other than gonadal hypertrophy and dysfunction, the *bmpr2a* mutant did not exhibit much difference in the sex ratio compared with the controls (Fig. 3D), again similar to the *amh* mutant (Zhang et al., 2020).

Further evidence for Bmpr2a as a putative Amh type II receptor in zebrafish

As suggested by the above results, Amh in zebrafish might signal through Bmpr2a in the absence of its cognate Amhr2, because the mutants of *amh* and *bmpr2a* phenocopied each other in terms of gonadal development and gametogenesis in both sexes. To test this hypothesis further, we generated an *amh* and *bmpr2a* double mutant ($amh^{-/-};bmpr2a^{-/-}$). At 3 mpf, single mutants of either *amh* ($amh^{-/-};bmpr2a^{+/-}$) or *bmpr2a* ($amh^{+/-};bmpr2a^{-/-}$) showed similar abnormalities in folliculogenesis, with accumulation of PG follicles (Fig. 4A). Interestingly, the ovary of the double mutant ($amh^{-/-};bmpr2a^{-/-}$) was comparable to those of *amh* and *bmpr2a* single mutants, without any visible additive effects, and the ovarian weights, as indicated by GSI, were also comparable among the three genotypes (Fig. 4C).

Similar to females, the males of all three genotypes, i.e. single mutants of *amh* ($amh^{-/-};bmpr2a^{+/-}$) or *bmpr2a* ($amh^{+/-};bmpr2a^{-/-}$) and the double mutant ($amh^{-/-};bmpr2a^{-/-}$), showed the same phenotypes, including enormous testicular hypertrophy with accumulation of spermatogonia and decreased entry into meiosis (Fig. 4B). Similar to females, the weights of testes (GSI) were also comparable among the three mutant groups, but they were all much higher than that in the control (Fig. 4C). Both males and females of the double mutant ($amh^{-/-};bmpr2a^{-/-}$) were subfertile at a young age and able to produce viable offspring (Fig. 4D).

In addition to gonadal development, the *bmpr2a* mutant also phenocopied the *amh* mutant in terms of *fshb* expression in the pituitary. As we reported recently, the expression of *fshb* in adult *amh* mutant males was dramatically reduced (Zhang et al., 2020). As shown in Fig. 4E, the expression of *fshb* in males was also significantly reduced in both the *bmpr2a* single mutant and the double mutant with *amh* ($amh^{-/-};bmpr2a^{-/-}$) (Fig. 4E).

Deficiency of *bmpr2b* leads to defective folliculogenesis in the ovary

Owing to genome duplication in teleosts, genes in zebrafish often have duplicated copies. In addition to *bmpr2a*, the zebrafish also has a duplicated copy of the *bmpr2* gene (*bmpr2b*) (Fig. S1A). To evaluate the roles of *bmpr2b*, we also generated a mutant line with a 14-base insertion ($bmpr2b^{+14/+14}$) (Fig. S1B). In contrast to *bmpr2a*, the loss of *bmpr2b* had no effect on male development at 3 mpf, and the mutant males ($bmpr2b^{-/-}$) showed normal body shape and testicular morphology, with normal fertility. Further histological analysis revealed a normal process of spermatogenesis in the mutant (Fig. 5A).

In contrast to the males, the *bmpr2b*-deficient females showed severe reproductive defects at 3 mpf. Histological analysis showed that despite comparable morphology at 3 mpf, the follicles in the mutant ovaries ($bmpr2b^{-/-}$) were much smaller than those in the control ovaries (+/+ and +/−), with a maximum of the mid-vitellogenic (MV) stage (Fig. 5B). The follicles from the controls ranged from 150 to 700 µm, indicating normal folliculogenesis. By contrast, the follicles in *bmpr2b* mutant ovaries ranged from 150 µm to a maximum of only 400 µm (Fig. 5C), suggesting an arrest of follicular growth at the early vitellogenic (EV) and/or MV stage, which have average diameters of ∼350 and ∼450 µm, respectively (Zhou et al., 2011). The folliculogenesis in the mutant ovary remained arrested at 5 mpf (Fig. 5B), and most of the leading follicles exhibited atretic structures, such as granulosa cell hypertrophy and abnormal chorion (Fig. 5D). To confirm the malfunction of the mutant females, we tested their fertility. The mutant females ($bmpr2b^{-/-}$) could ovulate only dozens of eggs each time during

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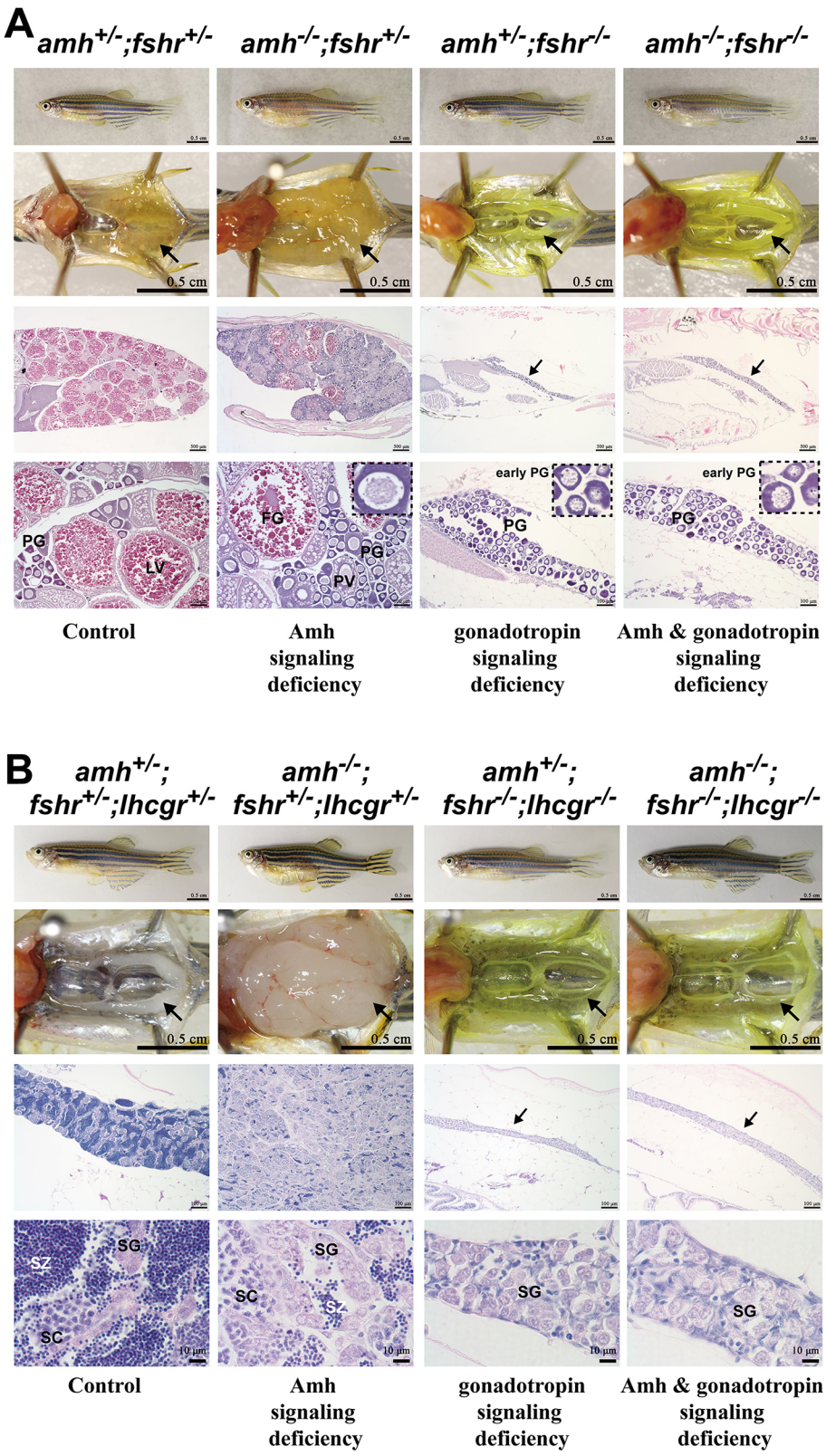

Fig. 2. Evidence for involvement of gonadotropin signaling in *amh* deficiency-induced gonadal hypergrowth and dysfunctional gametogenesis. (A) Ovaries of different genotypes at 5 mpf. The *amh* single mutation ($amh^{-/-};fshr^{+/-}$) caused ovarian hypertrophy with accumulation of PG follicles, whereas the *fshr* single mutant ($amh^{+/-};fshr^{-/-}$) showed ovarian hypotrophy with much fewer, underdeveloped PG follicles only. Double mutation of *amh* and *fshr* ($amh^{-/-};fshr^{-/-}$) completely abolished the phenotype of *amh* mutant. (B) Testes of different genotypes at 5 mpf. The *amh* mutation alone ($amh^{-/-};fshr^{+/-};lhcgr^{+/-}$) caused testicular hypertrophy and dysfunctional spermatogenesis with limited meiosis, whereas the loss of gonadotropin signaling in the *fshr* and *lhcgr* double mutant ($amh^{+/-};fshr^{-/-};lhcgr^{-/-}$) led to testicular hypotrophy and dysfunctional spermatogenesis, with no meiosis. The loss of all the three genes in the triple knockout ($amh^{-/-};fshr^{-/-};lhcgr^{-/-}$) completely abolished the hypertrophic phenotype of *amh* mutation. LV, late vitellogenic; SC, spermatocytes; SG, spermatogonia; SZ, spermatozoa. Arrows indicate ovaries or testes.

mating, in contrast to hundreds by control siblings (Fig. 6A), and the ovulated eggs from the mutant females were <550 µm in diameter, much smaller than those from the controls (~750 µm) (Fig. 6B). All eggs from the mutant females were unable to undergo normal cleavage and eventually died, indicating failure of fertilization; therefore, the *bmpr2b* mutant females were infertile (Fig. 6A,B).

Loss of Bmpr2 signaling results in juvenile mortality

Considering the importance of BMP signaling in animal development and the severe early embryonic lethality in *Bmpr2* null mice (Beppu et al., 2000), we were surprised by the lack of significant developmental defects in *bmpr2* single mutants, except in reproduction ($bmpr2a^{-/-}$ and $bmpr2b^{-/-}$). To address this issue

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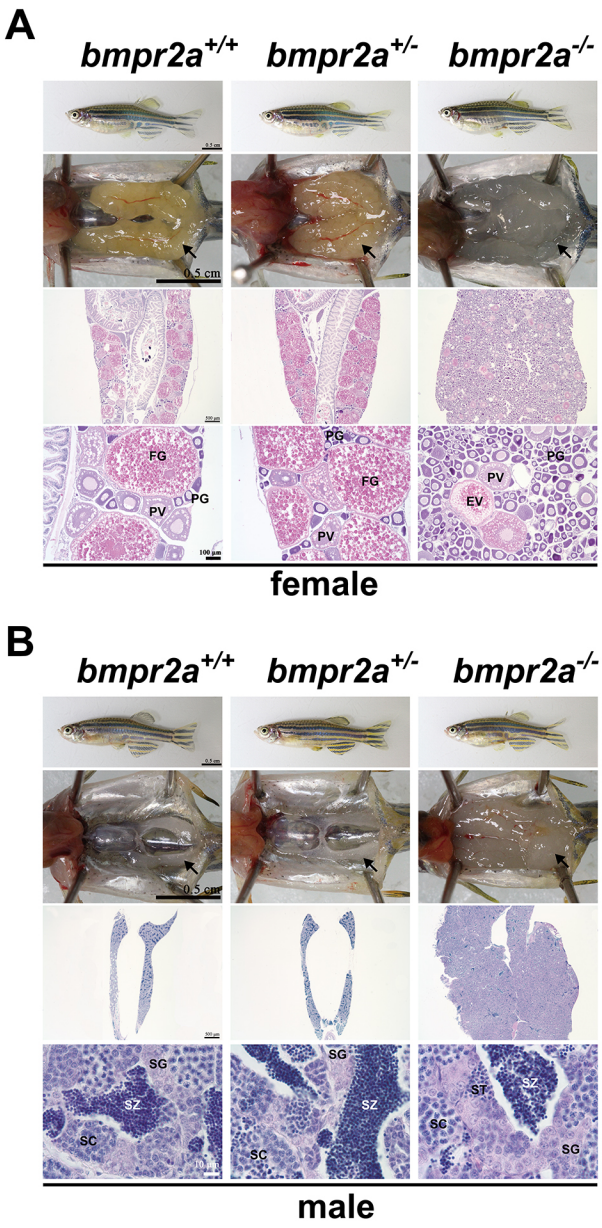

Fig. 3. Gonadal hypertrophy in *bmpr2a* mutant. (A) Ovaries of different genotypes at 5 mpf. The *bmpr2a* mutation (*bmpr2a*$^{-/-}$) caused significant enlargement of the ovary, which contained a large number of PG follicles, with only a few entering PV and vitellogenic growth. (B) Testes of different genotypes at 5 mpf. The *bmpr2a* mutation also induced hypertrophic growth of the testis, which contained abundant spermatogonia with limited meiosis. (C) Body length, body weight and GSI of different genotypes (n=5; *P<0.05, **P<0.01; two-tailed Student's t-test for unpaired data). (D) Sex ratios in different genotypes. SC, spermatocytes; SG, spermatogonia; ST, spermatids; SZ, spermatozoa. Arrows indicate ovaries or testes.

further, we generated a Bmpr2-deficient double mutant (*bmpr2a*$^{-/-}$; *bmpr2b*$^{-/-}$). The double mutant fish could develop normally until 15 days postfertilization (dpf), but died progressively afterwards. Most individuals died before 60 dpf, with only a few survivors, indicating the importance of Bmpr2 signaling for postembryonic development. Details of developmental defects and the potential cause of death are described in Figs S2-S4. Owing to the high juvenile mortality and low vitality of the survivors, it was difficult to obtain sexually mature double mutants for analysis of reproductive performance.

DISCUSSION

AMH was first identified by its induction of Müllerian duct regression during male differentiation (Behringer et al., 1994). Further studies have shown that it is also involved in the control of gonadal development and gametogenesis in both males and females (Durlinger et al., 2002; Rehman et al., 2017; Visser and Themmen, 2005). Interestingly, Amh/*amh* is also present in teleosts, which do not have Müllerian ducts (Pfennig et al., 2015). Genetic analyses in fish species have provided evidence for its primary roles in gonadal development and function. Disruption of the *amh* gene in zebrafish led to gonadal hypertrophy (Lin et al., 2017; Yan et al., 2019; Zhang et al., 2020), which is confirmed in the present study. Likewise, mutation of both *amh* and *amhr2* in tilapia also resulted in ovarian hypertrophy and accumulation of PG follicles (Liu et al., 2019). These results agree well with the report in medaka on the *amhr2* mutant (*hotei*) (Morinaga et al., 2007). Despite these studies, the mechanisms of action of Amh still remain largely unknown in fish. More intriguingly, the zebrafish does not even have the cognate type II receptor for Amh (Amhr2) (Morinaga et al., 2007; Yan et al., 2019). In this study, we have provided genetic evidence for modulation by Amh of gonadotropin signaling in the control of gonadal homeostasis and gametogenesis, specifically at the points of germ cell proliferation and differentiation. Furthermore, we have also presented evidence that Amh in zebrafish is most likely to signal through a noncanonical pathway involving bone morphogenetic protein receptor, type II a (serine/threonine kinase) (Bmpr2a/*bmpr2a*), but not Bmpr2b/*bmpr2b*.

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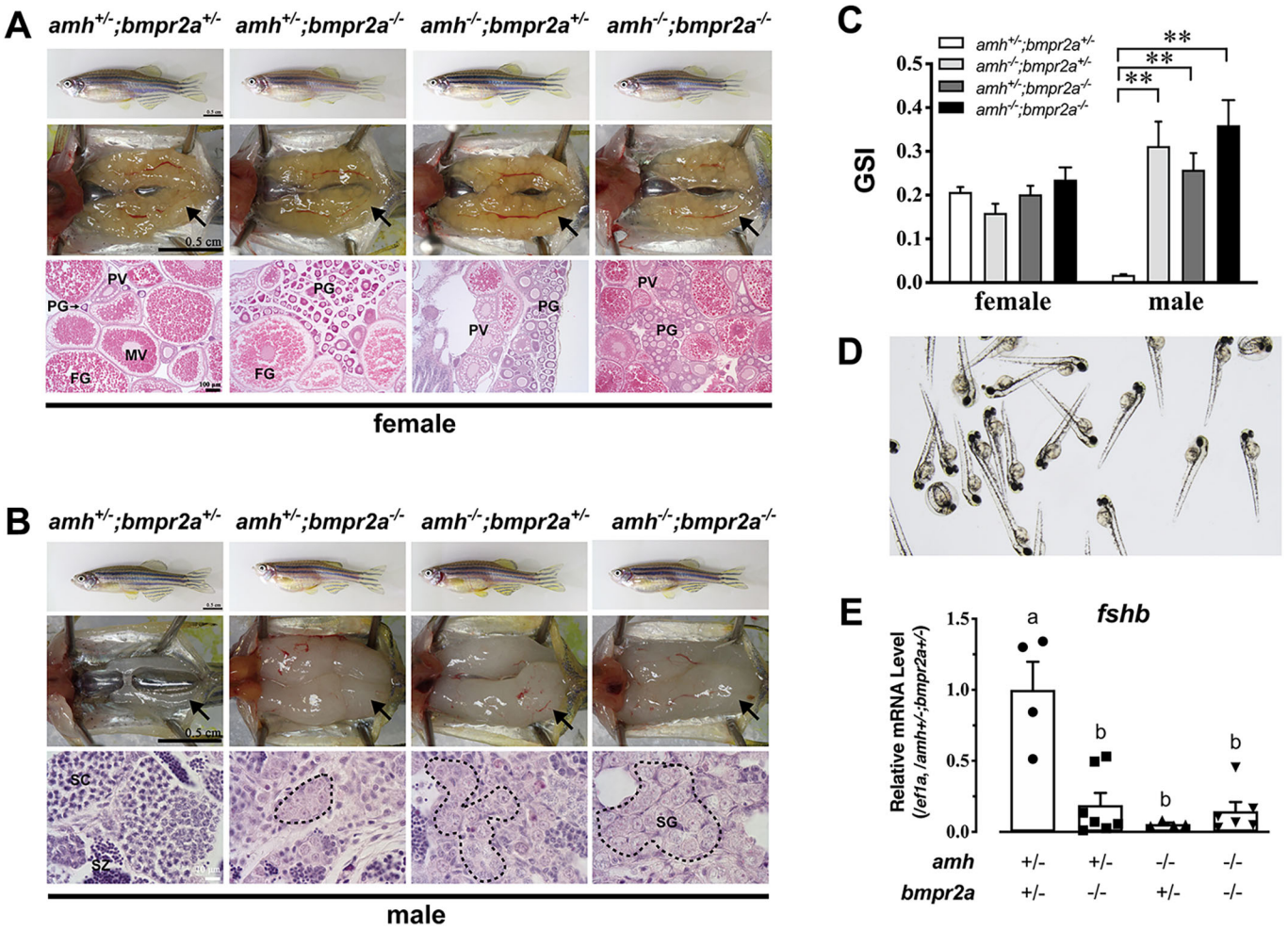

Fig. 4. Phenotype analysis of *amh* and *bmpr2a* double mutant at 3 mpf. (A) Ovaries of different genotypes. There was a significant accumulation of PG follicles in the *amh* single mutant (*amh*$^{-/-}$;*bmpr2a*$^{+/-}$), *bmpr2a* single mutant (*amh*$^{-/-}$;*bmpr2a*$^{-/-}$) and *amh* and *bmpr2a* double mutant (*amh*$^{-/-}$;*bmpr2a*$^{-/-}$) in comparison with the control (*amh*$^{+/-}$;*bmpr2a*$^{+/-}$). (B) Testes of different genotypes at 3 mpf. All three mutants (*amh* and *bmpr2a* single mutants and the double mutant) displayed tremendous testicular hypertrophy, with abundant spermatogonia (circled with dotted lines) and without any additive effect. (C) GSI of the different genotypes (n=5; **P<0.01; two-tailed Student's t-test for unpaired data). (D) Viable offspring (3 dpf) from male and female double mutants (*amh*$^{-/-}$;*bmpr2a*$^{-/-}$). (E) Expression of *fshb* in the pituitary of male *amh* and *bmpr2a* single and double mutants. The groups with different letters indicate that they are statistically significant (P<0.05) [n=4 (*amh*$^{+/-}$;*bmpr2a*$^{+/-}$), 7 (*amh*$^{+/-}$;*bmpr2a*$^{-/-}$), 4 (*amh*$^{-/-}$;*bmpr2a*$^{+/-}$) and 6 (*amh*$^{-/-}$;*bmpr2a*$^{-/-}$)]. Data are mean±s.e.m.

Interaction of gonadotropin and Amh signaling pathways in control of germ cell proliferation and differentiation

As reported by others in different models, we also demonstrated that a lack of Amh in zebrafish significantly increased proliferation of germ cells but decreased their differentiation. The increased germ cell proliferation resulted in accumulation of PG follicles in the ovary and spermatogonia in the testis, whereas their decreased differentiation was manifested by reduced exit of PG follicles to the PV stage (follicle activation) in the *amh* mutant ovary and limited meiotic entry to spermatocytes in the testis. The enormous growth of *amh* mutant gonads was therefore attributable to hyperproliferation of the germ cells and their limited differentiation to advanced stages.

Considering that pituitary gonadotropins are the master hormones that control gonadal growth and development and that AMH stimulates FSH expression and secretion (Barbotin et al., 2019; Garrel et al., 2016; Kadokawa, 2020) but suppresses gonadal sensitivity or responsiveness to FSH (Durlinger et al., 2001; Visser and Themmen, 2014), we hypothesized that the enormous growth of *amh* mutant gonads might involve gonadotropin signaling. As we reported recently, the loss of FSH (*fshb*) or its receptor (*fshr*) resulted in a significant suppression of gonadal growth and gametogenesis (Zhang et al., 2015a,b). However, the loss of *fshb* had no impact on gonadal hypertrophy in the adult *amh* mutant (*amh*$^{-/-}$;*fshb*$^{-/-}$). This could be attributable to the rescue of the lost function of FSH by LH through Fshr at high concentrations (So et al., 2005). To test this hypothesis, we went on to generate double and triple mutants of *amh* and gonadotropin receptors (*fshr* and *lhcgr*). Interestingly, the loss of *fshr* completely abolished the phenotype of ovarian hypertrophy in the female *amh* mutant (*amh*$^{-/-}$;*fshr*$^{-/-}$), and the loss of *fshr*/*lhcgr* abolished testicular hypertrophy in the male *amh* mutant (*amh*$^{-/-}$;*fshr*$^{-/-}$;*lhcgr*$^{-/-}$). These results strongly suggest that Amh might act in the ovary and testis by suppressing gonadal responsiveness to gonadotropins. The loss of Amh would lead to over-activation of gonadotropin signaling by FSH and/or LH, resulting in increased generation of PG follicles in the ovary and proliferation of spermatogonia in the testis (Fig. 7). The inhibition of gonadotropin signaling, especially FSH, by AMH has also been reported in mammals (Durlinger et al., 2001; Visser and Themmen, 2005, 2014). AMH reduced the sensitivity of ovarian follicles to FSH in mice, as evidenced by an inhibitory effect of AMH on FSH-stimulated follicle

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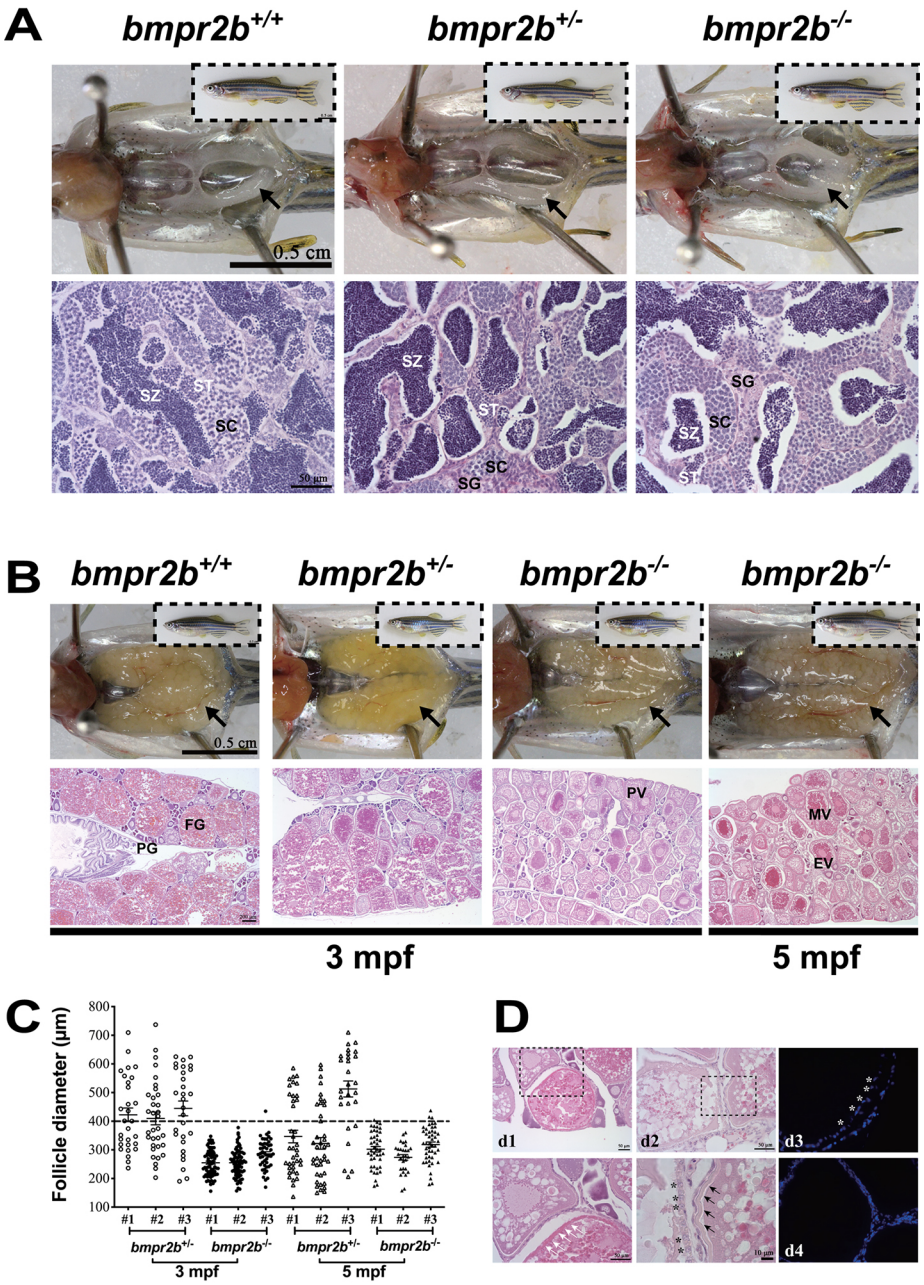

Fig. 5. Analysis of the phenotype of the *bmpr2b* mutant. (A) Normal testicular growth and spermatogenesis in male *bmpr2b* mutant at 3 mpf. (B) Ovarian defects in female *bmpr2b* mutant at 3 and 5 mpf. Folliculogenesis was arrested at the MV stage in the *bmpr2b* mutant at both 3 and 5 mpf. (C) Follicle diameters measured on histological section. Two distant sections from each individual were chosen for quantification of PV and vitellogenic follicles with germinal vesicle visible, and three individuals of each genotype (#1-#3) were analyzed at 3 and 5 mpf. Data are mean±s.e.m. (D) Follicular atresia in the ovary of *bmpr2b* mutants showing abnormal chorion (arrows in bottom panels of d1 and d2) and hypertrophic granulosa cells (asterisks in bottom panel of d2). Staining with DAPI showed the hypertrophic granulosa cells (asterisks in d3) in comparison with normal follicles (d4). Bottom panels in d1 and d2 show higher magnification of the boxed areas above. SC, spermatocytes; SG, spermatogonia; ST, spermatids; SZ, spermatozoa. Arrows indicate ovaries or testes.

growth *in vitro* (Durlinger et al., 2001), and this inhibition was attributable, in part, to reduced expression of aromatase and FSHR (Grossman et al., 2008; Pellatt et al., 2011).

However, the suppression of gonadotropin signaling by Amh in zebrafish might not involve changes of gonadotropin expression in the pituitary and Fshr in the ovary. First, the expression of *fshb*, but not *lhb*, decreased, instead of increasing, in the pituitary of the adult *amh* mutant (Zhang et al., 2020), which agrees well with the stimulatory effect of AMH on FSH expression and secretion in mammals (Barbotin et al., 2019; Garrel et al., 2016; Kadokawa, 2020). Second, no significant changes in *fshr* expression were observed in ovarian follicles of different stages in the *amh* mutant compared with controls. Likewise, we did not see any changes of expression in the *amh* mutant ovary in *cyp19a1a* (*cytochrome P450, family 19, subfamily A, polypeptide 1*; ovarian aromatase) and *inhbaa* (*inhibin subunit beta Aa*), which are two potential downstream genes for follicle activation (Fig. S5). Therefore, the suppression of Fshr signaling by Amh is likely to take place at the post-receptor level. This will be an interesting issue to explore in future studies. The inhibition of gonadotropin signaling by AMH/Amh at the gonadal level in both mammals and fish suggests a conserved local mechanism that keeps gonadotropin action in check to maintain gonadal homeostasis. The loss of Amh in the gonads would remove such inhibition, therefore intensifying gonadotropin signaling, which would, in turn, induce hyperproliferation of germ cells.

What is puzzling is why an enhanced gonadotropin signaling in the *amh* mutant was associated with reduced germ cell differentiation, viz. meiosis in the testis and PG-PV transition in the ovary. Our hypothesis is that although Amh suppresses gonadotropin-stimulated germ cell proliferation, it plays a permissive role in gonadotropin-stimulated germ cell differentiation, viz. meiosis in the testis and follicle activation in the ovary. This idea is supported by our evidence that without *fshr* (−/− female) or *fshr/lhcgr* (−/−;−/− male), the

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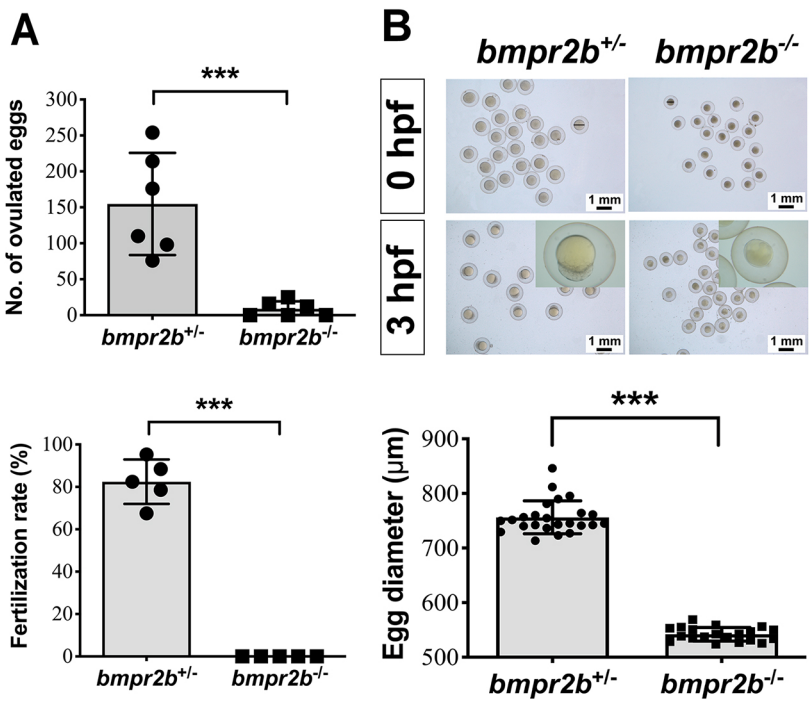

Fig. 6. Fertility test of *bmpr2b* mutant. (A,B) Mutant (*bmpr2b*$^{-/-}$) and control (*bmpr2b*$^{+/-}$) females were crossed with wild-type male partners. (A) The number of ovulated eggs (0 hpf) and fertilization rate (3 hpf). (B) Diameters of the eggs released. ***P<0.001 (two-tailed Student's t-test for unpaired data). Data are mean±s.e.m.

PG-PV transition in the ovary and meiosis in the testis stopped completely, regardless of the presence or absence of Amh (+/− or −/−), suggesting that Amh itself is not sufficient to drive germ cell differentiation. These activities resumed, but only to a very limited extent, in the presence of *fshr* (+/− female) or *fshr/lhcgr* (+/−;+/− male) but the absence of *amh* (−/−). Full-scale differentiation (meiosis and PG-PV transition) occurred in the presence of all genes (*amh*$^{+/-}$;*fshr*$^{+/-}$ in the female and *amh*$^{+/-}$;*fshr*$^{+/-}$;*lhcgr*$^{+/-}$ in the male). The dual roles played by Amh in modulation of gonadotropin signaling (inhibitory for germ cell proliferation but stimulatory for differentiation) make Amh a crucial gonadal factor in the maintenance of gonadal homeostasis and normal gametogenesis.

Evidence for Bmpr2a-mediated Amh signaling in zebrafish

As a member of the TGF-β superfamily, AMH is well known for its signaling through a specific type II receptor, AMHR2 (Mishina et al., 1996). Knockout of *Amhr2* in mice showed that the male mutant developed both male and female reproductive tract systems, including the uterus and oviduct, fully phenocopying the AMH ligand mutant (Mishina et al., 1996). In teleosts, a previous study in medaka characterized a mutant (*hotei*) that showed enormous gonadal sizes, and the mutant gene was identified as *amhr2* (Morinaga et al., 2007). Similar phenotypes have also been reported in *amh* mutants of zebrafish (Lin et al., 2017; Yan et al., 2019; Zhang et al., 2020) and *amh*/*amhr2* mutants in tilapia (Liu et al., 2019), suggesting Amh-Amhr2 signaling in teleosts. However,

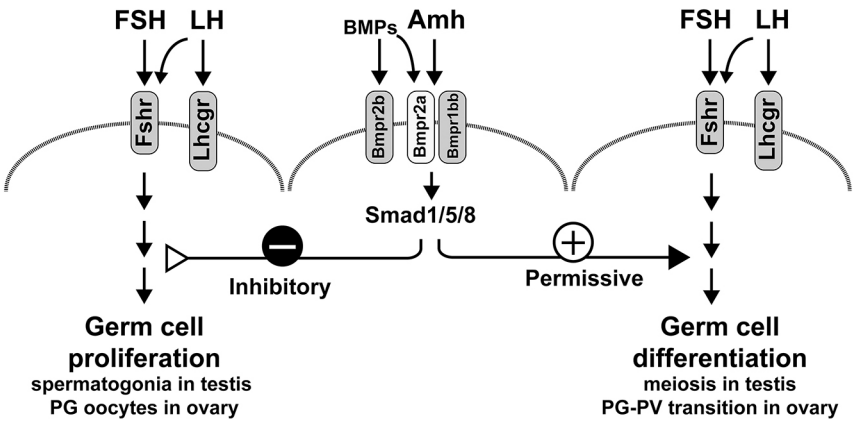

Fig. 7. Hypothetical model for interactions of Amh and gonadotropin signaling pathway in zebrafish gonads. Unlike other species, the zebrafish lacks the Amh cognate type II receptor (Amhr2). Instead, Amh in zebrafish is likely to signal through a closely related BMP type II receptor, Bmpr2a, which then recruits Bmpr1bb as its type I receptor for activation of the canonical Smad1/5/8 pathway. Mutations of *amh*, *bmpr2a* and *bmpr1bb* in zebrafish produced identical phenotypes in the gonads, viz. gonadal hypertrophy with increased proliferation of germ cells (spermatogonia in the testis and PG follicles in the ovary) and decreased differentiation or exit of germ cells to advanced stages (meiosis in the testis and PG-PV transition in the ovary). Amh in the gonads plays a negative role of inhibiting germ cell proliferation by suppressing gonadotropin signaling while playing a positive role of promoting germ cell differentiation (meiotic division in the testis and follicle activation or PG-PV transition in the ovary) by being permissive to gonadotropin signaling. By controlling both germ cell proliferation and their exit for maturation, Amh serves as a crucial local factor in the maintenance of gonadal homeostasis for steady production of mature gametes in both sexes.

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what is intriguing is that no homolog of *amhr2* has been found in the zebrafish genome. How, then, would Amh signal in zebrafish? Our hypothesis is that without its cognate type II receptor, Amh might bind and signal through a closely related receptor in the TGF-β family. Phylogenetic analysis showed that BMP type II receptors (*bmpr2a/bmpr2b*) have the closest genetic relation with the Amhr2 cluster among all the type II receptors of TGF-β family ligands.

To test this hypothesis, we generated two mutant zebrafish lines, for *bmpr2a* and *bmpr2b*, respectively. Analysis of the phenotype demonstrated distinct functions for *bmpr2a* and *bmpr2b* in zebrafish, suggesting neofunctionalization and/or subfunctionalization for these genes after genome duplication. Unexpectedly, neither *bmpr2a* nor *bmpr2b* single mutants exhibited any developmental defects, despite the well-known importance of the BMP family in embryogenesis and organogenesis (Chen et al., 2004); however, the double mutant of *bmpr2a* and *bmpr2b* (*bmpr2a*$^{-/-}$;*bmpr2b*$^{-/-}$) exhibited severe significant developmental defects, with juvenile mortality after 15 dpf, suggesting functional complementation of Bmpr2a and Bmpr2b in zebrafish embryonic and larval development. By comparison, *Bmpr2* null mice died much earlier, at the embryonic stage (Beppu et al., 2000).

Despite the lack of phenotypes of *bmpr2a* and *bmpr2b* single mutants in development and growth, they both exhibited unique phenotypes in reproduction. Surprisingly, the loss of *bmpr2a* fully phenocopied those of the *amh* mutant in both males and females. The female mutant (*bmpr2a*$^{-/-}$) exhibited abnormal folliculogenesis, with accumulation of PG follicles and gradual depletion of advanced follicles, and the male mutant had an enormous testis, with abundant spermatogonia. These phenotypes were identical to those of the *amh* mutant in zebrafish (Lin et al., 2017; Yan et al., 2019; Zhang et al., 2020) and tilapia (Liu et al., 2019) and those of the *amhr2* mutant in medaka (Morinaga et al., 2007) and tilapia (Liu et al., 2019). In addition, both *amh* and *bmpr2a* mutants had significantly reduced *fshb* expression in the pituitary (Zhang et al., 2020). These results strongly suggest a potential role for *bmpr2a* to function as the Amh type II receptor (Amhr2) in zebrafish, therefore completing a missing link in this species. In support of this idea is that the double mutant of *amh* and *bmpr2a* (*amh*$^{-/-}$;*bmpr2a*$^{-/-}$) showed no additive phenotypic effects in comparison to the single mutants of *amh* and *bmpr2a*, and the three genotypes (*amh*$^{-/-}$;*bmpr2a*$^{+/-}$, *amh*$^{+/-}$;*bmpr2a*$^{-/-}$ and *amh*$^{-/-}$;*bmpr2a*$^{-/-}$) showed no significant phenotypic difference in terms of both GSI and histological morphology, further suggesting that they are working in the same signaling pathway. This agrees well with a study in mice, in which the double mutant of *Amh* and *Amhr2* showed indistinguishable phenotypes from those of single mutants (Mishina et al., 1996). The adoption of Bmpr2a as Amhr2 to mediate Amh signaling in zebrafish is not surprising, because TGF-β family members often show a high degree of ligand-receptor promiscuity (Santibanez et al., 2011), and both BMP and AMH are known to suppress the sensitivity of follicles to FSH in mammals (Visser and Themmen, 2014). In zebrafish, our previous study showed that recombinant zebrafish Bmp2b and Bmp4 significantly suppressed *fshr* but stimulated *lhcgr* expression in cultured follicle cells (Li et al., 2012).

Interestingly, a mutation in a putative BMP type I receptor, *bmpr1bb* (*alk6b*), also caused similar gonadal defects in zebrafish (Neumann et al., 2011). Based on this and our results, we propose that as a gonad-specific growth factor, Amh might act through the signaling cascade of Amh-Bmpr2a-Bmpr1bb to control gonadal development in zebrafish (Fig. 7).

Distinct roles of Bmpr2b in folliculogenesis

Our previous study reported spatiotemporal expression profiles of BMP ligands (*bmp2a*, *bmp2b*, *bmp4*, *bmp6* and *bmp7a*) and BMP type II receptors (*bmpr2a* and *bmpr2b*) in zebrafish follicles. The ligands examined were expressed primarily in the oocyte, whereas their type II receptors, *bmpr2a* and *bmpr2b*, were localized exclusively in the surrounding follicle cells, suggesting a BMP-mediated paracrine regulation between the oocyte and somatic follicle cells. The two receptors, *bmpr2a* and *bmpr2b*, showed increased expression during folliculogenesis, with the highest levels reached at the FG stage (Li and Ge, 2011), suggesting roles for BMP signaling in follicular growth and maturation.

After demonstrating that *bmpr2a* might serve as the receptor for Amh in the control of gonadal development and function, we turned our attention to *bmpr2b*. The results showed entirely different phenotypes between *bmpr2a* and *bmpr2b* mutants. In contrast to *bmpr2a*, the loss of *bmpr2b* (*bmpr2b*$^{-/-}$) had no effect on males, which showed normal testicular development and spermatogenesis. By contrast, the female *bmpr2b* mutant showed severe reproductive defects. The mutant females were infertile because the follicles could grow only to the EV stage or a maximum of the MV stage, not the full-sized FG stage, and the ovulated eggs could not be fertilized. This suggests that the BMP-Bmpr2b signaling pathway plays an important role in the control of vitellogenic growth in zebrafish.

The distinct phenotypes of *bmpr2a* and *bmpr2b* mutants strongly suggest neofunctionalization of the two receptors after genome duplication. Bmpr2a takes up a new role to serve as the Amh type II receptor, therefore completing the missing link for Amh signaling in zebrafish, which lacks the cognate Amh type II receptor (Amhr2). By contrast, Bmpr2b has nothing to do with Amh signaling but plays a crucial role in folliculogenesis, most probably by mediating the BMP signals from the growing oocytes.

In conclusion, the present study confirmed the functions of Amh reported in zebrafish and provided crucial genetic evidence for its interaction with gonadotropin signaling in the gonads. The key discoveries can be summarized as follows: (1) Amh deficiency induces severe disturbance in gonadal homeostasis and gametogenesis in both males and females; (2) Amh inhibits germ cell proliferation by suppressing the action of gonadotropins but stimulates germ cell differentiation by promoting gonadotropin signaling; and (3) Amh might signal via Bmpr2a in the absence of its cognate receptor, Amhr2.

MATERIALS AND METHODS

Fish and maintenance

The AB strain of zebrafish was used in the present study. The larval fish were initially raised in an environmental chamber (model 3949; Thermo Scientific) with *Paramecium*. They were transferred to the ZebTEC Multilinking Rack Zebrafish System (Tecniplast) after starting to feed on brine shrimp (*Artemia*). The system was under a photocycle of 14 h light and 10 h dark, with temperature, pH and conductivity being 28±1°C, 7.5 and 400 µS/cm, respectively. All experiments were performed according to the protocols approved by the Research Ethics Committee of University of Macau.

Establishment of mutant lines

Zebrafish mutant lines were established using the CRISPR/Cas9 method according to the protocols reported previously (Lau et al., 2016). The online tool ZIFIT Targeter (http://zifit.partners.org/zifit) was used to design CRISPR target sites. The single guide RNAs (sgRNAs) and *Cas9* RNA were prepared using MEGAscript T7 and mMESSAGE mMACHINE SP6 kits (Life Technologies), respectively. About 100 pg of sgRNA and 400 pg of *Cas9* mRNA were co-injected into embryos at the one- or two-cell stage with the Drummond Nanoject system (Drummond Scientific). Mutagenesis was first screened at 24 hours postfertilization (hpf) using both high-resolution melting analysis (HRMA) and a heteroduplex mobility assay (HMA) (Zhang et al., 2015a,b). The F0 adults were genotyped on DNA extracted from the caudal fin for mutagenesis followed by sequence

confirmation (Zhang et al., 2015b). The sibling F1 females and males carrying the same frameshift mutation (+/−) were crossed to obtain homozygous F2 (−/−) offspring for analysis of phenotypes. The primers used for HRMA/HMA are listed in Table S1.

Sampling and histological examination

The fish were anesthetized with MS222 (Sigma) before handling. Each fish was photographed with a digital camera (Canon EOS 700D) to record gross morphology before measurement of body weight, standard body length and the GSI (gonad weight/body weight). For histological analysis, the entire fish or dissected gonads were fixed in Bouin's fixative for 24 h before processing on the ASP6025S Automatic Vacuum Tissue Processor (Leica). After paraffin embedding, the samples were serially sectioned at 5 µm, stained with Hematoxylin and Eosin (H&E), and viewed with a Nikon ECLIPSE Ni-U microscope (Nikon, Tokyo, Japan). The images were photographed with a Digit Sight DS-Fi2 digital camera (Nikon).

Fertility and survival test

The fish were crossed with wild-type partners for either individual or group tests. The fecundity of the females refers to the number of the ovulated eggs at 0-1 hpf, and the fertility refers to normal fertilization and embryogenesis in addition to viability of the offspring. The survival rate was calculated based on the ratios of different genotypes at different time points from 0 to 60 dpf at intervals of 15 days.

Fin regeneration

The caudal fins were amputated at 35 dpf (day 0), and the process of regeneration was recorded by photographing at days 3 and 6.

RNA extraction and quantitative real-time PCR

Total RNA was extracted from the ovary or testis using TRIzol (Invitrogen) according to the manufacturer's protocol. The extracted RNA was reverse transcribed to complementary DNA using MMLV reverse transcriptase (Invitrogen) as previously reported (Zhang et al., 2015a,b). The primers used for quantitative real-time PCR are listed in Table S1. The expression levels of target genes were normalized to that of the housekeeping gene *ef1a* and expressed as the fold change compared with the control group.

Immunofluorescence

Sections from paraffin-embedded samples were mounted on slides, followed by de-paraffinization in xylene and rehydration with serially diluted ethanol. Antigen retrieval was performed by placing the slides in sub-boiling citrate buffer for 10 min. The sections were washed with phosphate-buffered saline Tween (PBST) buffer three times, for 5 min each, then blocked with 10% heat-inactivated horse serum for 1 h at room temperature in a humidified chamber. The slides were incubated with primary antibody (1:100, anti-pSMAD1/5/8; AB3848-1; Millipore) overnight at 4°C. After washing with PBST buffer five times for 5 min each, the slides were incubated with the secondary antibody (1:1000, Alex Fluor 488; Cell Signaling Technology) for 2 h at room temperature. After washing with PBS five times for 5 min each, the sections were stained with 4′,6-diamidino-2-phenylindole (DAPI; 0.5 µg/ml; Roche) for 5 min. The sections were then mounted with ProLong Gold antifade reagent (Invitrogen) and sealed with commercial nail oil.

Data analysis

All the values in this study were expressed as the mean±s.e.m., and statistical significance was analyzed by ANOVA or unpaired two-tailed Student's *t*-test using Prism v.8 (GraphPad). The level of significance is indicated as follows: *P<0.05, **P<0.01 and ***P<0.001.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: W.G.; Methodology: Z.Z., K.W., Z.R.; Validation: Z.Z.; Formal analysis: W.G., Z.Z.; Investigation: W.G., Z.Z.; Resources: W.G.; Data curation: Z.Z., K.W., Z.R.; Writing - original draft: Z.Z.; Writing - review & editing: W.G.; Visualization: W.G.; Supervision: W.G.; Project administration: W.G.; Funding acquisition: W.G.

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Supplementary information

Supplementary information available online at
https://dev.biologists.org/lookup/doi/10.1242/dev.189811.supplemental

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