Loss of growth differentiation factor 9 causes an arrest of early folliculogenesis in zebrafish–A novel insight into its action mechanism

Weiting Chen, Yue Zhai, Bo Zhu, Kun Wu, Yuqin Fan, Xianqing Zhou, Lin Liu, Wei Ge

1 Department of Biomedical Sciences and Centre of Reproduction, Development and Aging (CRDA), Faculty of Health Sciences, University of Macau, Taipa, Macau, China, 2 Department of Toxicology and Hygienic Chemistry, School of Public Health, Capital Medical University, Beijing, China, 3 School of Life Science, South China Normal University, Guangzhou, China

☯ These authors contributed equally to this work.
* weige@umac.mo/gezebrafish@gmail.com

Abstract

Growth differentiation factor 9 (GDF9) was the first oocyte-specific growth factor identified; however, most information about GDF9 functions comes from studies in the mouse model. In this study, we created a mutant for Gdf9 gene (gdf9-/-) in zebrafish using TALEN approach. The loss of Gdf9 caused a complete arrest of follicle development at primary growth (PG) stage. These follicles eventually degenerated, and all mutant females gradually changed to males through sex reversal, which could be prevented by mutation of the male-promoting gene dmrt1. Interestingly, the phenotypes of gdf9-/- could be rescued by simultaneous mutation of inhibin α (inha-/-) but not estradiol treatment, suggesting a potential role for the activin-inhibin system or its signaling pathway in Gdf9 actions. In gdf9-null follicles, the expression of activin βAα (inhbaa), but not βAb (inhbab) and βB (inhbb), decreased dramatically; however, its expression rebounded in the double mutant (gdf9-/-;inha-/-). These results indicate clearly that the activation of PG follicles to enter the secondary growth (SG) requires intrinsic factors from the oocyte, such as Gdf9, which in turn works on the neighboring follicle cells to trigger follicle activation, probably involving activins. In addition, our data also support the view that estrogens are not involved in follicle activation as recently reported.

Author summary

Follicles are the basic structural and functional units of the ovary. Each follicle consists of an oocyte and surrounding somatic follicle cells. The growth and maturation of follicles or folliculogenesis is controlled by a variety of hormones and local factors. It has been known for decades that the oocyte in the follicle plays an active role in controlling folliculogenesis by releasing various regulatory factors, among which the growth differentiation factor 9 (GDF9) is the best characterized one. In this study, we used gene editing method to delete the gene for GDF9 (gdf9) in the zebrafish. We discovered that without gdf9 gene...
(mutant), the follicles could not develop to advanced stages and the females were therefore infertile. Interestingly, when inhibin (a hormone from the ovary) was lost simultaneously, the follicles in the gdf9 mutant resumed growth and development. We hypothesized that the resumption of follicle growth in the double mutant of gdf9 and inha was likely due to increased activity of activin.

Introduction
Follicles, each consisting of an oocyte and surrounding somatic follicle cells, are the basic structural and functional units of the ovary. The growth and maturation of follicles or folliculogenesis is primarily controlled by gonadotropins (follicle-stimulating hormone, FSH; and luteinizing hormone, LH) from the pituitary in both mammals and fish [1–4]. However, evidence has accumulated in various species that local ovarian factors also play critical roles in controlling folliculogenesis in paracrine and/or autocrine manners [5–9].

In the past two decades, there has been substantial progress in understanding the mechanisms underlying folliculogenesis, especially its early stage [10–12]. Since this stage of follicle development is considered less dependent on gonadotropins, most studies have focused on intraovarian growth factors that work locally through paracrine and/or autocrine pathways [10,11,13,14]. Among numerous local factors characterized, those released by the oocyte have caught much attention in research, particularly growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) [15–18]. Malfunction of these factors has been implicated in human reproductive disorders. For example, some POCS or POF cases in humans have been associated to mutations or expression abnormalities of GDF9 [19–21] or BMP15 [20,22].

As a TGF-β family member, GDF9 was first discovered in 1993 as an ovarian growth factor [23,24]. Subsequent studies have demonstrated that the expression of GDF9 mRNA and protein is restricted to the oocyte in several mammalian species including mice and humans [25]. As a novel oocyte-specific factor, GDF9 is now recognized as a critical player to orchestrate folliculogenesis by signaling the surrounding follicle cells, namely the granulosa and theca cells [14,26,27]. Studies using recombinant GDF9 showed that GDF9 stimulated initial follicle recruitment in the rat ovary [28] and promoted progression of human primordial follicles to the secondary follicle stage in vitro [29]. Both the granulosa and theca cells surrounding the oocyte are the targets of GDF9 action, and its effects vary during folliculogenesis depending on the stage of follicle development [27]. GDF9 promoted granulosa cell proliferation and FSH-induced cumulus expansion in vitro, and increased inhibin and progesterone secretion from these cells [28,30–33]. GDF9 also stimulated the expression of a variety of genes in the granulosa cells including Kit ligand (KL) [34], cyclooxygenase 2 and steroidogenic acute regulatory (StAR) protein [31]. In the theca cells, GDF9 seemed essential for the expression of some theca cell-specific genes such as CYP17 and LH receptor [35,36]. In cultured granulosa-lutein cells of humans, GDF9 alone showed weak stimulatory effects on expression of activin βA and βB subunits; but it dramatically increased the activin-induced βA and βB expression [37]. On the other hand, GDF9 inhibited FSH-induced steroidogenesis and LH receptor expression in the granulosa cells [28]. The physiological importance of GDF9 has also been documented by in vivo studies. The follicles in GDF9 knockout mice can develop normally to the stage of primary follicles but not beyond, causing female infertility [38,39]. A missense GDF9 mutation (V371M) in sheep was strongly associated with litter size [40]. In humans, a mutation in GDF9 diminished ovarian reserve in young women [41].
Unlike other members of the TGF-β superfamily with well-documented serine/threonine kinase receptors (type I and II) and intracellular signaling pathways involving specific Smads, the signaling mechanism of GDF9 still remains elusive. Some studies have implicated bone morphogenetic protein (BMP) type II receptor (BMPR-II) in GDF9 signaling [42,43], which recruits and activates a type I receptor ALK5 (activin receptor-like kinase 5; TGFBR1) [44]. The activation of ALK5 in turn induces phosphorylation of Smad2/3 for intracellular signaling, which can be blocked by inhibitory Smad7, but not Smad6 [30,37,44,45], a mechanism shared by TGF-β and activin.

Compared with studies in mammals especially rodents, the information about GDF9 in other species remains limited. Our laboratory was the first to isolate gdf9 gene in teleosts in 2007 and characterize its expression patterns in the zebrafish. As in mammals, zebrafish gdf9 is also expressed exclusively in the oocyte and its expression is the highest in early pre-vitellogenic follicles [46]. Similar expression patterns have also been reported in other vertebrates. In the chicken, GDF9 is also expressed primarily in the oocytes and its expression level is the highest in small follicles (< 1 mm) [47]. In lizards, gdf9 is highly expressed in the regressed ovary, but its expression level decreases progressively during recrudescence and afterwards [48]. These studies all suggest a conserved role for GDF9/gdf9 in controlling early folliculogenesis; however, the exact function of Gdf9 remains largely unknown in non-mammalian vertebrates.

Our recent study in zebrafish demonstrated that recombinant zebrafish Gdf9 could act on cultured follicle cells, stimulating Smad2 phosphorylation and expression of activin subunit genes (inhbaa and inhbb) but suppressing the expression of anti-Müllerian hormone gene (amh) [49]. A study using recombinant mouse GDF9 showed that GDF9 might regulate tight junction protein expression during zebrafish folliculogenesis [50], and immunization with synthetic zebrafish GDF9 peptide caused abnormal oocyte development [51]. In Japanese flounder, overexpression of gdf9 in an ovarian cell line stimulated expression of most steroidogenic genes [52]. Despite these studies, the functional importance of GDF9 in fish is unknown due to the lack of genetic approaches such as gene knockout in teleosts. In recent years, a new generation of gene editing technologies, in particular TALEN and CRISPR/Cas9, has revolutionized research in biological fields [53], and these technologies are now easily available in fish, in particular the model species such as zebrafish [54].

As a popular model organism, zebrafish offers unique advantages for studying sex determination and differentiation as well as gametogenesis in mature gonads (ovary and testis). First, unlike most mammalian species and many fish species, zebrafish does not have any master sex-determining genes such as SYR in mammals and Dmy/dmrt1bY in medaka [55]. The sex-determining mechanism is therefore considered polygenic in zebrafish [56]. Second, due to the lack of sex-determining genes, zebrafish shows high plasticity in sex differentiation, making it an excellent model for dissecting roles of various internal and external factors in sex differentiation. Third, zebrafish breeds continuously in the laboratory condition without seasonality, making it an ideal model for studying gametogenesis and its control year-round.

During zebrafish gonadal differentiation, which we divide into three stages: pre-differentiation (15–25 dpf, days post-fertilization), differentiation (25–35 dpf) and post-differentiation (35–45 dpf) [57], gdf9 expression starts to increase as early as 16 dpf before morphological differentiation starts, suggesting a role for Gdf9 in ovarian formation [49]. In adult zebrafish, the expression of gdf9 mRNA in the ovary was the highest in the follicles of primary growth (PG) stage, which is functionally analogous to the stage of primary follicles in mammals; however, the expression decreased significantly when the follicles entered the secondary growth (SG) phase. The significant change of expression at PG-SG transition led us to hypothesize that
Gdf9 plays an important role in controlling zebrafish folliculogenesis especially its early stage [46].

To test our hypothesis, we undertook this study to knock out gdf9 gene in zebrafish using TALEN method. Our data demonstrated a complete arrest of follicle development at PG stage in the mutant, indicating an essential role for Gdf9/gdf9 in follicle activation to initiate the secondary growth. In contrast to the mutant in mice, the gdf9-null zebrafish females eventually changed sex to males due to the high plasticity of gonadal differentiation and imbalanced pathways that control the process. This was supported by the evidence that simultaneous mutation of dmrt1 gene (a male-promoting factor) prevented the sex change or reversal.

**Results**

**Generation of gdf9 knockout zebrafish by TALEN**

We generated gdf9 mutant fish (gdf9-/-) by transcription activator-like effector nucleases (TALEN) method. The TALEN target site of gdf9 is located in the first exon downstream of the ATG start codon. A mutant fish with 5-bp deletion in the gdf9 gene was generated (gdf9-5/-5) (ZFIN line number: umo18), which resulted in a frameshift mutation predicted to produce a truncated protein of 32 amino acids with 15 being the N-terminal part of the Gdf9 precursor. The mutation was also confirmed at mRNA level in the ovary by RT-PCR using a wild type (WT)-specific primer with the 3'-end overhanging the deleted sequence, which would generate a positive product in the control fish with WT sequence (+/-), but not the mutant fish (-/-) (Fig 1A).

**Role of Gdf9 in gonadal differentiation**

Gdf9 is an oocyte-specific growth factor in both mammals and fish [24,46] and its expression in zebrafish increases significantly when the gonads differentiate towards ovaries during sex differentiation together with ovarian aromatase (cyp19a1a) [49]. This had led us to hypothesize that Gdf9 might play a critical role in driving ovarian differentiation in zebrafish. To test this hypothesis, we examined sex ratio and its changes during and after gonadal differentiation from 35 to 130 dpf. To our surprise, the lack of Gdf9 did not have any impact on gonadal differentiation. The sex ratios of the mutant (gdf9-/-) were normal and comparable to those in the age-matched control fish (gdf9+/-) (~50%) during the post-differentiation period from 35 to 55 dpf. However, the female ratio declined progressively afterwards, and nearly all fish examined were males at 130 dpf except a few still containing some oocytes in the gonads (~3%), suggesting sex reversal from females to males (Fig 1B). The change of sex ratio was not due to the death of females as we closely monitored fish mortality during the period of observation. Furthermore, we often observed ovotestes in the mutant by histology at different time points (Figs 2A and 3).

**Arrest of follicle development at PG stage in gdf9 mutant females**

To find out the impact of gdf9 mutation (gdf9-/-) on gonadal development and gametogenesis, we first examined mature males and females at 90 dpf using gdf9+/+ as the control (we did not observe any difference between gdf9+/- and gdf9+/-). The control fish already reached maturity with all stages of follicles present in the ovary and full scale of spermatogenesis in the testis. The spermatogenesis in the mutant testis seemed normal compared to the control with abundant mature spermatozoa in the tubular lumen. However, the follicles in the mutant ovary were completely arrested at the PG stage without any signs of transition to the SG phase, which begins with the pre-vitellogenic (PV) stage characterized with formation of cortical alveoli (vesicles) (Fig 1C).
Fig 1. Disruption of gdf9 gene in zebrafish and evidence for its roles in gonadal differentiation and gonadal development. (A) Generation of gdf9 mutant by TALEN. The target site on chromosome 14 for left and right TALE proteins are boxed. A mutant with 5-bp deletion was obtained with a premature stop codon (TGA), which generated a truncated protein with 32 amino acids (15 from the Gdf9 precursor). RT-PCR analysis on mRNA from the ovary using a WT-specific primer (F) showed a positive signal in the control ovary (gdf9+/−), but not in the mutant (gdf9−−). (B) Sex ratios during and after gonadal differentiation in gdf9 mutant fish. The offspring from one crossing (gdf9+/− female X gdf9−− male) were sampled at different times from 35 to 130 dpf for genotyping and sex identification by histology. The sex ratios in the post-differentiation period from 35 to 55 dpf remained relatively constant and comparable to those in the control fish. However, the ratio changed gradually but significantly afterwards towards all males. The number of fish examined is shown at the bottom of each column. **P < 0.001 (X² test). (C) The ovary and testis in the control (gdf9+/−) and mutant (gdf9−−) at 90 dpf. The control females had reached sexual maturity with all stages of follicles present in the ovary (PG, primary growth; PV, pre-vitellogenic; MV, mid-vitellogenic; LV, late vitellogenic; and FG, full-grown) while the mutant ovary contained PG follicles only. The testis development and spermatogenesis showed no difference between mutant and control males. SG, spermatogonia; SC, spermatocytes; SZ, spermatozoa. The number in each photo indicates the number of fish examined with the same phenotype.

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To follow the developmental process of gonads between gonadal differentiation and sex maturation, we performed a systematic analysis on both ovary and testis by histology from gonadal differentiation to sexual maturation (35–80 dpf). At 35 dpf, the gonads had completed...
differentiation with ovary and testis well formed in both control (gdf9+/−) and mutant (gdf9−/−) fish. The ovaries contained PG follicles (stage I) and the testes were at pre-luminal stage (PL; stage I) with signs of meiosis according to our recent categorization of spermatogenic stages [58]. At 45 dpf, the leading follicles in the control females began accumulating cortical alveoli in the oocytes (PV; stage II), a morphological marker for follicle activation and transition from PG to SG phase (PG-PV transition) [59]. These follicles continued to grow through vitellogenic growth (from early vitellogenic to full-grown, EV-FG; stage III) from 55 to 80 dpf. In contrast, the follicles in the mutant ovary remained arrested at PG throughout the period without any signs of follicle activation such as formation of cortical alveoli in the oocytes. At 80 dpf, the control females had reached sexual maturity with all stages of follicles present in the ovary. However, the mutant ovaries were undergoing degeneration; the follicles were well separated and much part of the ovary including interfollicular spaces was occupied by infiltrating stromal tissues, a sign of masculinization and sex reversal (Fig 2A). What should be noted is that although most mutant females were arrested at the PG stage, the follicles in some individuals were able to enter very early PV stage with formation of rudiment cortical alveoli; however, this was not common (Fig 2B).

As for males, although gdf9 expression could also be detected in adult testis [46], we did not see any abnormalities in testis development and spermatogenesis in mutant males (gdf9−/−) at any time points of examination compared to age-matched control males. At 45 dpf, the testes in both control and mutant started to produce small amount of mature spermatozoa in the tubular lumen (mid-luminal stage, ML; stage III), which we defined as the marker for puberty
onset in males [58]. The post-pubertal spermatogenesis in the mutant males remained normal throughout the examination period compared to the control (Fig 2A).

**Sex reversal of gdf9 mutant from female to male**

The change of sex ratio after post-differentiation period suggested that the female mutant (gdf9-/-) might undergo sex reversal from females to males after being arrested at PG stage for some time. This was confirmed by histological analysis. The mutant ovary started to degenerate after a long time of follicle arrest at PG stage. While the ovarian tissues/follicles were degenerating and receding, the stromal tissues containing gonial (spermatogonia) or early meiotic germ cells (spermatocytes) gradually infiltrated the interfollicular spaces followed by emergence of testicular tissues with active spermatogenesis (Fig 3A–3C). The process of sex reversal began around 45 dpf or earlier based on histology, and it took about 2–3 months for all female mutants to change to males (Fig 3A). The sex-reversed males were functional with normal spermatogenesis, and they could spawn with WT females to produce normal offspring (Fig 3D).

Recently we demonstrated that the loss of ovarian aromatase (cyp19a1a-/-) also resulted in all-male phenotype, which was due to the failure of ovarian differentiation and sex reversal from juvenile ovary to testis [60]. Surprisingly, double mutation of cyp19a1a and male-promoting gene dmrt1 (cyp19a1a-/--;dmrt1-/-) prevented sex reversal from juvenile females to males in cyp19a1a mutant and therefore rescued the all-male phenotype of cyp19a1a-/-, resulting in normal sex ratio and normal ovarian formation [61]. To see if the loss of dmrt1 has any effect on the sex reversal in gdf9 mutant, we created a double mutant (gdf9-/--;dmrt1-/-) and examined its gonadal development up to 190 dpf. Interestingly, mutation of dmrt1 (dmrt1-/-) (ZFIN line number: umo15) also prevented sex reversal in gdf9 mutant (gdf9-/-) with the double mutant (gdf9-/--;dmrt1-/-) showing a female-biased sex ratio and the ovaries containing PG follicles only without any signs of sex reversal such as infiltrating stromal tissues (Fig 4).

**Expression analysis of key regulatory factors in the follicles of gdf9 mutant**

To investigate the mechanism underlying Gdf9 actions, we examined the expression of several key ovarian factors at 45 dpf when some follicles were entering the PV stage in the control fish. We isolated the PG and early PV follicles from the control fish (gdf9+/-) and PG follicles from gdf9-/fish (no PV follicles in the mutant) to analyze expression of genes by real-time qPCR, including gonadotropin receptors (fshr and lhcr), ovarian aromatase (cyp19a1a), epidermal growth factor receptor (egfra) and members of the activin-inhibin family (inha, activin β subunits inhbaa, inhbab and inhbb). Most of these genes showed increased expression during the PG-PV transition in the control fish (fshr, cyp19a1a, egfra, inha, inhbaa and inhbab) except lhcr and inhbb, which agreed well with our previous studies [62–66]. However, their expression showed little change in mutant PG follicles compared to stage-matched PG follicles from the control except activin βAa (inhbaa). Among all the genes examined, inhbaa was the only one that decreased expression dramatically in the mutant PG follicles (gdf9-/) compared to the PG follicles from the control (gdf9+/+) (Fig 5).

**Effect of estrogen exposure on follicle development in gdf9 mutant females**

During PG-PV transition, the expression of cyp19a1a increased significantly [66]. To test if the arrest of follicles at PG stage in gdf9-null females was due to the lack of sufficient estrogens, we treated the juvenile mutant fish (gdf9-/+) with estradiol (E2) either via water-borne exposure (10 nM) for 20 days (40–60 dpf) or oral administration (feeding) (20 μg/g diet) for 17 days (45 to 62 dpf). E2 administration via water-borne exposure induced all-female sex ratio (100%
Fig 4. Prevention of sex reversal of gdf9 mutant by dmrt1 mutation. (A) Gonadal histology at 60 and 120 dpf. Ovary could form in the control (dmrt1+/-;gdf9+/-), single mutants (dmrt1+/- and gdf9+/-) and double mutant (dmrt1+/-; gdf9+/-; gdf9-/-) as seen at 60 dpf. The gdf9-/- single mutant became all males at 120 dpf (16/16 fish examined in total) whereas most individuals of the double mutant were females (8/14 examined), similar to that in the control (9/15). The numbers shown in the photos indicate the total number of fish examined (lower) and the number of female fish exhibiting similar phenotype to that shown (upper). (B) Change of sex ratio during 60–190 dpf. The offspring from one crossing (dmrt1+/-;gdf9+/- female X dmrt1+/-;gdf9+/- male) were sampled at different times from 60 to 190 dpf for genotyping and sex identification by histology. The gdf9-/- fish showed a male-biased sex ratio at 60 dpf, and it became all-male from 120 dpf due to sex reversal. Double mutation with dmrt1 rescued the sex ratio to the control level with even higher female ratios at 150 and 190 dpf. PG, primary growth; PV, pre-vitellogenic; FG, full-grown; SC, spermatocytes; SZ, spermatozoa.

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females), in contrast to the male-biased sex ratio in the control (~70% males) (Fig 6A). Furthermore, E2 also significantly increased the vitellogenin levels in both serum and liver of the mutants (gdf9-/-), especially the serum level (Fig 6B). However, histological examination showed that E2 had little effect on follicle blockade in mutant females except that some follicles showed rudiment signs of cortical alveoli (Fig 6C), the same as what we occasionally observed in some mutant fish without treatment (gdf9-/-) (Fig 2B). Similar to water-borne exposure,
oral administration of E2 by feeding had no effect on follicle activation either. All follicles remained arrested at the PG stage without any signs of transition to PV stage. Both control and E2-treated mutant females showed well-developed genital papilla (a female secondary sex characteristic) with no breeding tubercles on the pectoral fins (a male secondary sex characteristic) (Fig 6D).

Rescue of gdf9 mutant phenotypes by inha deficiency

The dramatic decrease of activin βAa (inhaaa) in gdf9 mutant (gdf9-/-) led us to hypothesize that the activin-inhibin system could be part of the underlying mechanism for Gdf9 actions in
zebrafish follicle development. To test this hypothesis, we made a double mutant fish (gdf9-/-; inha-/-) by crossing the gdf9 mutant with an inha mutant we reported recently (umo19) [67]. Surprisingly, the loss of inhibin, a natural activin antagonist, could significantly rescue the phenotypes of gdf9 mutant. The double mutant (gdf9-/-;inha-/-) could overcome follicle blockade and resume vitellogenic growth, phenocopying inha single mutant (inha-/-) in several aspects. First, the follicles in the double mutant could undergo follicle activation or PG-PV transition to enter the fast SG phase. As the marker for puberty onset, cortical alveoli could form normally in the oocytes (PV stage). Second, the activated follicles could continue to grow to the next stage by accumulating yolk granules (vitellogenic growth). Third, the double mutant also phenocopied inha single mutant (inha-/-) in that the yolk mass in the oocytes had a sharp boundary compared to that in the control oocytes (Fig 7) [67].

Interestingly, although inha mutation could rescue the follicle blockade in the gdf9 mutant, the restoration of folliculogenesis was not complete. Analysis of follicle composition in the ovaries showed that while folliculogenesis resumed in the double mutant, the follicles could only grow to the mid-vitellogenic (MV) stage (~450 μm), not the full-grown (FG) stage (>650 μm) as seen in the control and inha single mutant (Fig 8A and 8B). The double mutant females were not able to spawn normally with WT males in most fertility tests, similar to the infertility seen in the inha mutant (Fig 8C). When tested in the iSpawner (Techniplast), the double mutant females could sometime release some eggs; however, these eggs were smaller and could not be fertilized (Fig 8D), consistent with the histological observation that the follicles could only develop to the MV stage.

In addition to rescuing follicle blockade, inha mutation also delayed sex reversal in the gdf9 mutant. At 90 dpf, the single mutant (gdf9-/-) showed a male-biased sex ratio as described earlier, but this was prevented in the double mutant (gdf9-/-;inha-/-), indicating no sex reversal at this time point (Fig 8E). Despite this, the double mutant females still underwent sex reversal to males at later stage. At 7 months post-fertilization (mpf), most oocytes in the double mutant ovary gradually degenerated and the ovarian tissues were infiltrated by stromal and testicular tissues. The sex reversed males had lost the genital papilla, and they could spawn with WT females to produce normal offspring (Fig 8F).

Rescue of inhbaa expression in gdf9 mutant follicles by inha deficiency

The decreased expression of inhbaa in gdf9-null PG follicles and the rescue of gdf9 mutant phenotypes by the loss of inhibin both suggested that activin could be involved in Gdf9 actions in controlling early folliculogenesis. To test this hypothesis, we examined the expression of inhbaa in PG follicles from gdf9 single mutant (gdf9-/-) and double mutant (gdf9-/-;inha-/-), together with fshr, cyp19a1a, inhbab (activin βAb) and inhbb (activin βB). As shown in Fig 9, fshr, cyp19a1a and inhbaa all increased their expression significantly in the PG follicles from inha-/- females, in agreement with our recent report [67]. In gdf9 mutant (gdf9-/-), however, inhbaa but not fshr and cyp19a1a showed a significant decrease in expression. Interestingly, in the double mutant (gdf9-/-;inha-/-), the expression of all three genes (fshr, cyp19a1a and inhbaa) was raised to the high levels as observed in the inha single mutant. The decreased expression of inhbaa was therefore not only reversed by the loss of inha but increased to a higher level (Fig 9A–9C). By comparison, inhbab and inhbb did not show any changes in different mutants (Fig 9D and 9E). These results suggest a potential role for activin βA, especially inhbaa, in mediating actions of oocyte-derived Gdf9.

Our previous study showed that activin subunits including inhbaa were exclusively expressed in the follicle cells whereas activin receptors and its intracellular signaling molecules Smad2/3 were abundantly expressed in the oocyte, suggesting an activin-mediated paracrine
pathway in the follicle for follicle cell-to-oocyte signaling [68,69]. The increased inhibin α (inha/-) expression and loss of inhibin in the gdf9 and inha (gdf9/-;inha/-) double mutant would enhance such signaling by activin. To provide further evidence for direct actions of activin on oocytes, we examined Smad2/3 phosphorylation in PV oocytes in response to activin treatment in
Fig 8. Phenotype analysis of single (gdf9-/ and inha-/+) and double mutants (gdf9-/-;inha-/+). (A and B) Follicle composition in different genotypes at 90 dpf. The follicles in gdf9-/- were arrested at PG stage. Although the PG-PV transition and yolk accumulation resumed in the double mutant (gdf9-/-;inha-/-), the vitellogenic growth could not proceed to the FG stage, and the follicles were arrested at the MV-LV transition. * P < 0.05; ** P < 0.001 (X² test, n = 6). (C) Fertility test on females of single (gdf9-/ and inha-/+) and double mutants (gdf9-/-;inha-/+) at 90 dpf. Five mutant and control females were tested 10 times consecutively every other day by natural pair breeding with WT males (n = 5/test). The test was conducted over about 20 days. In each test, the number of fertilized eggs or embryos was counted for each female and the data point represents the average of the five fish tested. (D) Eggs spawned by the control (gdf9+/-;inha+/+) and double mutant (gdf9-/-;inha-/+) at 3 mpf. The eggs from the double mutant were smaller than those from the control. (E) Prevention of sex reversal in gdf9-/- by double mutation with inha-/- at 90 dpf. The gdf9-/- fish showed a male-biased sex ratio (82% males), and this was corrected in the double mutant (47% males). *** P < 0.001 (X² test, n = 11–14). (F) Sex reversal of the gdf9 and inha double mutant (gdf9-/-;inha-/+) at 7 mpf. (1) Double mutant female at 3 mpf with female-specific genital papilla (arrow); (2) Ovary of 3-mpf double mutant; (3) Sex
vitro. Exposure to activin B (5 U/ml, 2 h) significantly increased the level of p-Smad2/3 in PV oocytes. In addition, the phosphorylated Smad2/3 were mostly concentrated in the germinal vesicles (nuclei), indicating nuclear translocation upon activin stimulation (Fig 9F).

Fig 9. Expression of fshr, cyp19a1a and activins (inhbaa, inhibb and inhibb) in PG follicles from single (gdf9/- and inha/-) and double mutant (gdf9-/-;inha-/-). (A-E) The PG follicles were isolated from the fish of different genotypes at 43 dpf, shortly before puberty onset or PG-PV transition occurs in the control fish (normally around 45 dpf). The expression of inhbaa was significantly reduced in gdf9-/- mutant; however, it was reversed by inha mutation in the double mutant together with fshr and cyp19a1a, but not inhibb and inhibb. Different letters indicate statistical significance (P < 0.05, n = 3 biological replicates). (F) Stimulation of Smad2/3 phosphorylation (p-Smad2/3) in the PV oocytes by activin in vitro. The PV follicles were isolated and treated with recombinant goldfish activin B (5 U/ml) for 2 h followed by immunofluorescent staining for p-Smad2/3.

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Discussion

Folliculogenesis in vertebrates is tightly controlled by both endocrine hormones such as pituitary FSH and LH and local ovarian growth factors [70–72]. In the past decades, evidence has accumulated that local intraovarian paracrine factors play critical roles in orchestrating folliculogenesis [10,11,73]. This concept is now being generally accepted largely due to the discovery and characterization of growth differentiation factor 9 (GDF9) [23]. The discovery of GDF9 has triggered tremendous interest in research and numerous studies have demonstrated an essential role for GDF9 in mammalian folliculogenesis [18,38,74]. GDF9 is also present in non-mammalian vertebrates including fish, and its oocyte-specific expression seems to be highly conserved [46,47,75–80]. However, the functional importance of GDF9 in folliculogenesis remains largely unknown in non-mammalian species.

Using genome editing method TALEN, we successfully knocked out the gdf9 gene in the zebrafish. Surprisingly, despite its oocyte origin and increased mRNA expression during ovarian differentiation [49], the loss of Gdf9 had no impact on gonadal differentiation as shown by normal sex ratio in young zebrafish in the post-differentiation period. This suggests that although gdf9 mRNA is expressed early during ovarian formation, its protein may not be translated or secreted until follicle activation at PG-PV transition. In our previous study, we reported that gdf9 mRNA level was the highest at PG stage and its level declined progressively during zebrafish folliculogenesis. The decline could be the result of increased mRNA turnover and protein translation [46].

Phenotype analysis of zebrafish gdf9 mutant (gdf9-/-) showed that similar to GDF9 in the mouse, Gdf9 also plays an essential role in controlling early folliculogenesis in fish. The disruption of gdf9 gene caused a complete arrest of follicle development at the PG stage without any signs of follicle activation to enter the fast-growing SG phase. The follicles could grow to nearly full size of PG stage; however, further development to PV stage, which is the beginning of the SG phase characterized with the formation of cortical alveoli in the oocytes, was completely blocked. Since the appearance of cortical alveoli is considered a marker for follicle activation and puberty onset [59], the arrest at PG-PV transition also resulted in failed puberty onset and female maturation, and therefore infertility. Our observation in zebrafish agrees well with the report in the mouse model. First, the primordial and one-layer primary follicles could form normally in Gdf9-null female mice; however, further development to two- and multiple-layer secondary follicles was blocked [38]. The blockade at the primary-secondary follicle transition in Gdf9-null mice is surprisingly similar to the blockade at the PG-PV (primary-secondary growth) transition in zebrafish gdf9 mutant. Second, both Gdf9-null mice and gdf9-null zebrafish showed lack of cortical alveoli (granules) in the oocytes [38]. These results strongly suggest that the fundamental functions of GDF9 in controlling early follicle development are highly conserved in vertebrates. Another striking similarity between mutant mice and zebrafish is that the phenotype of follicle blockade could be partially rescued in both models by the loss of inhibin (see below for detailed discussion). Our results, however, contrast with a recent study in zebrafish, which reported no phenotypes in gdf9-null zebrafish [81]. The reason for such discrepancy is unknown. Similar situations have also been reported in mice. For example, the Sirt1-null mice generated by different groups exhibited different phenotypes, which might be due to different genetic backgrounds of the mice used [82,83].

In addition to controlling PG-PV transition or follicle activation, our data also suggest an important role for Gdf9 in maintaining feminization including the ovary although it was not involved in primary gonadal differentiation. The loss of Gdf9 caused gradual degeneration of the follicles followed by transformation of the ovary to testis, resulting in all-male phenotype in the end. This process seems due to the change of dominance of the two antagonistic gonadal
differentiation pathways, which are present in each individual of vertebrate species [55,84]. The loss of Gdf9 weakens the female pathway in zebrafish, resulting in a switch of dominant pathway from female to male. This idea is supported by our evidence that deletion of \textit{dmrt1}, a critical gene in male-promoting pathway [85], prevented sex reversal in the \textit{gdf9} mutant. Interestingly, although \textit{gdf9} expression could also be detected in adult testis [46], its loss had no impact on spermatogenesis in males. In \textit{Gdf9}-null mice, the oocytes arrested at the primary follicle stage also underwent degeneration followed by collapse of the zona pellucida and luteinization of the granulosa cells [38], but not sex reversal as we observed in zebrafish. This is likely due to the lack of master sex-determining genes and the high plasticity of sex differentiation in zebrafish.

Our observation in \textit{gdf9}-null zebrafish indicates that although pituitary gonadotropins play pivotal roles in driving follicle growth and maturation [1,2], an intrinsic regulatory mechanism exists in the follicle that orchestrates folliculogenesis in zebrafish, and that the oocyte plays an active role in timing the developmental process. As an oocyte-derived factor, Gdf9 is expected to act on the surrounding follicle cells where it may interact with the endocrine hormones and other local factors to control follicle development. The exact functions of Gdf9 in zebrafish follicles are largely unknown. Future studies using recombinant zebrafish Gdf9 and in vitro follicle or follicle cell culture will shed light on the biological activities of Gdf9 in the follicle. Using this approach, we recently demonstrated that recombinant zebrafish Gdf9 could activate the Smad2 signaling pathway in cultured follicle cells and it significantly increased the expression of activin subunits (\textit{inhbaa} and \textit{inhbb}) but decreased that \textit{amh}, a male-promoting factor in the gonads [49]. It is therefore conceivable that the oocyte-derived Gdf9 may act on the follicle cells to stimulate the factors that promote follicle growth and maintenance such as activin but suppress those involved in masculinization such as Amh, which increases its expression significantly during female-to-male sex reversal [2].

Follicle activation or PG-PV transition is a critical stage in zebrafish folliculogenesis, which involves multiple endocrine, paracrine and autocrine factors as well as various signaling pathways [66]. Pituitary gonadotropins (FSH and LH) are undoubtedly the master hormones that control this process. The FSH receptor (\textit{fshr}), which is co-activated by both FSH and LH in zebrafish [86], increases its expression significantly during PG-PV transition [62,87], suggesting an important role for gonadotropin signaling. This has been confirmed recently by the observation that deletion of pituitary \textit{fshb} gene (FSH\(\beta\)) significantly delayed the PG-PV transition and therefore puberty onset [1] and knockout of \textit{fshr} suppressed ovarian growth and completely blocked follicle activation [2,88]. As reported in mammals, local ovarian factors are also implicated in controlling follicle activation in zebrafish. Activin subunits especially \textit{inhbaa} increased expression significantly during the PG-PV transition [65,66]. Similarly, epidermal growth factor (EGF) family ligands including EGF (\textit{egf}) and transforming growth factor \(\alpha\) (TGF-\(\alpha\)/\textit{tgfa}) as well as their receptor EGFR (\textit{egfra}) also showed an increased expression at the PG-PV transition [68]. In zebrafish follicles, EGF family ligands are primarily expressed in the oocyte whereas their receptor \textit{egfra} is mainly expressed in the somatic follicle cells [68], which respond to EGF strongly by both ERK/MAPK and AKT phosphorylation, increased expression of activin subunits (\textit{inhbaa}, \textit{inhbab} and \textit{inhbb}), and decreased expression of follistatin (an activin binding protein) [89,90]. Like Gdf9, EGF ligands and EGFR may represent another paracrine pathway within the follicle that mediates signaling from oocyte to follicle cells. Our recent study showed that disruption of \textit{egfra} in zebrafish also blocked follicle development at PG-PV transition followed by sex reversal to males [63], which is similar to the phenotypes of \textit{gdf9} mutant observed in the present study. These results indicate that follicle development in zebrafish ovary is finely controlled by multiple factors from the oocyte and that the regulation of folliculogenesis involves many checkpoints for regulation by both internal and external
signals. This view is further supported by a recent study in zebrafish showing that mutation of BMP15 (bmp15), also an oocyte-specific factor from TGF-β family, caused an arrest of follicle growth at PV stage [81], in contrast to the arrest at PG stage as seen in the gdf9 and egfra mutants.

Having defined the functional role and importance of Gdf9 in controlling folliculogenesis in zebrafish, we went on to explore the mechanism underlying its actions. Much needs to be learnt to unravel how GDF9 acts, which remains an issue for further research in mammalian models as well [91]. One important clue from our experiments was the dramatic decrease in the expression of activin subunit inhbaa in gdf9 mutant follicles. It agrees well with our previous in vitro study showing stimulation of inhbaa expression in cultured follicle cells by recombinant zebrafish Gdf9 [49]. This has led us to hypothesize that part of the mechanism for Gdf9 to regulate follicle development is to increase the production of activin in the follicle cells, which in turn acts on the oocyte that expresses activin receptors and signaling molecules Smad2/3/4 [69]. Our observation that the PV oocytes responded strongly to activin treatment in terms of Smad2/3 phosphorylation and nuclear translocation further supports the idea that the oocyte is a direct target for activin actions. The loss of oocyte-derived Gdf9 would lead to decreased expression of activin subunits, which compromises an important signaling from the follicle cells to oocyte, resulting in the arrest of follicle development.

As a key local factor from the follicle cells targeting the oocyte, activin has been proposed to play a central role in mediating both endocrine hormones such as gonadotropins [92] and local paracrine factors such as EGF ligands [89,90] in zebrafish follicles. The present study suggests that activin may also mediate Gdf9 actions. In support of this hypothesis was our evidence that knockout of inha could partially reverse the phenotypes of gdf9 mutant. Inha is also a member of TGF-β family and it dimerizes with an activin β subunit to form inhibin (αβ), which acts as an activin antagonist. The loss of inha advanced PG-PV transition and therefore puberty onset in female zebrafish [67], in contrast to the PG-PV blockade in gdf9 mutant. Double mutations of inha and gdf9 (gdf9-/-;inha-/-) not only resumed follicle growth to MV stage with formation of cortical alveoli and accumulation of yolk granules, but also delayed sex reversal of gdf9-/- females to males. Our discovery is surprisingly similar to a previous study in mice. The loss of Gdf9 in mice blocked follicle development at the primary follicle stage without thecal layers [38]. Interestingly, double mutant of Gdf9 and Inha partially rescued the phenotypes of Gdf9 single mutant. The follicles in the double mutant ovary (Gdf9-/-;inha-/-) contained not only primordial and one-layer primary follicles, but also two- and multiple-layer secondary follicles with thecal layers [93]. Interestingly, although EGF also stimulated expression of all activin subunits in zebrafish follicle cells [90] and the loss of egfra gene led to similar phenotypes to those of gdf9 mutant, viz. blockade at PG-PV transition followed by sex reversal to males, inha mutation could not rescue the phenotypes of egfra mutant [63], suggesting differential mechanisms underlying actions of Gdf9 and EGF family ligands.

How the loss of inhibin rescued the defective phenotypes of gdf9 mutant is not clear. Interestingly, three genes (fshr, cyp19a1a and inhbaa) that are considered functionally important in promoting folliculogenesis were all increased dramatically in the double mutant (gdf9-/-;inha-/-) to the high levels seen in inha single mutant. The increased expression of these genes would suggest increased gonadotropin signaling, estrogen biosynthesis and activin production, all of which could enhance follicle development, therefore explaining how mutation of inha could rescue the defects of gdf9 mutant. Activin is of particular interest because inhbaa was the only gene among the three that decreased expression significantly in gdf9-null PG follicles and its expression rebounded dramatically in the double mutant (gdf9-/-;inha-/-). In addition to increased expression, the bioactivity of activin is also expected to increase in the inha mutant for two reasons. First, as a potent activin antagonist, the loss of inhibin is expected to cause a
reduced antagonism against activin. Second, sharing the same β subunits, activin and inhibin compete for β subunits in their biosynthesis; therefore, the loss of inhibin α subunit (inha) may likely increase the formation of activin molecules. Whether the rescue of gdf9 mutant phenotypes by inha mutation involves activin alone or together with gonadotropins and estrogens would be an interesting issue to explore in future studies. As for cyp19a1a, our recent study has provided clear evidence for its critical role in ovarian differentiation; however, cyp19a1a was not involved in promoting PG-PV transition [61], which agrees with the finding in medaka fish [94]. This view is further supported by our evidence that treatment with E2 could not overcome the blockade of follicle development in the gdf9 mutant although E2 could prevent sex reversal and induce vitellogenin production in the liver as expected.

In summary, using genome editing method, we performed a genetic analysis on roles and functional importance of gdf9 in zebrafish folliculogenesis. Our data demonstrated that gdf9 is critical for early follicle development, especially at follicle activation or transition from PG to SG phase. The loss of gdf9 resulted in a complete cessation of follicle development at the PG stage. Gene expression analysis and genetic study with inha and gdf9 double mutant provided critical insight into how Gdf9 may work in zebrafish follicles. We hypothesize that the oocyte-derived Gdf9 works on the surrounding follicle cells to stimulate biosynthesis of activins, which in turn act back on the oocyte in a paracrine manner to activate the Smad2/3 signaling pathway, promoting formation of cortical alveoli and therefore follicle activation (Fig 10). This study provides a strong support to the view that oocytes play active and important roles in orchestrating folliculogenesis and Gdf9 has conserved functions across vertebrates.

Materials and methods

Ethics statement

The animals were handled according to the Animal Protection Act enacted by the Legislative Council of Macao Special Administrative Region under Article 71(1) of the Basic Law and the experimental protocols approved by the Research Ethics Panel of the University of Macau (AEC-13-002).

Animals and maintenance

The AB strain of wild type (WT) zebrafish (Danio rerio) was used to generate mutant lines, and the fish were kept in the flow-through ZebTEC multilinking zebrafish system (Tecniplast,

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Fig 10. Hypothetical model on roles of oocyte-derived GDF9 (gdf9) in controlling zebrafish folliculogenesis. (A) Intrafollicular distribution of GDF9 and other important genes (fshr, lhcr, cyp19a1a, inhbaa/ab, inhbb, inha, acvr1b, acvr2a/b and smad2/3/5/7). (B) GDF9 from the oocyte plays an important gating role in controlling zebrafish follicle activation or PG-PV transition and therefore puberty onset. PV-I, early PV with one single layer of small cortical alveoli; PV-II, mid-PV with one single layer of large cortical alveoli; PV-III, late PV with multiple layers of cortical alveoli.

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Buguggiate, Italy) on 14L:10D lighting cycle. The system condition was maintained as follows: temperature 28°C, pH 7.3 and conductivity 400 mS/cm. The fish were fed twice per day with Otohime fish diet (Marubeni Nisshin Feed, Tokyo, Japan), which was delivered by the Tritone automatic feeding system (Tecniplast).

**Generation of gdf9 mutant zebrafish**

To create mutant zebrafish, the whole sequence containing gdf9 gene (ID: ENSDARG00000003229) was retrieved from the Ensembl database for target site identification. The sequence in the first exon downstream of the ATG start codon was chosen for targeting by TALEN. Both left and right TALEN arms were designed by the online software (TAL Effector Nucleotide Targeter 2.0 Tools; https://tale-nt.cac.cornell.edu/node/add/talen) as we previously reported [1], and the sequences are: TAGTGCGCTTTGTTACC (left TALE), TTGTCCTCGCAGTTCTCGGA (right TALE), and TACCCAGATCAT TAA (spacer sequence) (Fig 1). Gene-specific TALEN constructs were assembled using the TALEN Golden Gate assembly system as described [95] with the two backbone plasmids pCS2TAL3DD and pCS2TAL3RR (Addgene, Cambridge, MA). The TALE mRNAs were generated by in vitro transcription using the mMESSAGE mMACHINE SP6 kit (Ambion, Austin, TX), and co-injected (50 pg each) into one or two-cell stage embryos. To detect mutations, the genomic DNA was extracted from each embryo or caudal fin cut and analyzed by high resolution melt analysis (HRMA) as we previously reported [12]. The primers for HRMA analysis are listed in S1 Table. The reaction was performed with EvaGreen Supermix on the CFX96 real-time PCR machine, and the data were analyzed with the precision melt analysis software (Bio-Rad, Hercules, CA). The mutations were confirmed by sequencing (Tech Dragon, Hong Kong).

**Genomic DNA isolation**

Genomic DNA was isolated from embryos or caudal fins of WT or mutant fish as described [1]. Briefly, a piece of caudal fin or an embryo was incubated in 40 μl NaOH (50 mM) at 95°C for 10 min. After cooling down to room temperature, 4 μl Tris-HCl (pH 8.0) was added to each sample. After centrifugation, the supernatant was then used directly as the template for HRMA.

**Real-time qPCR quantification**

Total RNA was isolated from the follicles with Tri-Reagent (Molecular Research Center, Cincinnati, OH) according to the protocol of the manufacturer and our previous reports [46,96]. The RNA was reverse transcribed into cDNA at 37°C for 2 h in a total volume of 10 μl reaction solution that contains total RNA, reverse transcription buffer, DTT, oligo-dT and reverse transcriptase (Invitrogen, Hercules, CA).

The expression levels of target genes in the follicles from WT or mutant fish were determined by real-time qPCR as described [96]. The standard for each gene was prepared by PCR amplification of cDNA fragments with specific primers (S1 Table). These amplified amplicons were used to construct standard curves in the assay. Real-time qPCR was carried out on the CFX96 Real-Time PCR Detection System (Bio-Rad) in a volume of 20 μl that contained 10 μl diluted RT reaction mix, 1× PCR buffer, 0.2 mM of each dNTP, 2.5 mM MgCl₂, 0.2 μM of each primer, 0.75 U Taq polymerase, 0.5× EvaGreen (Biotium, Hayward, CA). The reaction profile consisted of 38 cycles of 94°C for 30 sec, 60°C for 30 sec, 72°C for 1 min, and 80°C for 7 sec for signal detection. A melt curve analysis was performed at the end of amplification to demonstrate reaction specificity.
Histological analysis of gonads

The gonadal development and morphology were examined by histology as described [59]. Briefly, the body trunk of each fish was fixed in Bouin’s solution overnight at room temperature. Following dehydration and embedding in paraffin, the samples were sectioned on a Leica microtome (Leica, Wetzlar, Germany) and stained with hematoxylin and eosin (H&E). For the convenience of analysis, we divide zebrafish follicle development into two phases: primary growth (PG) (<150 μm; stage I) and secondary growth (SG) (~150–750 μm). The SG phase is further divided into pre-vitellogenic (PV, ~250 μm; stage II), early vitellogenic (EV, ~350 μm; early stage III), mid-vitellogenic (MV, ~450 μm; mid-stage III), late vitellogenic (LV, ~550 μm; late stage III) and full-grown (FG, >650 μm; full-grown stage III) stages, based on size and morphological markers such as cortical alveoli and yolk granules [87,97]. To quantify follicle composition in the ovary, we performed serial longitudinal sectioning of the whole fish at 5 μm and measured diameters of follicles on three largest sections spaced at least 60 μm apart by the NIS-Elements BR software (Nikon, Tokyo, Japan). To ensure accuracy of diameter measurement for follicle staging, we only measured the follicles with visible nuclei (germinal vesicles) on the section.

Estradiol treatment in vivo

To examine if the phenotype of gdf9 mutant could be rescued by estrogens, we treated the mutant fish with E2 (Sigma-Aldrich, St. Louis, MO) followed by sampling for histological analysis. Two approaches were used for estrogen treatment: water-borne exposure and oral administration by natural feeding. For water-borne exposure, E2 stock in ethanol was added to the water in fish tank to the final concentration of 10 nM, and the juvenile fish were treated for 20 days from 40 to 60 dpf. The water was replaced by half every day with E2 supplement with ethanol being used as the vehicle control. For oral administration, E2 was incorporated into the diet. Briefly, the Otohime fish feed was mixed with E2 stock solution in ethanol and dried overnight at 60˚C. The mutant females were fed with E2-containing diet (0 or 2 μg/g diet) twice a day for 17 days from 45 to 62 dpf, each at 5% of total fish body weight in the tank (10% per day in total). In addition, the fish were also supplemented with brine shrimp larvae. During the treatment period, the water was renewed daily to maintain good water quality.

SDS-PAGE examination for vitellogenin production

The whole blood was collected from each fish by cutting the tail to cause bleeding. The blood was collected by a pipette, left in a microtube at room temperature for clotting, and then centrifuged to obtain the serum. The sera were subject to standard sodium dodecyl sulfate (SDS) PAGE analysis (1 μl per lane) for vitellogenin proteins. We also examined vitellogenin production in the liver. Briefly, the liver was dissected from each fish and lysed in 400 μl SDS sample buffer (62.5 mM Tris-HCl [pH 6.8] at 25˚C, 1% [wt/vol] SDS, 10% glycerol, 5% 2-mercaptoethanol). All samples were heated to ~95˚C for 10 min, cooled on ice, centrifuged for 5 min, and then loaded to 12% SDS-PAGE gel for electrophoresis.

Immunofluorescent staining for Smad2/3 phosphorylation

To demonstrate responsiveness of oocytes to activin, PV follicles were isolated from the ovary and incubated in 60% Leibovitz L-15 medium (Gibco, Thermo Fisher Scientific, Waltham, MA) in the presence or absence of recombinant goldfish activin B (5 U/ml, 2 h) prepared in our laboratory as reported [98,99]. The follicles were fixed after incubation in 2% formaldehyde–phosphate-buffered saline (PBS), permeabilized with 0.02% TritonX-100 in PBS,
incubated with primary anti-p-Smad2/3 (Cell Signaling, Danvers, MA) and then secondary goat anti-rabbit IgG conjugated with Alexa Fluor 594 (Thermo Fisher Scientific). The follicles were mounted on a slide for observation with the FluoView FV1000 IX81 confocal microscope (Olympus, Tokyo, Japan).

**Data analysis**

The expression levels of target genes were normalized to the house keeping gene eif1a. All values were expressed as the mean±SEM, and the data were analyzed by Dunnett’s Multiple Comparison Test with Prism (GraphPad Software, San Diego, CA).

**Supporting information**

S1 Table. Primer list.

(DOCX)

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**Author Contributions**

**Conceptualization:** Wei Ge.

**Data curation:** Weiting Chen, Yue Zhai, Bo Zhu, Kun Wu.

**Formal analysis:** Weiting Chen, Yue Zhai, Kun Wu, Wei Ge.

**Funding acquisition:** Wei Ge.

**Investigation:** Weiting Chen, Yue Zhai, Bo Zhu, Kun Wu, Yuqin Fan, Lin Liu, Wei Ge.

**Methodology:** Weiting Chen, Yue Zhai, Bo Zhu, Kun Wu, Xianqing Zhou, Wei Ge.

**Project administration:** Wei Ge.

**Resources:** Wei Ge.

**Supervision:** Wei Ge.

**Validation:** Yue Zhai, Kun Wu, Wei Ge.

**Visualization:** Wei Ge.

**Writing – original draft:** Weiting Chen, Wei Ge.

**Writing – review & editing:** Wei Ge.

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