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Decreased Oocyte DAZL Expression in Mice Results in Increased Litter Size by Modulating Follicle-Stimulating Hormone-Induced Follicular Growth[1]

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ABSTRACT

While the germ cell-specific RNA binding protein, DAZL, is essential for oocytes to survive meiotic arrest, DAZL heterozygous (het) mice have an increased ovulation rate that is associated with elevated inhibin B and decreased plasma follicle-stimulating hormone (FSH). The relationship between decreased oocyte DAZL expression and enhanced follicular development in het mice was investigated using in vitro follicle cultures and in vivo modulation of endogenous FSH, by treating mice with inhibin and exogenous FSH. In vitro, follicles from het mice are more sensitive to FSH than those of wild-type (wt) mice and can grow in FSH concentrations that are deleterious to wild-type follicles. In vivo, despite no differences between genotypes in follicle population profiles, analysis of granulosa cell areas in antral follicles identified a significantly greater number of antral follicles with increased granulosa cell area in het ovaries. Modulation of FSH in vivo, using decreasing doses of FSH or ovine follicular fluid as a source of inhibin, confirmed the increased responsiveness of het antral follicles to FSH. Significantly more follicles expressing aromatase protein confirmed the earlier maturation of granulosa cells in het mice. In conclusion, it is suggested that DAZL expression represses specific unknown genes that regulate the response of granulosa cells to FSH. If this repression is reduced, as in DAZL het mice, then follicles can grow to the late follicular stage despite declining levels of circulating FSH, thus leading to more follicles ovulating and increased litter size.

FSH, FSH receptor, follicular development, granulosa cells, inhibin, ovulation rate

INTRODUCTION

The number of offspring born at any one time is determined by the ovulation rate, defined as the number of follicles that develop sufficiently to allow ovulation of competent fertilizable oocytes. From the developing ovary in the fetus, when the full complement of germ cells is defined, there is a fine balance between survival and death of these germ cells throughout the reproductive lifespan of the animal. While studies of inbred mouse strains have indicated differences in follicle numbers in the neonatal period due to different rates of activation and atresia [1], these differences do not influence the final number of ovulating follicles, and hence, the ovulation rate is similar. However, it has been shown in mice that genetic differences are a major source of variation in ovarian responsiveness to gonadotropins and hence ovulation rate [2].

Folliculogenesis is the highly regulated maturation process initiated when primordial follicles are activated from the quiescent pool laid down in fetal life, and either one or more, depending on the species, complete their maturation and ovulate. Therefore, factors that control the activation and maturation of follicles determine ovulation rate. Furthermore, ovulation rate is determined by selection of ovulatory follicles from the initially large activated follicular cohort. As follicles grow, they acquire factors at precise times, which determine whether they are able to proceed in their development. Failure to express the appropriate factors leads to loss of the follicle by atresia [3].

Initial activation of primordial to primary follicles involves complex oocyte–somatic cell signaling pathways and does not require extraovarian factors; it is gonadotropin-independent, relying for development on intraovarian factors including TGFB growth factor family members, especially oocyte-specific growth differentiation factor 9 (GDF9) and bone morphogenic protein 15 (BMP15), although studies in mice and sheep have shown that the functions of BMP15 are species-specific, exhibiting different roles within the developing follicle [4]. However, as the follicle matures, it must acquire the ability to respond to endocrine factors such as the pituitary gonadotropins, follicle-stimulating hormone (FSH), and luteinizing hormone (LH). The subsequent secretion of steroids and growth factors by the follicles crucially regulates pituitary gonadotropin secretion, and only the follicles that are now gonadotropin-dependent will survive and become preovulatory follicles. Hence, gonadotropins and the ability of the follicles to respond to them are essential for follicle survival, maturation, and ovulation [5, 6].

Whereas studies in sheep have shown that the growth of follicles to an ovulatory size is dependent on FSH, with the total number of follicles that develop determined by the amount of FSH and the time of exposure [7, 8], there are no similar studies in mice. However, in transgenic mice, which lack functional FSH signaling, i.e., the *Fshb* knockout gene [9], the FSH receptor (*Fshr*) knockout genes [10, 11], or the naturally occurring hypogonadal (*hpg*) mutant mouse [12] gene, which lacks both LH and FSH, follicles arrest at the preantral stage, confirming that further development is gonadotropin-dependent. Furthermore, treatment with exogenous FSH in the *hpg*

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mutant mouse will induce growth and maturation to produce preovulatory follicles, with the number of follicles ovulating dependent on the amount of FSH [13]. In the wild-type (wt) mouse, it is known that survival and subsequent maturation of follicles depend on the acquisition of functional receptors at the appropriate time. Hence, the number of follicles that remain sensitive to FSH and, hence, acquire LH receptors and express aromatase and, thus, are resistant to apoptosis will determine the number of oocytes that will ovulate [5, 14].

Previous studies have shown that the oocyte plays a crucial role in regulating follicular growth, with oocyte-specific GDF9 and BMP genes implicated at various stages of development [4, 14, 15]. However, no DAZL-associated oocyte-specific genes that regulate follicular sensitivity to gonadotropins have been identified. The RNA binding protein, DAZL, is expressed specifically in germ cells. In our female DAZL null mouse, fetal germ cells proliferate and enter meiosis normally, but there is substantial loss of oogonia from Embryonic Day (ED) 17.5 onward, and by Postnatal Day (PND) 4, there are no germ cells remaining in the ovaries due to failure to progress through meiotic prophase [16, 17]. Timing of the loss of germ cells is affected by strain background and inbreeding. Significant germ loss occurred by ED14.5 both in an inbred line of mice [18, 19] and in a different DAZL null mouse line [20]. Despite this total loss of oocytes, we have previously reported the presence in the remaining ovarian tissue of steroidogenically active cells that secrete inhibins A and B and sufficient estrogen to induce uterine hypertrophy in adult mice at 12 weeks of age [21]. Mice heterozygous (het) for the *Dazl* gene were reported to have similar numbers of germ cells in fetal life, and there was a minimal effect on meiosis compared with that of wt mice [20]. Unexpectedly, in female *Dazl* het mice, a significant increase in litter size was noted together with increased plasma inhibin B and decreased plasma FSH, suggesting the apparent survival of more small follicles. The aim of the present study was to determine how decreased oocyte DAZL expression could enhance follicle development in heterozygous ovaries despite the presence of physiologically reduced plasma FSH concentrations. Our in vitro and in vivo results show that follicles in het mice are more sensitive to FSH. This sensitivity results in more rapid follicle maturation and reduced atresia rates of preovulatory follicles, resulting in increased ovulation rates and litter sizes.

MATERIALS AND METHODS

Animals

The *Dazl* knockout (KO) MF1 mouse line was generated as described previously [16] and maintained by het male-to-female matings. Ear notches were collected for genotyping and identification as previously described [17, 21]. These wt matings, using siblings generated by the DAZL line, were set up as controls for litter size studies. To determine the robustness of the increased litter size phenotype, the MF1 line was backcrossed for four generations and was also rederived onto a 129 background and litter sizes were assessed. Animals were kept on a 14L:10D cycle in temperature- and humidity-controlled rooms and had free access to food and water. All studies were approved by the University of Edinburgh's Biological Services Ethical Review Committee and were performed under a Project Licence as required by the United Kingdom Animals (Scientific procedures) Act 1986.

DAZL Expression, Ovulation Rates, and Pregnancy

The level of *Dazl* mRNA was determined initially by PCR in three separate pools of 10 ovaries (n = 5 mice) from Day 10 (D10) wt, het, and KO mice. Initial PCR assays confirmed there was no *Dazl* mRNA in the KO ovaries. Subsequently, the levels of *Dazl* mRNA in wt mice were compared with those in het mice by using QuantiTect SYBR Green PCR (Qiagen, Crawley, U.K.) in these pools of D10 ovaries and in three separate pools of 50 oocytes from D21 wt and het mice. The forward primer used in both PCR assays was TCCAAATGCTGAGACTTACATG; and the reverse primer was GTCTGTCTGCTTCGGTCCAC.

The relationship between the number of implantation sites and the number of fresh corpora lutea (CLs) in wt and het *Dazl* females was determined. Following timed mating of female mice (n = 10 for each genotype), the animals were culled at ED8, and the numbers of implantation sites and CLs were counted at collection. All ovaries were then fixed in Bouin fixative, processed to paraffin wax, and serially sectioned at 5-μm thicknesses, and every 30th section was counterstained with hematoxylin. The number of CLs was then counted by an independent assessor, and the correlation with the implantation sites was determined for each genotype.

Ovarian Follicle Cultures

Ovaries were removed from D21 wt and het female mice and placed in Leibovitz L-15 (Gibco-BRL, Irvine, U.K.) medium supplemented with 3 mg of bovine serum albumin (BSA)/ml (Fraction V; Sigma, Poole, U.K.) at 37°C. Individual preantral follicles (180 ± 10 mm) were microdissected using fine acupuncture needles and placed in individual wells of a 96-well plate (U wells; Iwaki, Sterilin, Caerphilly, U.K.) containing 30 ml of minimum essential medium (aMEM; Invitrogen, Renfrew, U.K.) supplemented with 5% (v/v) mature F1 (adult female) mouse serum and 140 mM ascorbic acid (Sigma, Poole, U.K.) as described previously [22]. Recombinant human FSH (rhFSH) (300 IU, Puregon, batch 311209; N.V. Organon, The Netherlands) was added from the start of culture at 1.0, 0.1, or 0.01 IU/ml, giving an FSH concentration of 3.0, 0.3, or 0.03 mU per well. Each well was covered with 75 μl of silicon oil (Dow Corning, VWR, U.K.) and incubated at 37°C in 5% CO_2 for 6 days.

Follicle morphology was noted daily, and growth was determined by measuring the diameter with a calibrated ocular micrometer at 40× magnification. Each follicle was then transferred to a new well with fresh medium containing FSH at the appropriate concentration. No culture lasted more than 6 days, and all cultures contained FSH as the absence of FSH leads to follicle death at approximately 2 days of culture [22]. Medium samples were collected from each well at transfer and frozen at −20°C for measurement of estradiol and inhibins A and B. The number of cultures performed was 16, using 24 wt and 21 het D21 mice, which yielded 168 and 166 follicles, respectively.

In Vivo Studies

Morphology. Animals (D21 and 10- to 12-week-old adult wt and het females [n = 6 mice for each group and age]) were killed by CO_2 asphyxiation, and ovaries were collected, weighed, and then fixed in Bouin fixative and processed to paraffin wax stage. All ovaries were serially sectioned (5-mm slices), and every 10th serial section was stained with hematoxylin prior to assessment. Follicles containing a nucleus within the oocyte were classified [23] and counted using an Olympus BH-2 microscope fitted with a Prior automatic stage (Prior Scientific Instruments Ltd., Cambridge, U.K.), and the area of granulosa cells was measured using Image-Pro Plus version 4.5.1 software with Stereologer-Pro plug-in software (Media Cybernetics U.K., Wokingham, Berkshire, U.K.). In addition, ovaries were collected and weighed from all transgenic females culled during routine breeding.

At culling, blood samples were collected by cardiac puncture following CO_2 asphyxiation and centrifuged at 8000 × *g* for 15 min, and plasma was collected and stored at −20°C for measurement of FSH, LH, estradiol, progesterone, and inhibins A and B.

Treatment with FSH and inhibin. The wt and het D21 females (n = 5 per group) were injected intraperitoneally twice daily for 3 days with either FSH (10 or 1 IU of rhFSH in 0.1 ml of phosphate-buffered saline) or charcoal-stripped ovine follicular fluid (oFF) as a source of steroid-free inhibin. We have previously demonstrated that administering oFF in this regimen will significantly suppress plasma FSH concentrations in mice [24]. Animals were culled 24 h after the final injection, and ovaries were collected and processed to paraffin wax stage. Follicles were classified and counted as previously described.

Immunoassays

Plasma FSH and LH concentrations were measured by radioimmunoassay using reagents supplied by the National Hormone and Peptide Program (Dr. A.F. Parlow, Harbor-UCLA Medical Center, CA), with all samples for each hormone assayed in duplicate in one assay. The reference preparations were rat FSH RP-3 and rat LH RP-1, and the minimum detectable concentrations were 1.0 and 0.1 ng/ml, respectively. The intra-assay coefficients of variation were <6% [21, 25].

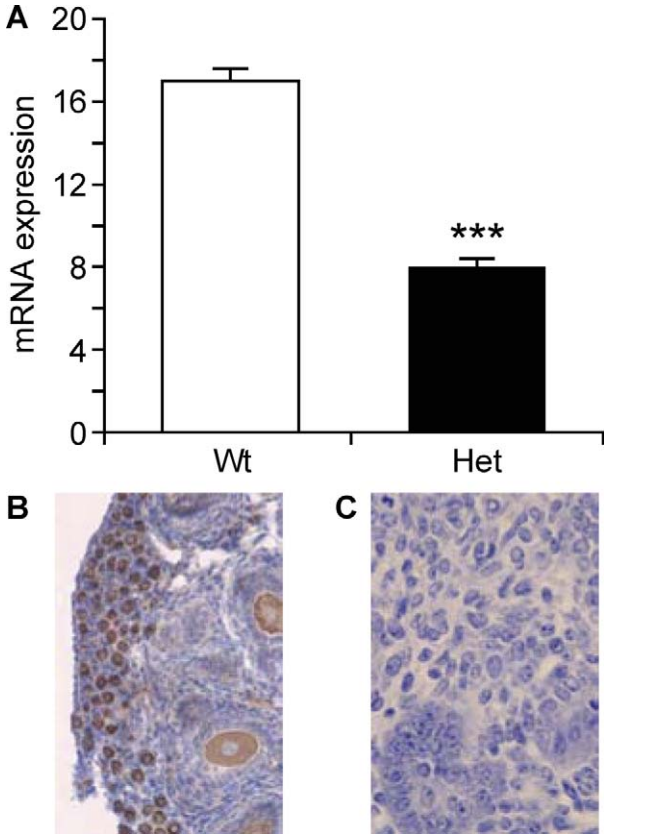

FIG. 1. Levels of expression of *Dazl* mRNA in pools of oocytes from D21 wt and het ovaries (**A**), the presence of DAZL in oocytes in a D21 het ovary (**B**), and the absence of oocytes and DAZL staining in a D21 KO ovary (**C**) are shown. ***Statistical significance of $P < 0.001$.

Inhibins A and B were measured using two-site ELISA [26, 27], previously validated for mouse plasma (21). All samples were measured in a single assay with medium samples diluted in culture medium. The minimum detectable concentrations were 1 pg/ml (inhibin A) and 8 pg/ml (inhibin B), and the intra-assay coefficients of variation were <10%.

Estradiol and progesterone in plasma were assayed following solvent extraction using sensitive radioimmunoassay methods modified for mouse plasma as previously described [28, 29]. All samples were assayed in a single assay with intra-assay coefficients of variation of <8%. The minimum detectable concentrations for estradiol and progesterone were 4.6 and 100 pg/ml, respectively. Estradiol in culture medium was measured by a sensitive ELISA without solvent extraction, using an antiestradiol antibody that had been previously validated [30].

Immunohistochemistry

Ovaries were collected from D21 wt, het, and KO females (n = 5 mice per group) and processed to paraffin wax stage and then serially sectioned at 5-mm thicknesses, and every 10th section was analyzed as previously described. Following antigen retrieval using 0.01 M citrate, pH 6, all sections were incubated with 1) methanol-hydrogen peroxide to block endogenous peroxidases; 2) avidin biotin (Vector Laboratories, Inc., Burlingame, CA) to block endogenous biotin; and then 3) serum (normal goat serum at 1:5 dilution in Tris-buffered saline containing 5% BSA). Sections were then incubated overnight at 4°C with primary antibody (mouse monoclonal antibody to DAZL [clone 311/A] or aromatase [31] at 1:50 dilution [kindly provided by Prof. N. Groome, Oxford Brookes University, U.K.]). Positive staining was detected by biotinylated goat anti-mouse secondary antibody (DAKO Corp., Copenhagen, Denmark), followed by streptavidin-horseradish peroxidase (Vector Laboratories, Inc., Burlingame, CA), and visualized by using a diaminobenzidine detection kit (DAKO Corp., Copenhagen, Denmark). Sections were counterstained with hematoxylin and then dehydrated and mounted in Pertex (Cell Path, Hemel Hempstead, U.K.). Sections were photographed using an Olympus Corp. Provis model microscope (New Hyde Park, NY) and a Kodak digital camera (Eastman Kodak, Inc., Rochester, NY). For each ovary, the number of follicles in each section with positive aromatase staining was counted.

Statistical Analysis

All data shown are expressed as means ± SEM and were analyzed using GraphPad Prism 5 software (GraphPad Software, Inc., San Diego, CA). Plasma hormone concentrations, litter sizes, and ovarian weights were log transformed prior to comparison between two genotypes by using unpaired one-way ANOVA. Parametric one-way ANOVA followed by Tukey post hoc test was used to determine differences in follicle count, granulosa cell area data, and estradiol and inhibin A and B concentrations between genotypes, following follicle incubation. A P value of <0.05 was considered significant.

RESULTS

DAZL Expression, Litter Size, and Plasma Hormones

DAZL expression. In an initial screen, *Dazl* mRNA was detected by PCR in ovaries from wt and het mice, but no signal was detected in KO ovaries. Quantitation of DAZL mRNA by SYBR Green quantitative PCR in both D10 ovaries and D21 oocyte pools is shown in Figure 1A and confirms that expression was decreased to 45% in het compared to that in wt ovaries and oocytes (Fig. 1A). Furthermore, while there were no quantified subjective differences between levels of DAZL expression in wt and those in het oocytes after immunohistochemistry testing for DAZL (data not shown), there were no oocytes and no DAZL protein present in KO ovaries (Fig. 1, B and C). Several attempts were made to quantify levels of DAZL protein by Western blotting using up to 60 μg of protein from pools of D10 ovaries and D21 oocytes (n > 500), with D10 and adult testis (20 μg of protein) used as a control. While a band of the correct size for DAZL (33 kDa) was easily detected in both testis extracts, no signal was present in any of the ovary extracts (n = 3 pools of D10 ovaries and D21 oocytes).

Litter size. The numbers of pups born per litter from wt × wt and het × het matings (n = 35 and 105, respectively) are shown in Figure 2A. Significantly ($P < 0.01$) greater numbers of pups per litter were born to het females following both first and subsequent matings. All animals were between 8 and 10 weeks of age at first mating, and males were left with females until final culling after 6–7 litters. In randomly selected groups of 10 wt and 10 hets culled during pregnancy, the number of CLs (7.5 ± 0.5, wt; and 12.3 ± 0.6, het) and implantation sites (6.9 ± 0.4, wt; and 11.5 ± 0.5, het) were directly correlated within each animal in both wt and het genotypes (n = 10 per group; $r^2 = 0.91$ for wt and 0.94 for het; $P < 0.001$). The increased litter size phenotype was confirmed after backcrossing the MF1 line for four generations (wt mice had 8.1 ± 0.4 pups per litter, and DAZL het mice had 12.4 ± 0.9 pups per litter; n = 13; $P < 0.001$). Furthermore the MF1 line was rederived onto a 129 background and, despite the lower litter size in this in-bred line, the increased litter size was maintained (wt mice had 4.2 ± 0.37 pups per litter versus 8.83 ± 1.14 pups per litter for het mice; $P < 0.001$).

Plasma hormones. Plasma FSH concentrations in adult wt and het 10–12-week-old females are shown in Figure 2B. While there were no differences in plasma LH levels between the genotypes (data not shown), plasma FSH levels were significantly ($P < 0.01$) lower in het females than in wt females. Plasma inhibin B levels were significantly ($P < 0.05$) elevated in het females compared to that in wt females (Fig. 2F). No significant differences in plasma estradiol, progesterone, or inhibin A concentrations were observed between wt and het females (Fig. 2, C, D, and F, respectively).

In Vitro Follicle Cultures

Follicle growth. The growth rates of wt and het follicles, cultured with decreasing concentrations of rhFSH for 6 days,

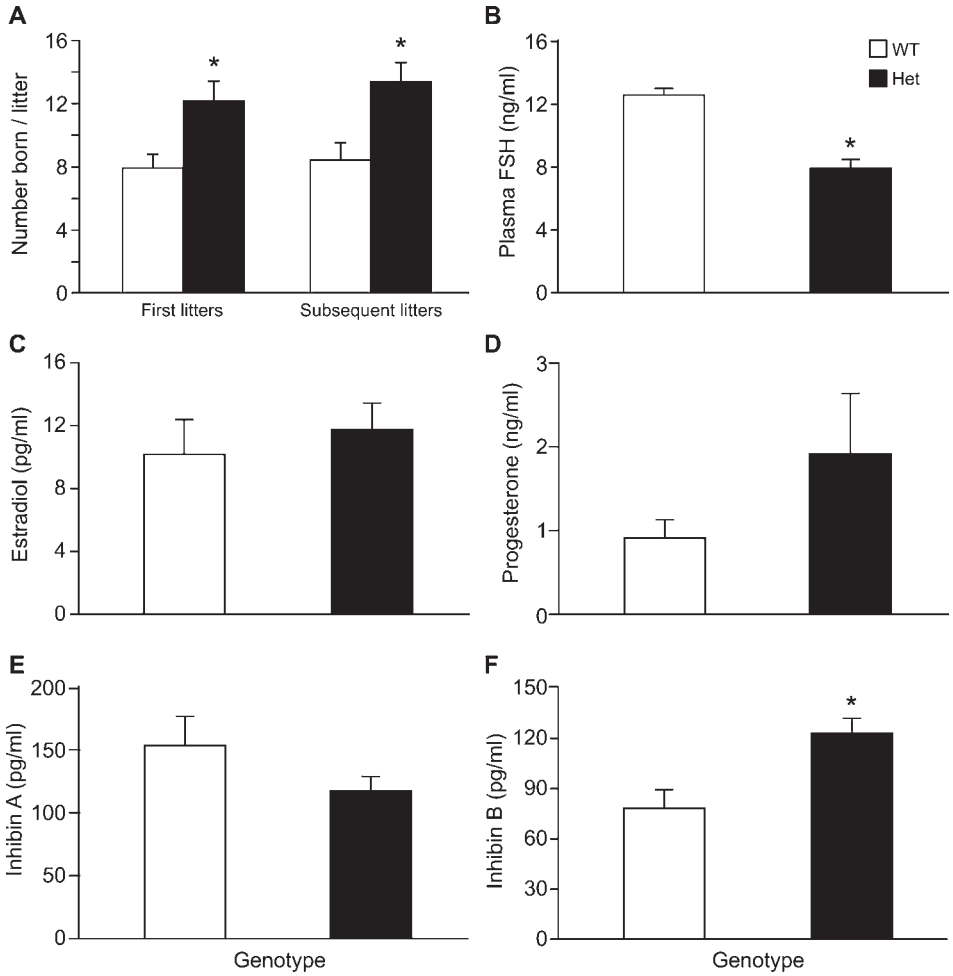

FIG. 2. Phenotypic data from adult wt and het DAZL mice are shown. **A**) The numbers of pups born per litter from wt × wt and het × het matings (n = 35 and 105, respectively) are shown. Mean plasma concentrations of (**B**) FSH, (**C**) estradiol, (**D**) progesterone, (**E**) inhibin A, and (**F**) inhibin B are shown. All data are expressed as means ± SEM. *Statistical significance of at least $P < 0.05$.

are shown in Figure 3, A–C. When follicles were cultured with 1.0 IU of FSH (Fig. 2A), there were no significant differences in follicle diameters on Days 2 and 3, but het follicles on Day 4 were significantly ($P < 0.05$) larger than wt follicles. However, this difference was not maintained on Days 5 and 6. The final diameters of both wt and het follicles in this treatment group were the same (>350 mm). In contrast, in cultures treated with 0.1 IU of FSH (Fig. 3B), het follicles grew faster and by Day 3 had significantly ($P < 0.05$) greater diameters than wt follicles. These differences had increased by Day 4 ($P < 0.01$) and were maintained over the next 2 days. The maximum follicle diameters in both wt and het on Day 6 were 314 ± 4.1 mm and 343 ± 3.3 mm, respectively. At the lowest concentration of rhFSH, 0.01 IU (Fig. 3C), all follicles grew at a similar rate for the first 24 h (D2). Thereafter, the rate of growth of het follicles was greater than that of wt follicles, with het follicles continuing to grow throughout the culture period. However, by Day 4, wt follicles reached their maximum diameters, which were maintained over the next 2 days. On Days 5 and 6, het follicles were significantly ($P < 0.001$) larger than wt follicles, achieving final diameters of 304 ± 3.8 mm and 254 ± 6.3 mm, respectively. The final diameters of both wt and het follicles after 6 days of culture in the lowest concentration of rhFSH (0.01 IU) were significantly ($P < 0.001$) smaller than follicles cultured with either 1.0 or 0.1 IU rhFSH.

Interestingly, it was noted that a number of follicles had ruptured by Day 3 when cultured with either 1.0 IU or 0.1 IU of rhFSH, with a significantly ($P < 0.05$) greater proportion of wt follicle rupture (15%) than het follicle rupture (5%). When follicles were cultured with 0.01 IU of rhFSH, there was no difference in the rate of follicular rupture between genotypes.

Inhibin A and B and steroids. Inhibin A and B concentrations in culture medium on D6 of follicle culture are shown in Figure 3, D and E. While there were no differences in inhibin A concentrations between genotypes following culture with either 1.0 or 0.1 IU rhFSH, medium from het follicles incubated with 0.01 IU rhFSH had significantly ($P < 0.05$) increased inhibin A compared to wt follicles (Fig. 3D).

In contrast, inhibin B concentrations in medium on D6 were significantly ($P < 0.05$) higher in het than in wt follicles following culture with 1.0 and 0.1 IU rhFSH. However, in cultures with the lowest concentration of FSH (0.01 IU), there was significantly less inhibin B in the culture medium from het follicles ($P < 0.05$) (Fig. 3E). In order to determine whether the significant differences in inhibin secretion were related to the differences in rate of growth of follicles, inhibin A and B concentrations in media from wt and het follicles of equivalent sizes (348–350 mm), cultured with 1.0 IU rhFSH, were measured. No differences in either inhibin A or B concentrations were observed for equivalent sized wt and het follicles (Fig. 3F).

In all treatment groups at D6, there were no differences in estradiol and progesterone between genotypes (data not shown).

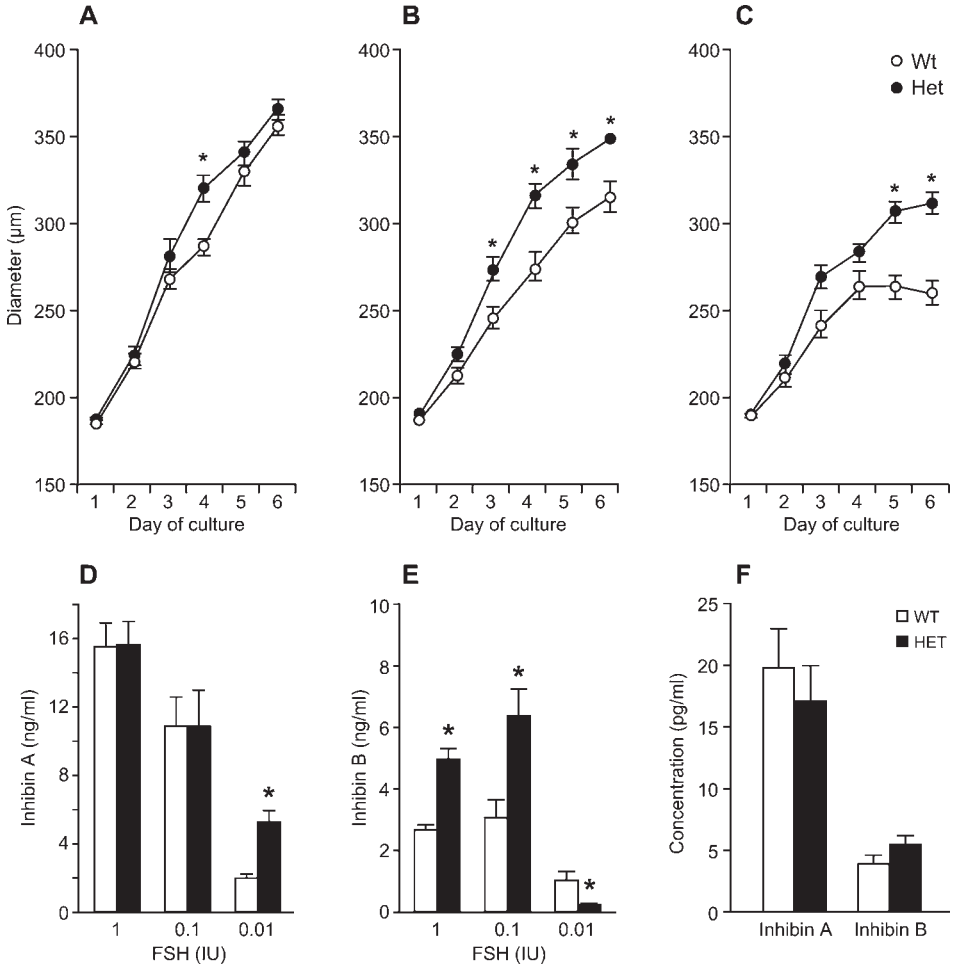

FIG. 3. In vitro cultures of follicles from DAZL wt and het ovaries are shown. Follicle diameter measurements (μm) from Day 1 to Day 6 of culture in medium containing 1.0 (**A**), 0.1 (**B**), and 0.01 (**C**) IU of rhFSH (wt, n = 78 [1.0 IU]; n = 58 [0.1 IU], and n = 32 [0.01 IU]; het, n = 82 [1.0 IU], n = 45 [0.1 IU], and n = 39 [0.01 IU]). All data are shown as means ± SEM. *Statistical significance of at least $P < 0.05$. Inhibin A and B concentrations on D6 of culture in media samples containing 1.0, 0.1, and 0.01 IU rhFSH are shown in **D** and **E**, respectively. **F**) Concentrations of inhibin A and B in media samples from wt and het follicle cultures where the follicle diameters are similar (348–350 μm) following culture in 1.0 IU rhFSH are shown (wt, n = 13; het, n = 18). All data are shown as means ± SEM. *Statistical significance of at least $P < 0.05$.

In Vivo Studies

Follicle populations in wt and het ovaries. There were no differences in individual ovary weights between genotypes or with age (data not shown). The number of follicles containing a nucleus within the oocyte in every 10th section of each ovary (n = 6/genotype) were counted and then multiplied by 10 and classified according to their granulosa cell morphology [23, 32, 33]. Distribution of follicle populations at Day 21 is illustrated in Figure 4. No differences were identified between the genotypes. A similar population profile was noted in 10- to 12-week-old adult ovaries (data not shown).

Follicle morphology. As there were no differences in follicle numbers between genotypes, the morphology of individual follicles at all stages of development was assessed. As follicle maturation is dependent on granulosa cell proliferation, granulosa cell number was used as an indicator of maturity. A preliminary study demonstrated that the relationship between granulosa cell area, excluding oocyte and antral cavity areas, and granulosa cell numbers was highly correlated in both genotypes ($r^2 = 0.98$ for wt and 0.92 for het; $P < 0.001$; data not shown), and, hence, granulosa cell area was used as a marker of granulosa cell number. No differences in follicle size or granulosa cell area were observed in primary, transitionary, or secondary follicles (data not shown), nor were there any differences between oocyte area or volume of follicular fluid in antral follicles between genotypes (data not shown). Analysis of granulosa cell areas in wt and het antral follicles is shown in Figure 5A. It can be seen that the distribution of granulosa cell areas in wt antral follicles ranges from 2×10^4 to 7×10^4 mm^2, with the majority (>90%) of follicle areas between 2×10^4 and 5×10^4 mm^2. However, in het antral follicles, whereas the majority (>80%) of follicles were in the same range, approximately 9% were larger, up to 10.1×10^4 to 11×10^4 mm^2, than only 1% in this range in wt ovaries.

Modulation of FSH

Treatment with oFF. The distribution of granulosa cell area in antral follicles following the inhibition of secretion of endogenous FSH by oFF, a rich source of inhibin, is shown in Figure 5B. The population profiles of both genotypes showed similar distribution patterns, with the exception of follicles with granulosa areas in the range of 5.1×10^4 to 6×10^4 mm^2, where there was a significantly higher percentage of follicles in het ovaries than in wt ovaries ($P < 0.05$). However, the absence of follicles with granulosa cell areas of $>8 \times 10^4$ mm^2 in het ovaries indicates that their growth beyond this size, as seen in the untreated het mice, is FSH dependent.

Treatment with rhFSH. The distribution profiles of antral granulosa cell areas in wt and het ovaries following treatment with 1 IU and 10 IU of rhFSH are shown in Figure 5, C and D, respectively. As expected, treatment with 10 IU of FSH in both genotypes significantly ($P < 0.01$) increased the number of large follicles compared with that of untreated controls. Apart from small antral follicles with a granulosa cell area in the range 5.1×10^4 to 6×10^4 mm^2, where there were significantly ($P < 0.01$) more in wt than in het, no other significant differences were observed between the genotypes.

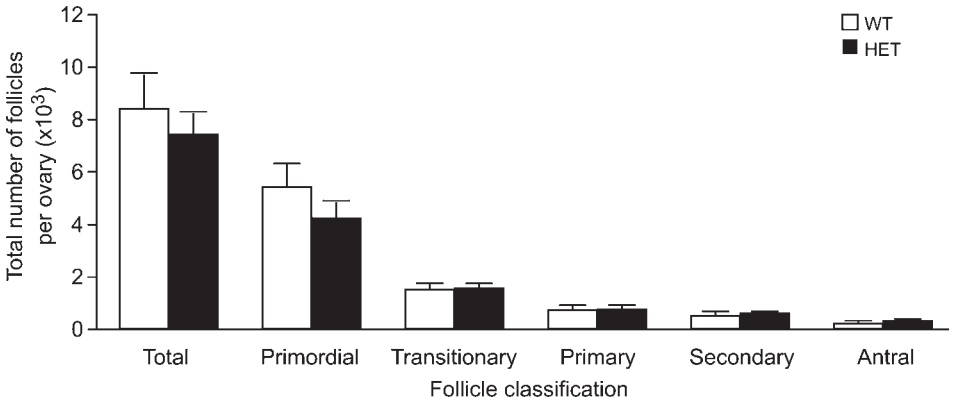

FIG. 4. Follicle population classification in D21 DAZL wt and het ovaries are shown according to reference [19]. All data are shown as means ± SEM (n = 6 for each genotype).

Following their treatment with 1 IU of FSH, a distribution pattern of granulosa cell areas was observed that was similar to the in vivo control nontreated mice with endogenous FSH (Fig. 5C). The majority of follicles in both genotypes had granulosa cell areas in the range of 2.1×10^4 to 7×10^4 mm^2, with no differences in percentages of follicles between genotypes at any point, apart from those with a granulosa cell areas of 5.1×10^4 to 6×10^4 mm^2, where there were significantly ($P < 0.01$) more wt than het follicles. Similarly, there were significantly (8% het versus 1% wt; $P < 0.01$) more follicles with granulosa cell areas greater than 7×10^4 mm^2 in het ovaries than in wt ovaries. There were no follicles with granulosa cell areas of $>9.1 \times 10^4$ to 10×10^4 mm^2 in ovaries from either genotype. Interestingly, treatment with 1 IU of FSH altered the distribution profile only in the wt ovaries, shifting the median point of the curve from 3.1×10^4 to 4×10^4 mm^2 (for control, nontreated ovaries) to 5.1–6 mm^2. This area of granulosa cells is in the same size range as that of small antral follicles, 240–280 mm in diameter, with an equivalent area of follicular fluid and the same size of oocytes in wt and het mice (wt, 66.1 ± 1.1 mm; het, 66.7 ± 1.5 mm).

Aromatase immunohistochemistry. The number and distribution of aromatase-positive follicles in wt and het D21 ovaries are shown in Figure 5. While there are aromatase-positive follicles in ovaries from both genotypes (Fig. 6, A and B), there are significantly ($P < 0.001$) more positive follicles in het than in wt ovaries at this age (Fig. 6C).

DISCUSSION

In the present study, we have shown reduced oocyte *Dazl* mRNA expression in het females, and this is related to in an increase in the sensitivity of granulosa cells to FSH. This change could account for the increase in litter size found in the study published previously [21] and is confirmed in the present, much larger study.

During the follicular phase of the cycle, FSH levels decrease as a result of increased estrogen and inhibin B secretion from

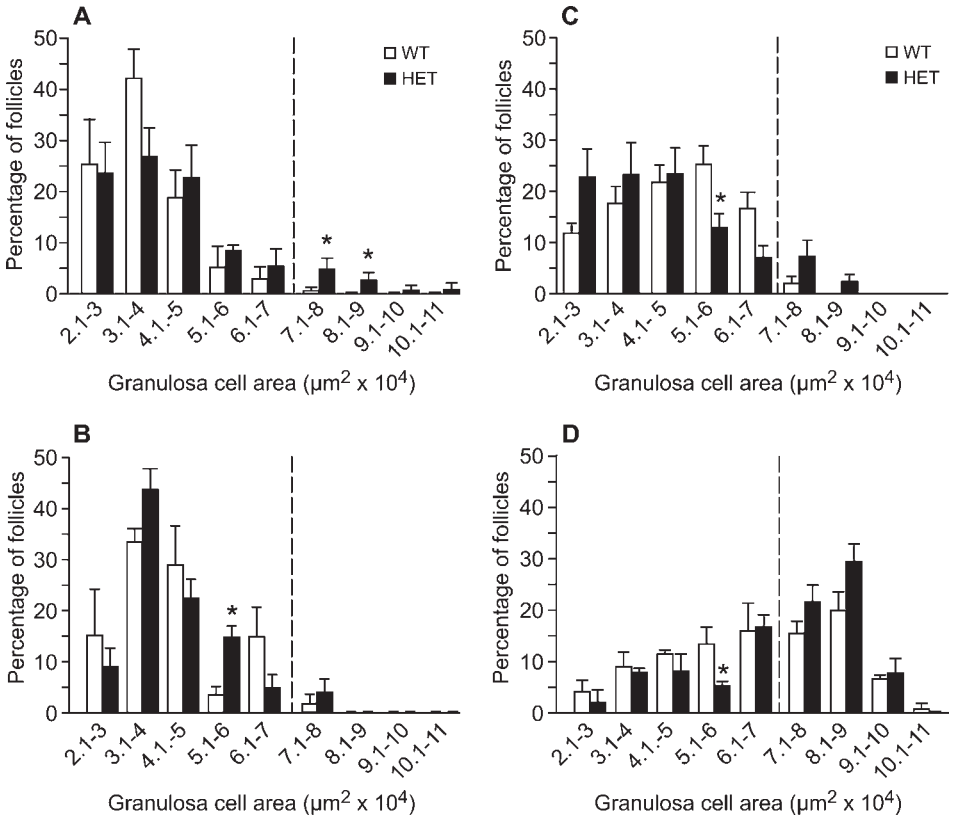

FIG. 5. Distribution profiles of granulosa cell areas ($\times 10^4$ μm^2) in (**A**) antral follicles from untreated DAZL D21 wt and het ovaries, (**B**) following treatment with oFF, and (**C** and **D**) following treatment with 1 and 10 IU rhFSH, respectively. All data are expressed as mean percentages of antral follicles ± SEM. *Statistical significance of at least $P < 0.05$. Dashed line indicates the granulosa cell area above which there are few (1%) larger follicles in wt ovaries. This is indicated for clarity only.

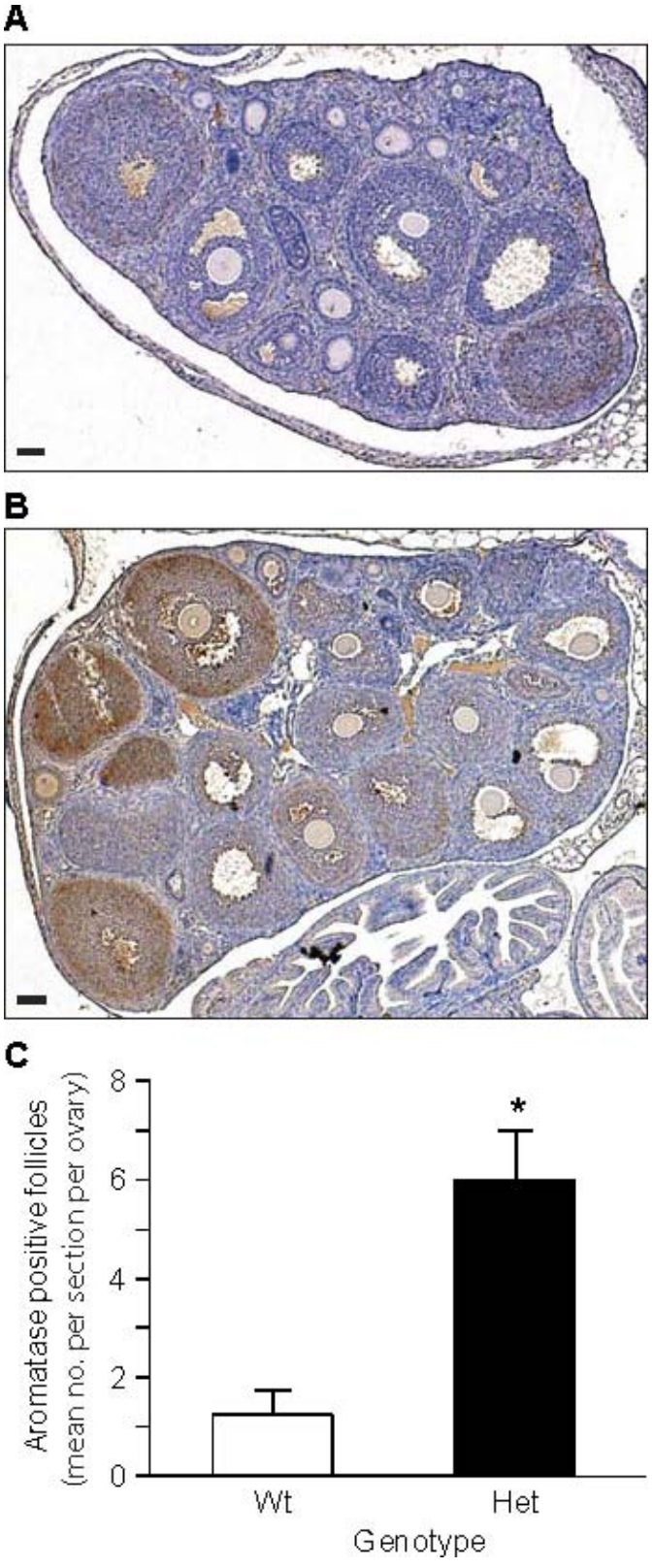

FIG. 6. Aromatase expression in D21 wt (**A**) and in het (**B**) ovaries is shown as detected by immunohistochemistry. Bars = 50 μm. **C**) Total number of aromatase-positive follicles are shown in untreated DAZL D21 wt and het ovaries (n = 5 for each genotype). Data are expressed as the mean number of positive staining follicles per ovary ± SEM when every 10th 5-μm section was analyzed. *Statistical significance of at least $P < 0.05$.

the growing cohort of small follicles [27]. Only follicles that can survive this decrease in circulating FSH in the follicular phase of the cycle, through additional utilization of LH via FSH-induced LH receptors on granulosa cells [14], will continue to grow and mature to become preovulatory follicles. Therefore, the ability to respond to FSH and then LH to enhance, e.g., cAMP production, at the appropriate time regulates the number of follicles attaining ovulatory competence. The ovary and, particularly, the developing follicles finely control FSH secretion by producing inhibin B from the small follicles and subsequently inhibin A and estradiol from the larger preantral and antral follicles [26]. Manipulation of FSH by abolishing secretion, using transgenic technology, resulted in infertility with a block in folliculogenesis at the preantral stage [9]. Similar ovarian morphology was demonstrated in the FSH receptor KO mouse, although lack of ovarian feedback at the pituitary resulted in elevated plasma gonadotropins and consequently severe ovarian pathologies in old mice [10, 11, 34]. In addition, reducing FSH with GnRH antiserum or hypophysectomy followed by an increase in plasma FSH levels has a dramatic effect on the number of follicles ovulating in rodents and sheep [35, 36].

However, in the *Dazl* het females, increased plasma inhibin B, not estradiol, appears to be associated with low FSH. For this to occur in the het ovary, it must either have more small preantral follicles secreting similar amounts of inhibin B per follicle or a similar number of follicles with each follicle producing more inhibin B. In fact, our in vitro study has demonstrated that the increased plasma inhibin B observed in het females is due to accelerated follicle maturation and not to the secreted product of additional follicles. Culturing preantral wt and het follicles in decreasing FSH concentrations showed that het follicles grow faster than wt follicles at each FSH dose and, crucially, have the ability to survive in FSH concentrations that are detrimental to equivalent wt follicles. This indicates that DAZL het follicles in which the oocytes express reduced DAZL protein (Y.A. Brown, unpublished results) are more sensitive to FSH at all concentrations investigated. As each het follicle grows faster, they secrete significantly more inhibin B, suggesting they are developmentally more advanced than the corresponding wt cohort, with no differences in estradiol output, data that concur with our previously reported preliminary in vivo results [21]. This result indicates that in the DAZL het ovary, FSH enhances granulosa cell proliferation and differentiation as demonstrated by increased inhibin B secretion. Further confirmation that the increase in inhibin B in het follicles is due to their significantly greater size is demonstrated by the observation that wt and het follicles of equivalent size secrete similar amounts of inhibin A and B and estradiol.

In vivo, this increased sensitivity to FSH per se could have increased the numbers or sizes of follicles in the DAZL het ovaries. Analysis of all size classes of follicles in D21 and adult ovaries from both genotypes showed no differences in the numbers of follicles at any stage in folliculogenesis, indicating that increased sensitivity to FSH has no effect on follicle activation. However, by using the highly correlated relationship between granulosa cell number and area, we could determine the number of granulosa cells in antral follicles and classify antral follicles by granulosa cell area. Consequently, the presence of significantly larger follicles in het ovaries than in wt follicles confirms our in vitro results that D21 DAZL het ovaries have antral follicles that are larger than those present in wt ovaries of the same age due to significantly increased numbers of granulosa cells. These follicles are more developmentally advanced as confirmed by the expression of

aromatase in significantly more follicles at D21. Furthermore, the effect of significantly reduced DAZL mRNA and protein is only in the granulosa cells and has no effect on the size of the oocyte or area of the antrum (data not shown), despite the fact that the *Dazl* gene is oocyte-specific. Hence, it is suggested that the increased sensitivity to FSH allows accelerated maturation of granulosa cells. This, together with more follicles being able to respond to low FSH concentrations, reduces the rate of atresia compared with that of wt, leading to more follicles becoming preovulatory. Interestingly, a similar increase in ovulation rate in Booroola sheep heterozygous for the *BMPR1B* mutation, otherwise known as *ALK6*, has been extensively studied, but, in that case, follicles attained ovulatory competence at a smaller follicle size due to a reduction in granulosa cell numbers associated with accelerated differentiation [37].

If the accelerated maturation of granulosa cells in antral follicles from DAZL het ovaries is entirely mediated by FSH, then manipulation of FSH should alter the distribution of these follicles, classified by size. Decreasing endogenous FSH by treatment with oFF completely abolished the large follicles, which were observed in the untreated het ovaries, demonstrating that this increase in the number of larger follicles is entirely dependent on their enhanced response to FSH. However, it should be noted that the actual decrease in plasma FSH following oFF treatment in the het mice is less drastic than in wt mice, as the levels of endogenous FSH in the het mice are significantly lower than that in the wt mice. Therefore, it would appear that the ability of the het ovary to respond to a narrow range of plasma concentrations of FSH is very finely tuned. In addition, following oFF treatment, similar granulosa cell area classification profiles confirmed that all differences between the genotypes are dependent on FSH.

Treatment of DAZL het mice with exogenous FSH did not perturb the enhanced granulosa cell maturation by increasing either the size or number of the large follicles. Low FSH concentrations had no effect on the distribution of follicular granulosa cell areas in het ovaries, which still had more large follicles than the wt ovaries. In contrast, in the wt mice, treatment with low FSH levels altered the population profile by increasing the median size of the follicles. Higher FSH levels increased the proportion of larger follicles in both het and wt ovaries but did not increase the size of the largest follicles. This suggests that a granulosa cell area of 10.1×10^4 to 11×10^4 mm^2 is the maximum area achievable in mouse follicles. In DAZL wt ovaries, the higher FSH level treatment was sufficient to transform the granulosa cell distribution profile to that of the het follicles. Our results also suggest that antral follicles with a granulosa cell area of 5.1×10^4 to 6×10^4 mm^2 appear to be most responsive either to increasing or reducing FSH. The reason for this is not known, but it is not related to differences in the sizes of the follicle, antrum, or oocyte, which are the same in both het and wt follicles with this granulosa cell area.

In this mouse model, the difference between wt and het ovaries is a single copy of the *Dazl* gene expressed in the oocytes. Therefore, the relationship between a single *Dazl* copy in oocytes and accelerated granulosa cell proliferation, which we have shown is FSH-dependent, must involve FSH receptors (FSHR) expression, activation, and subsequent signaling. KO experiments have shown that follicular growth is arrested in mice with no FSH [9] or FSHR [10, 11], which are present on granulosa cells. Simplistically, reduced DAZL expression could alter FSHR numbers expressed, but this does not appear to be the case, as preliminary data (Y.A. Brown, unpublished results) have shown no differences in FSHR mRNA expression between the two genotypes. However, this does not determine receptor functionality. FSH is known to regulate granulosa cell proliferation through multiple signaling pathways [38] such as the induction of cell cycle regulatory protein cyclin D2 expression, while simultaneously reducing the cell cycle inhibitor protein p27 kip [39, 40]. In our model, significantly reduced plasma FSH is the result of increased inhibin B feedback from developing small follicles. This must be an indirect effect of reduced DAZL, as DAZL proteins are germ-cell-specific RNA binding proteins affecting mRNA translational regulation [41].

In oogenesis, where the temporal regulation of gene expression is crucial, there are periods of increased mRNA expression when entering meiosis, followed by quiescence when the oocyte arrests at diplotene until completing meiosis following the LH surge. It has been assumed that DAZL functions to regulate intraoocyte gene expression at these particular stages. However, in our study, the increased sensitivity of the granulosa cells to FSH is likely to be through oocyte-secreted proteins, as there are few reports of DAZL expression in granulosa and theca cells in mouse and human ovaries [42–44]. Therefore, regulation of an oocyte-secreted protein must be either directly or indirectly related to DAZL expression. Of the oocyte-secreted proteins whose effects on granulosa cells are well documented [4, 45], GDF9 could be a candidate for DAZL interaction. However, the lack of a putative DAZL binding consensus sequence precludes any apparent direct interaction with DAZL, and, furthermore, by using real-time PCR, we have shown there are no differences in *Gdf9* mRNA expression between the genotypes (Y.A. Brown, unpublished observation). A possible indirect effect might be through modulation of anti-Müllerian hormone, which can affect the effects of FSH on follicular growth [46].

Efforts to identify in vivo mRNA targets of mammalian DAZL have focused mainly on testis-expressed mRNAs. Mouse vasa homolog (*Mvh* [official symbol, *Ddx4*]) and synaptonemal complex protein (*Sycp3*) genes were isolated by coprecipitation with DAZL protein from testis extracts [47, 48] but are also present in oocytes during early oogenesis. However, the connection between these genes, follicle sensitivity to FSH, and early follicular maturation does not seem obvious at the moment and needs further investigation. Embryonic stem cells derived from *Dazl* heterozygous mice appear to show aberrant gene expression and imprinting abnormalities, suggesting that a single copy of the *Dazl* gene may not be enough to support germ cell development in vitro [20]. However, in vivo, in the present study, there were no differences between the number of oocyte-containing follicles in het and those in wt mice, suggesting that there was no impairment in oocyte development or survival in fetal life. Furthermore, once formed in vivo, the germ cells and oocytes are perfectly viable, because in our studies, there was an increase in fertility and fecundity, and almost all ovulations appear to result in implantation and birth of viable young. Identification of oocyte-specific mRNAs with putative DAZL consensus sequences at later stages of oogenesis might help elucidate the complex signaling pathways that appear to be modified in DAZL het mice. Our unpublished results after attempting to use the DAZL monoclonal antibody that was used for immunohistochemistry failed to identify any products when used in cross-linking and immunoprecipitation assays with pools of up to 500 oocytes from D21 ovaries, even though DDX4 was successfully identified from testis extracts at the same time, as previously reported [47, 48]. Indeed Western blot analysis of proteins from pools of D21 oocytes ($n = 500$) or D10 ovaries, using the same monoclonal antibody, failed to

identify any protein of the expected size (33 kDa), although this was identified in extracts from both juvenile and adult wt testes. Thus, it was not possible to determine if there was a reduction in DAZL protein levels, even though this would be highly probable given the reduction in *Dazl* mRNA levels. Furthermore, it might be possible that DAZL could form dimers in the present KO model as, potentially, the RNA-binding domain may remain present if the protein were expressed. However, this was shown not to be the case when the original KO model was created as the mRNA was destabilized and no protein was detectable with a range of antibodies raised to different parts of the protein [16] (Prof. H. Cooke, personal communication).

In conclusion, it is suggested that in activated wt follicles, DAZL represses a specific gene or genes that, through unknown pathways, regulate the response of the granulosa cells to FSH. If the degree of repression is reduced, as in the DAZL het mice, then there is increased expression of this specific gene(s), allowing increased responsiveness of the granulosa cells to FSH. Thus, more follicles continue to grow in the face of declining plasma concentrations of FSH in the late follicular phase of the cycle, fewer become atretic, and more eventually ovulate, resulting in increased litter sizes.

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