BIOLOGY OF REPRODUCTION (2012) **87**(1):3, 1–11
Published online before print 18 April 2012.
DOI 10.1095/biolreprod.112.099929

Follicle-Stimulating Hormone Accelerates Mouse Oocyte Development In Vivo[1]

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ABSTRACT

During folliculogenesis, oocytes grow and acquire developmental competence in a mutually dependent relationship with their adjacent somatic cells. Follicle-stimulating hormone (FSH) plays an essential and well-established role in the differentiation of somatic follicular cells, but its function in the development of the oocyte has still not been elucidated. We report here that oocytes of *Fshb*$^{-/-}$ mice, which cannot produce FSH, grow at the same rate and reach the same size as those of wild-type mice. Consistent with this observation, the granulosa cells of *Fshb*$^{-/-}$ mice express the normal quantity of mRNA encoding Kit ligand, which has been implicated in oocyte growth. Oocytes of *Fshb*$^{-/-}$ mice also accumulate normal quantities of cyclin B1 and CDK1 proteins and mitochondrial DNA. Moreover, they acquire the ability to complete meiotic maturation in vitro and undergo transition from non-surrounded nucleolus to surrounded nucleolus. However, these events of late oocyte development are significantly delayed. Following in vitro maturation and fertilization, only a small number of embryos derived from oocytes of *Fshb*$^{-/-}$ mice reach the blastocyst stage. Administration of equine chorionic gonadotropin, which provides FSH activity, 48 h before in vitro maturation increases the number of blastocysts obtained subsequently. These results indicate that FSH is not absolutely required for oocyte development in vivo but that this process occurs more rapidly in its presence. We suggest that FSH may coordinate the development of the germline and somatic compartments of the follicle, ensuring that ovulation releases a developmentally competent egg.

developmental competence, follicle-stimulating hormone, follicular development, FSH, knockout, oocyte

INTRODUCTION

The production of a mature egg ready to be fertilized begins with the entry of a primordial follicle, consisting of a small oocyte surrounded by a small number of squamous granulosa cells (GCs), into the growth pool [1–6]. As the oocyte begins to grow in size, the GCs assume a cuboidal shape and begin to proliferate so that they continue to enclose the growing oocyte in a structure now termed a primary follicle. Proliferating GCs generate several layers around the oocyte, an extracellular matrix is laid down outside the GCs, and a new cell type, the theca, develops externally to this layer. Follicles containing more than one layer of GCs are termed secondary or preantral follicles. By the late preantral stage, the oocyte has (nearly) reached its full size and has acquired the ability to undergo the final stage of oogenesis, termed meiotic maturation, although it is not yet competent to develop as an embryo. Meanwhile, a fluid-filled cavity termed the antrum develops and expands within the GCs that differentiate into two populations, the cumulus GCs, which surround the oocyte, and the mural GCs, which line the wall of the antral follicle. During the antral stage of follicular growth, the fully grown oocyte becomes competent to develop as an embryo [7–11]. Expression of luteinizing hormone (LH) receptors on the mural GCs enables ovulation to occur in response to LH at this final stage of follicular development. Thus, the integrated and coordinated of programs of differentiation of the germline and the somatic compartments of the ovarian follicle ovulation produce a developmentally competent egg.

Follicle-stimulating hormone (FSH), a dimeric glycoprotein composed of an α and a β subunit, is produced by gonadotropic cells of the anterior pituitary and plays an essential role in follicular development [12–14]. FSH receptors can be detected on the GCs of primary follicles and remain present exclusively on these cells throughout subsequent folliculogenesis [15, 16]. FSH, acting principally through activation of cAMP-dependent signaling pathways, drives proliferation and differentiation of GCs, and many gene targets of FSH have been identified [12, 17, 18]. In the absence of FSH or its receptor, FSHR, follicles can develop to the early antral stage both in vivo and in vitro. However, they do not develop further—the antrum fails to enlarge and the follicle becomes atretic [14, 19–24]. Among other defects, the mural GCs of FSH-deprived follicles also do not produce LH receptors, so LH is unable to trigger ovulation, leading to infertility [14]. Thus, folliculogenesis up to the early antral stage is considered independent of FSH, whereas antral (late) folliculogenesis is strictly FSH-dependent [2, 25].

In contrast to its well-established role in differentiation of the somatic compartment of the follicle, the role of FSH in development of the oocyte is poorly understood. FSH regulates the degree of intercellular contact between the oocyte and its immediately surrounding GCs [26] and can alter gene expression patterns in the oocyte [9, 26, 27]. These observations, together with the fact that oocytes acquire full developmental competence during the FSH-

[1]Supported by the Canadian Institutes for Health Research, the Department of Obstetrics and Gynecology, McGill University, and the McGill University Health Centre Foundation. I.D. was supported by the Fonds de la Recherche Scientifique (FRSM 3.4546.07). E.M. was supported by the government of Saudi Arabia.
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Received: 13 February 2012.
First decision: 25 March 2012.
Accepted: 6 April 2012.
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eISSN: 1529-7268 http://www.biolreprod.org
ISSN: 0006-3363

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dependent period of follicular growth, provide circumstantial evidence that FSH regulates oocyte development. It may also be noted that the studies which have excluded an indispensable role for FSH in preantral folliculogenesis have generally focused on the somatic compartment rather than on the oocyte. Genetic studies have indicated that oocytes of mice lacking *Fshr* show reduced growth depending on the age of the female [28]. However, oocytes of mice lacking *Fshb*, which encodes the FSH β subunit, can give rise to 2-cell embryos after hormonal priming [19]. Although the subsequent development of these embryos has not been reported, most embryos derived from oocytes of mice carrying a mutation in the *Gnrh1* gene, which lack both FSH and LH, are unable to progress beyond early embryogenesis [29]. In vitro studies have yielded contrasting results. When follicles obtained at preantral stages in mice are grown in vitro, FSH is required for the oocytes to become meiotically and developmentally competent [24, 30–32], yet high levels of FSH inhibit oocyte growth in cultured follicles of the baboon [33]. With the use of a different culture system, one in which mouse preantral follicles are first subjected to a mild enzymatic digestion that removes the basement membrane and thecal layer to yield granulosa-oocyte complexes (GOCs) and are then grown in vitro, FSH is reported to be necessary [34, 35] or not necessary [36, 37] for them to acquire meiotic and developmental competence. In summary, the role of FSH in development of the oocyte remains remarkably little understood.

Defining the role of FSH is not only necessary for acquiring a more profound understanding of the nature of somatic-germ cell signaling that drives production of a fertilizable egg [3, 6] but also, in view of the routine administration of FSH to women undergoing in vitro fertilization (IVF) and widespread efforts to develop culture systems that will efficiently support oocyte development in vitro [38–41], is crucial for improving the success of assisted reproduction in humans. Therefore, we studied oocyte development in mice that carry a targeted mutation in the *Fshb* gene [19] and so do not produce FSH.

MATERIALS AND METHODS

Mice

All experiments were performed in compliance with the regulations and policies of the Canadian Council on Animal Care and were approved by the Animal Care Committee of the Royal Victoria Hospital. Mice bearing a targeted deletion in the *Fshb* gene [19] were obtained from Jackson Laboratories, and a colony was established at McGill University. Mice were housed and bred in a temperature- and light-controlled room and given food and water ad libitum. $Fshb^{-/-}$ mice were generated by mating $Fshb^{+/-}$ females with $Fshb^{+/-}$ or $Fshb^{-/-}$ males. $Fshb^{+/+}$ or $Fshb^{+/-}$ females of the same litter were used as controls. Genomic DNA was screened by PCR, allowing detection of the wild-type gene (*Fshb* primers TTCAGCTTTCCCCAGAA GAG and CTGCTGACAAAGAGTCTATG) and the replacement targeting vector (primers CTTGCGCTCATCTTAGGCTT and GGACCTCTC GAAGTGTTGGAT) in the same sample. DNA extraction and amplification were performed using a commercial kit (EZ Bioresearch, St. Louis, MO). PCR was performed using a program of 94°C for 60 sec, 55°C for 60 sec, and 72°C for 90 sec. PCR products were visualized on 1.5% agarose gels stained with ethidium bromide.

Preparation and Analysis of Ovarian Sections

Ovaries were fixed overnight at 4°C in freshly prepared 4% *para*-formaldehyde in phosphate-buffered saline (pH 7.5). Following subsequent dehydration and embedding in paraffin, sections were cut at 5 μm, mounted on glass slides, and stained using hematoxylin and eosin. Sections were examined using a Zeiss confocal microscope. Every fifth section was analyzed, and follicles containing an oocyte with a visible nucleolus were scored. Follicles containing a single layer of GCs or those in which a second layer covered less than half the circumference of the follicle were classified as primary follicles. Follicles containing more than one layer of GCs according to this criterion and no visible antrum were scored as preantral follicles. Follicles containing a visible antrum that occupied at least one-quarter of the area of the follicle were scored as large antral follicles; this threshold was aimed at reducing the possibility that fixation-induced intercellular spaces would be scored as antra follicles. To estimate the diameter of follicles and oocytes, image analysis software of the confocal microscope was used to draw a line around the circumference of the structure, and the diameter was calculated. Four ovaries of each genotype at each age were analyzed.

Collection and Culture of Oocytes

Experiments were design to take advantage of the synchronous follicular growth that occurs during the first 3 weeks of life in mice. Ovaries were removed from prepuberal mice at different ages, thus corresponding to different stages of follicular development, and from adults. Ovaries were transferred into 2.5 ml of Hepes-buffered minimal essential medium (Hepes-MEM; pH 7.2; Life Technologies, Burlington, ON, Canada) supplemented with sodium pyruvate (0.25 mM; Sigma Chemicals, Windsor, ON, Canada), penicillin G (63 mg/l; Sigma), streptomycin (50 mg/l; Sigma), and bovine serum albumin (BSA; 1 mg/ml; Sigma) at 37°C. In some experiments, dibutyryl cyclic AMP (0.1 mg/ml; Sigma) was added to maintain meiotic arrest. Follicles protruding from the surface of the ovaries were punctured, and cumulus-oocyte complexes (COCs) were collected. Complexes whose oocyte contained a visible germinal vesicle (GV) were selected for experiments. In some experiments, mice received an intraperitoneal injection of 5 IU of equine chorionic gonadotropin (eCG; Sigma) 48 h before oocyte collection.

To permit meiotic resumption, COCs were transferred into maturation medium and cultured for ~16 h in a humidified atmosphere of 5% CO_2 in air. Maturation medium was composed of bicarbonate-buffered MEM medium supplemented as above and also including 5% fetal bovine serum (Life Technologies) and 10 ng/ml recombinant epidermal growth factor (Becton Dickinson, Mississauga, ON, Canada). At the end of the culture period, cumulus cells surrounding the oocyte were removed using a mouth pipette, and the stage of nuclear maturation was determined using a dissecting microscope. Oocytes were classified as GV, germinal vesicle breakdown (GVBD) when no GV was visible, or metaphase II when a polar body was visible. The diameter of each oocyte excluding the zona pellucida was measured using images obtained with a Zeiss LSM 510 model confocal microscope.

To analyze chromatin configuration, we collected oocytes at the GV stage from 19- and 23-day-old mice by follicular puncture, mechanically freed of their surrounding cumulus cells, and incubated them in Hepes-MEM supplemented with 1 μg/ml Hoechst 33342 (Cell Signaling Technology, Pickering, ON, Canada) for 10 min. Oocytes were rinsed in fresh medium and individually placed into 5-μl droplets of medium covered with mineral oil. Oocytes were observed using a Zeiss LSM 510 confocal microscope and ultraviolet light and classified as containing a surrounded-nucleolus (SN) or non-surrounded nucleolus (NSN) chromatin configuration [42].

Analysis of Kitl mRNA

GOCs from 12- and 15-day-old mice were isolated enzymatically [43, 44], and COCs from 19-day-old mice were isolated by follicular puncture. Total RNA was purified using a Picopure RNA isolation kit (Arcturus, Life Technologies), and cDNA was synthesized using Superscript II (Gibco, Life Technologies) as previously described [45]. Primer sequences were designed using freely available software (Primer3 and BLAST) and obtained from Sigma. For each primer pair, a standard curve was generated using serial dilutions of cDNA prepared from ovarian RNA and used to determine the efficiency of amplification. Each PCR tube contained 10 μl of FastStart Master SYBR Green Mix (Qiagen, Toronto, ON, Canada), 7 μl of sterile dH_2O, 1 μl of primers (10 μM), and 2 μl of template. Real-time PCR was performed using a Corbett Rotorgene 6000 (Montréal Biotech, Montréal, QC, Canada). Melting curve analysis and electrophoresis of amplified products confirmed that only a single product of the expected size was generated. Data were analyzed using software provided by the manufacturer. Data were normalized to the value obtained for *Fst* (follistatin), which is expressed in GCs but not oocytes and whose expression is not changed in $Fshb^{-/-}$ mice [17]. Primers TATGT TACCCCCTGTTGCAG and CTTCCAGTATAAGGCTCCAA (predicted size, 166 nucleotides [nt]; efficiency, 0.91) were used for *Kitl1*; primers TTAGGTCCCGAGAAAGGGAA and CTTCCAGTATAAGGCTCCAA (the latter is the same as for *Kitl1*; predicted size, 126 nt; efficiency, 0.96) were used for *Kitl2*; and primers AGGAAGAGTGTTGCAGCACC and CAGGT GATGTTGGAACAGTC (predicted size, 226 nt; efficiency, 0.88) were used for *Fst*.

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Quantification of Mitochondrial DNA

A fragment of the mouse *mt-ND1* gene (GenBank no. NC005089.1) was amplified from mouse tail DNA, inserted into a plasmid by using standard techniques, and used to construct a standard curve for PCR amplification. For experimental analyses, 1 pg of a plasmid containing rabbit globin cDNA and 20 μg of glycogen were added to a microtube containing a single oocyte. DNA was purified using QIAmp DNA mini kits (Qiagen). Real-time PCR was performed using the Rotorgene unit. Each reaction tube contained 10 μl of FastStart Master SYBR Green Mix (Qiagen), 7 μl of sterile ddH_2O, and 1 μl of primers at a concentration of 10 μM and 2 μl of DNA template. Amplification conditions were 95°C for 5 min; and 40 cycles at 95°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec. Melting curve analyses confirmed that only one PCR product was generated. Results were normalized to the globin data to correct for between-sample differences in the efficiency of DNA purification and were converted to absolute quantities by using a sample of the mt-ND1 fragment that was amplified in the same experimental run. Primers CGATTAAAGTCC TACGTGATCTGA and CTGGGAGAAATCGTAAATAGATAGAAA (predicted size, 80 nt; efficiency, 0.95; designed by Dr. Lawrence C. Smith, Université de Montréal) were used for *mt-ND1*; and primers GTGGGACAG GAGCTTGAAAT and GCAGCCACGGTGGCGAGTAT (predicted size, 257 nt; efficiency, 0.98) were used for rabbit globin.

Immunoblotting

Immunoblotting was performed as previously described [45]. Primary antibodies were directed against cyclin-dependent protein kinase-1 (CDK1; 1:3000 dilution; product no. 06–966; Upstate Biotechnology, Millipore, Billerica, MA), cyclin B1 (1:1000 dilution; freshly diluted for each experiment; product no. 4135 [V152]; Cell Signaling) and α-tubulin (1:2000 dilution; Cedarlane Laboratories, Burlington, ON, Canada). Following incubation with appropriate secondary antibodies conjugated to horseradish peroxidase, immunoreactive species were detected using ECL+ (GE Healthcare, Baie d'Urfé, QC, Canada) and quantified using a Storm 860 phosphorimager (GE Healthcare).

In Vitro Fertilization

For IVF, the caudae epididymides from a 3- to 6-month-old male mice were isolated and placed in $NaHCO_3$-buffered modified human tubal fluid (mHTF) supplemented with 9 mg/ml BSA. The large tubules were cut to release spermatozoa into the medium, and the mixture was incubated for 2 h. Sperm at a concentration of 1×10^6/ml to 2×10^6/ml were then added to a dish containing the eggs in 500 μl of mHTF supplemented with 4 mg/ml BSA. After 4 h of incubation, oocytes were rinsed and transferred into 40-μl drops (~10/drop) of potassium simplex optimized medium (KSOM) supplemented with 1 mg/ml BSA and essential and nonessential amino acids (Life Technologies).

Intracytoplasmic sperm injection (ICSI) was performed as described previously [46]. A sperm suspension was prepared as described above and then mixed in equal parts with HEPES-buffered mHTF containing 12% (w/v) polyvinylpyrrolidone (360 kDa; Sigma). Micromanipulation was performed using an inverted microscope (Olympus model IX 70) equipped with a heating plate (Tokay Hit thermo plate) that maintained the temperature at 25°C during all manipulations. The sperm head was separated from the tail by applying piezo pulses from a Piezo model 150 PMM unit (Prime Tech). Five to 10 sperm heads were isolated and loaded into the injection pipette and then injected singly into individual oocytes, as described previously [47]. The sperm suspension was replaced every 30 min. Injected oocytes were placed in 20-μl drops of Hepes-buffered mHTF covered with mineral oil and kept at room temperature for 5 min to permit the plasma membrane to heal. Following an additional 20-min incubation at 37°C in a humidified atmosphere of 5% CO_2 in air, surviving oocytes were transferred to KSOM and cultured for 4 days in a humidified 5% CO_2 atmosphere at 37°C.

Statistical Analyses

Statistical tests used to compare data are described in the legend to each figure.

RESULTS

Follicular Growth in $Fshb^{-/-}$ Animals

Shortly after birth, a large cohort of follicles and their enclosed oocytes begin to grow, reaching full-size after approximately 3 weeks. To study the role of FSH in follicular growth in vivo, we examined the progression of this first cohort, as well as the distribution of follicles in adult (8-week-old) animals. Ovarian histological sections were prepared from $Fshb^{-/-}$ and wild-type ($Fshb^{+/+}$) or heterozygous ($Fshb^{+/-}$) littermates. No differences were observed between wild-type ($Fshb^{+/+}$) and heterozygous ($Fshb^{+/-}$) individuals (not shown). As shown in Figure 1A, which illustrates $Fshb^{+/-}$ and $Fshb^{-/-}$ individuals, a large number of follicles began to grow in prepuberal mice of both genotypes. Similarly, follicles at different stages of preantral growth were observed in adult mice of both genotypes. Corpora lutea were not observed in the ovaries of $Fshb^{-/-}$ mice, confirming their anovulatory phenotype. These observations are consistent with previous descriptions of the ovarian histology of $Fshb^{-/-}$ mice [19].

We quantified follicular growth in two ways. First, we counted the number of primary, secondary, and large antral follicles in ovaries from litter mates at different ages (Fig. 2A). By 12 days, approximately half of the growing follicles of wild-type mice had reached the secondary stage, whereas most growing follicles of $Fshb^{-/-}$ mice were at the primary stage. By 18 days, most growing follicles of wild-type mice had reached the secondary stage, and a small number contained a large antrum, whereas primary follicles remained predominant in $Fshb^{-/-}$ mice. At 24 days, large antral follicles were present in wild-type mice, whereas in $Fshb^{-/-}$ mice, many follicles had reached the secondary stage, but large antral follicles were not observed. Some follicles contained cavities which could be small antra (Fig. 1); however, as we cannot exclude fixation artifact, such follicles were classified as secondary. Nonetheless, mitotic chromosomes could be detected (Fig. 1B, upper right inset), confirming that GCs were continuing to proliferate. Similarly, in adults, $Fshb^{-/-}$ individuals contained fewer secondary follicles than wild-type individuals and no large antral follicles (Figs. 1 and 2A).

Second, we measured the size of growing follicles (Fig. 2B). Because increases in follicle size are most clearly detected during antral formation, we examined 18-day-old (mainly preantral) and 24-day-old (large antral present in wild-type) mice. We measured the size in sections that passed through the nucleolus of the oocyte. At 18 days, follicles of different sizes were present in both genotypes, but the average size was greater in the wild-type animals than in the $Fshb^{-/-}$ animals. During the following 6 days, follicles of both genotypes continued to grow; however, large antral follicles were observed only in wild-type individuals. Taken together, these results show that ovarian follicles of $Fshb^{-/-}$ individuals are able to grow but more slowly than in wild-type mice and that they do not develop large antra. These observations suggest that FSH stimulates growth of preantral, as well as antral follicles, although the former can develop in the absence of FSH.

Oocyte Growth in $Fshb^{-/-}$ Animals

To evaluate oocyte growth, we first measured the size of oocytes using the same histological sections described above. We examined secondary and antral follicles, which would contain oocytes that were nearing full size. Between 12 and 24 days, the average and maximum diameters of oocytes increased in both wild-type and $Fshb^{-/-}$ animals (Fig. 3A). We observed a statistically significant but quantitatively very small difference between the two genotypes in the prepuberal animals. In adult animals, however, there was no significant difference in oocyte diameter between the two genotypes. In both wild-type and mutant mice, there appeared to be a slight decrease in average oocyte size in adult animals compared to those in 24-

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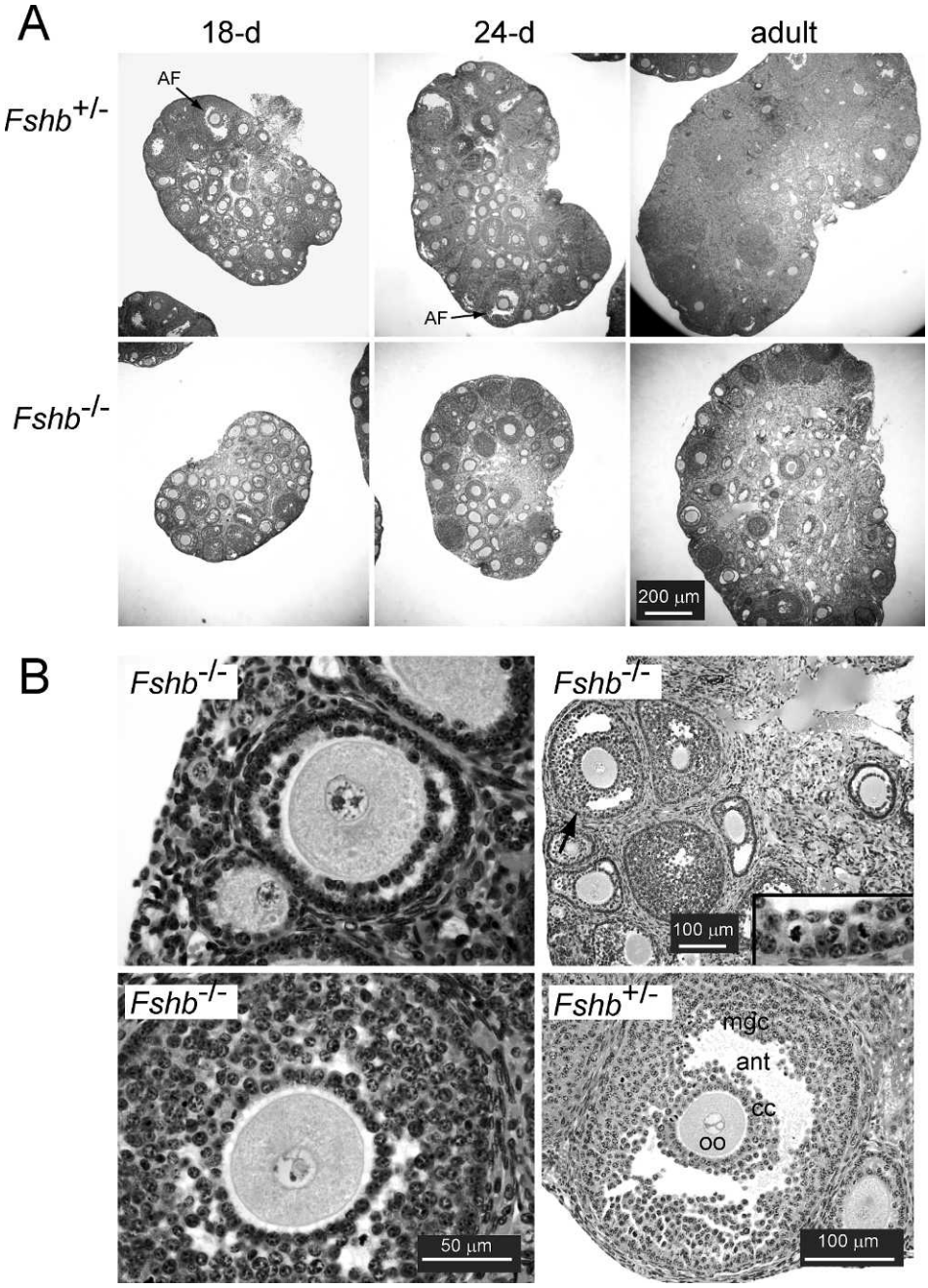

FIG. 1. Ovarian histology of *Fshb*$^{+/-}$ and *Fshb*$^{-/-}$ and mice. **A**) Low-power magnification images of ovaries of mice at the indicated ages. Many growing oocytes and follicles are visible at 18 days (d) and 24 days in both *Fshb*$^{+/-}$ and *Fshb*$^{-/-}$ mice, but large antral follicles (AF, arrow) are seen in *Fshb*$^{+/-}$ only. Ovaries of *Fshb*$^{-/-}$ adults show many growing follicles but no corpora lutea. **B**) High-power magnification images. In *Fshb*$^{-/-}$ animals, some large oocytes are enclosed by only 1 or 2 layers of GCs (upper left), whereas others are enclosed by several layers (upper right, lower left). Small antra-like structures may be present (upper right, arrow) but these do not grow to the size seen in *Fshb*$^{+/-}$ mice (lower right). Inset shows mitotic figures indicating that GCs of *Fshb*$^{-/-}$ individuals are mitotically active. Oo, oocyte; mgc, mural GCs; cc, cumulus cells; ant, antrum.

day-old animals; this may reflect the greater asynchrony among the population of growing oocytes in adults. To compare the size of living oocytes, we punctured ovarian follicles of adult animals and measured the diameter of the oocytes that were recovered. We observed no differences in the average oocyte diameters between wild-type and *Fshb*$^{-/-}$ animals. It is also worth noting that in histological sections, the oocytes of mutant mice were morphologically indistinguishable from those of *Fshb*$^{+/-}$ (Fig. 1B) or wild-type mice (not shown). These results indicate that oocytes follow (nearly) normal growth kinetics and reach full-size in the absence of FSH in vivo.

Kit ligand (KITL) can promote oocyte growth in vitro and is thought to play an important role in vivo also [3, 48]. The primary transcript of the *Kitl* gene can be alternatively spliced to generate two mRNAs, *Kitl1* and *Kitl2*, which encode soluble and membrane-bound forms, respectively, of KITL. In vitro studies have demonstrated that KITL2 promotes oocyte growth more efficiently than KITL1 and that FSH promotes the production of *Kitl2* [34, 48]. As our results indicated that FSH is not required for oocyte growth in vivo, we examined the expression of *Kitl1* and *Kitl2* in GCs of wild-type and *Fshb*$^{-/-}$ mice. GOCs were harvested from 12-day-old, 15-day-old, and

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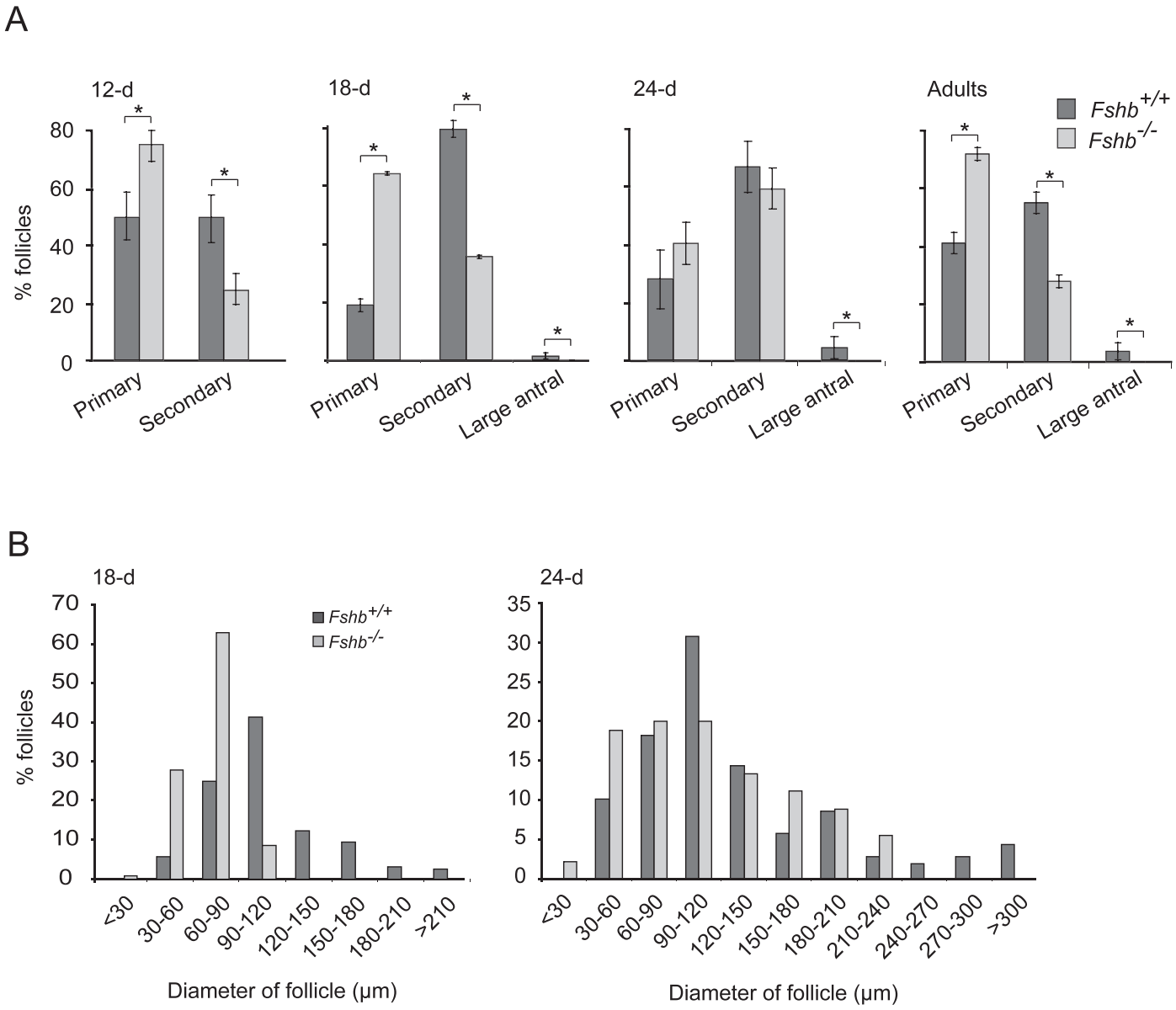

FIG. 2. Follicular growth in $Fshb^{+/+}$ and $Fshb^{-/-}$ mice. Ovaries were harvested from mice of the indicated age, and histological sections were prepared. Primary, secondary, and large antral follicles containing an oocyte with a visible nucleolus were counted, and the diameter of each follicle was measured. **A**) Proportions of follicles at different stages from ovaries of 12-, 18-, and 24-day-old and adult mice; 391, 257, 205, and 410 follicles, respectively, were analyzed from $Fshb^{+/+}$ mice; and 305, 258, 113, and 197 follicles, respectively, were analyzed from $Fshb^{-/-}$ mice. Mean ± SEM values are plotted. Each pair of means was compared using the *t*-test. *Means are statistically different ($P < 0.05$). **B**) Distribution of follicles according to diameter in 18-day-old and 24-day-old mice.

19-day-old mice of both genotypes. Following mRNA purification and conversion to cDNA, the relative amounts of *Kitl1* and *Kitl2* at each age were measured. To correct for differences in the number of GCs in different samples, all values were normalized to the quantity of *Fst* mRNA, which is expressed in GCs but not oocytes and whose abundance shows little or no change in $Fshb^{-/-}$ mice [17].

First, we measured the relative amounts of *Kitl1* and *Kitl2* in GOCs collected from 12-, 15-, and 19-day-old wild-type mice. Our method permits the amount of each mRNA to be compared at different ages but does not indicate whether the amount of *Kitl1* mRNA differs from the amount of *Kitl2* mRNA at any given age. We found a decrease in the amounts of both *Kitl1* and *Kitl2* in complexes obtained from 19-day-old mice (Fig. 4A). This result confirms earlier reports that *Kitl* expression decreases in follicles containing oocytes that have nearly reached full size [49]. We then compared amounts of *Kitl1* and *Kitl2* in GOCs collected from wild-type and $Fshb^{-/-}$ littermates. We found that both mRNA splice forms were equally abundant in wild-type and $Fshb^{-/-}$ GOCs at all three ages (i.e., ratio close to 1) (Fig. 4B). This indicates that neither the decrease in the amount of *Kitl* nor the *Kitl1*:*Kitl2* ratio at 19 days were altered in $Fshb^{-/-}$ GOCs. These results indicate that FSH does not regulate *Kitl* mRNA abundance or splicing in vivo.

Among the numerous intracellular changes that occur during oocyte growth, particular attention has focused recently on the enormous accumulation of mitochondria, which are necessary to support embryonic development and whose compromised function has been suggested to underlie age-dependent loss of fertility in human females [50–52]. As a

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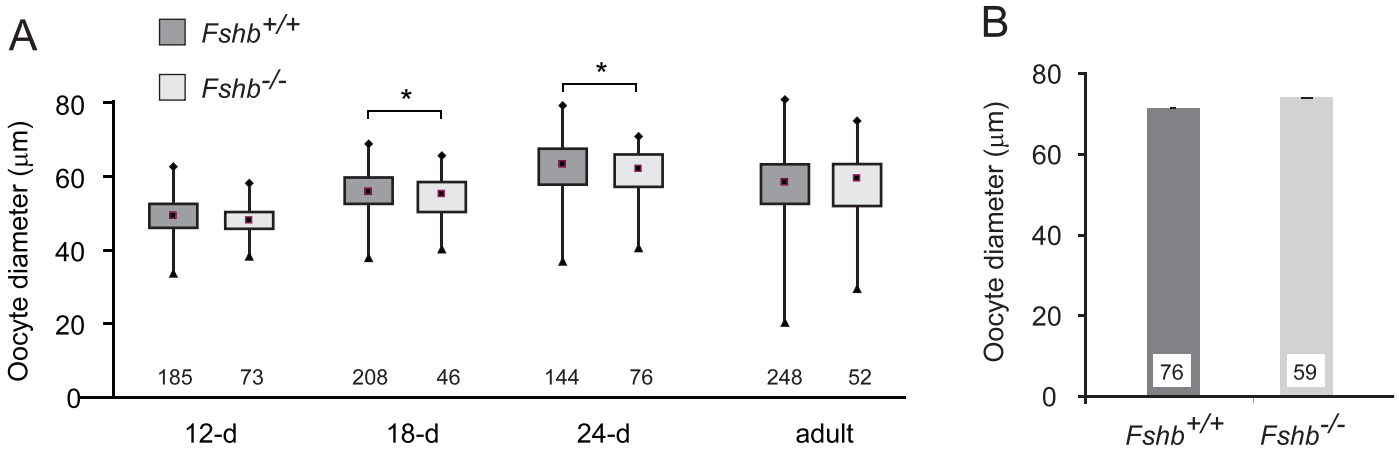

FIG. 3. Oocyte growth in *Fshb*$^{+/+}$ and *Fshb*$^{-/-}$ mice. **A**) Diameters of oocytes within secondary and antral follicles were measured in the ovarian sections shown in Figure 2 from 12-, 18-, and 24-day-old and adult mice. Box plot indicates the mean and 25th and 75th quartiles. For both genotypes, mean diameters at 12, 18, and 24 day were significantly different from each other (one-way ANOVA followed by Tukey HSD test, $P < 0.01$). At each age, mean diameters were compared between genotypes using the *t*-test. *Values are statistically different ($P < 0.05$). Numbers of oocytes analyzed are shown at the base of each group. **B**) Mean ± SEM diameters of living oocytes obtained by puncture of large follicles from adults. Means were compared using the *t*-test. No significant differences were detected. Numbers of oocytes analyzed are shown at the base of each bar.

surrogate for mitochondrial number, we used quantitative PCR to measure the amount of mitochondrial DNA (mtDNA). We found no difference in the amount of mtDNA in oocytes of *Fshb*$^{+/+}$ (mean, 141 877/oocyte; SEM, 15 804; n = 10) and *Fshb*$^{-/-}$ (mean, 145 242/oocyte; SEM, 8 946; n = 10) animals. Thus, like oocyte growth, the accumulation of mtDNA apparently occurs normally in the absence of FSH.

Meiotic Competence of Oocytes of Fshb$^{-/-}$ *Animals*

As the oocytes of *Fshb*$^{-/-}$ mice appeared to complete growth normally, we then tested whether they became meiotically competent. We recovered oocytes by follicular puncture as described above, selected those with a visible GV, and incubated these oocytes overnight. The next morning, we determined whether each oocyte had remained immature (intact GV), initiated maturation as indicated by GVBD, or completed maturation to metaphase II as indicated by the presence of the first polar body. Oocytes of *Fshb*$^{-/-}$ mice showed a reduced ability to undergo GVBD in 19-day-old animals (Fig. 5A), but no difference was apparent in older animals. In contrast, oocytes from *Fshb*$^{-/-}$ mice at all ages showed a modestly reduced ability to reach metaphase II (Fig. 5B). This difference compared to *Fshb*$^{+/-}$ animals was greatest at 19 days and then became progressively smaller in older animals. The oocytes that reached metaphase II were, however, morphologically indistinguishable from those of *Fshb*$^{+/-}$ (Fig. 5C) or wild-type mice (not shown). Because the ability to undergo maturation is

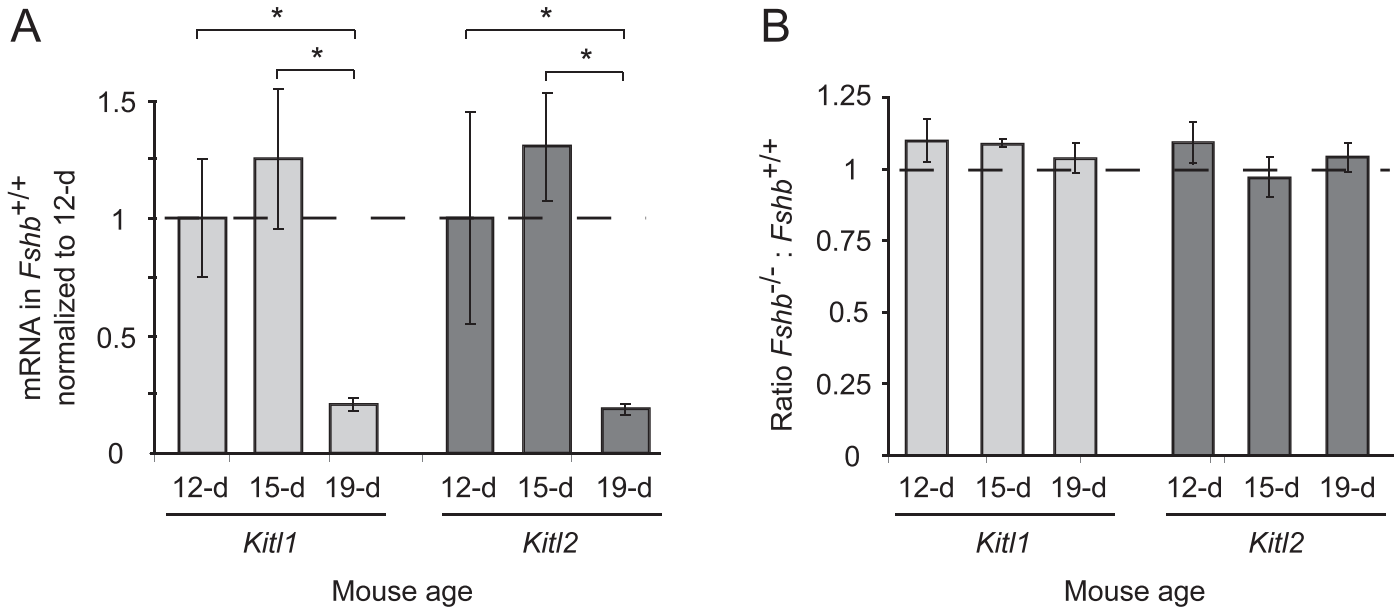

FIG. 4. Expression of *Kitl1* and *Kitl2* in GOCs of *Fshb*$^{+/+}$ and *Fshb*$^{-/-}$ mice. GOCs were isolated from mice at the indicated ages (COCs at 19 days), and the amounts of *Kitl1* and *Kitl2* mRNA were quantified. Mean ± SEM values are plotted. **A**) Expression in *Fshb*$^{+/+}$ mice. Data are presented relative to 12-day-old *Fshb*$^{+/+}$ GOCs in which expression level was set at 1. Mean values at 12, 15, and 19 days were compared using one-way ANOVA followed by Tukey HSD test. *Values are statistically different ($P < 0.01$). **B**) Expression in *Fshb*$^{-/-}$ mice relative to that in *Fshb*$^{+/+}$ mice. Means of each group were compared to the *Fshb*$^{+/+}$ value by using a single-sample *t*-test. No significant differences were detected.

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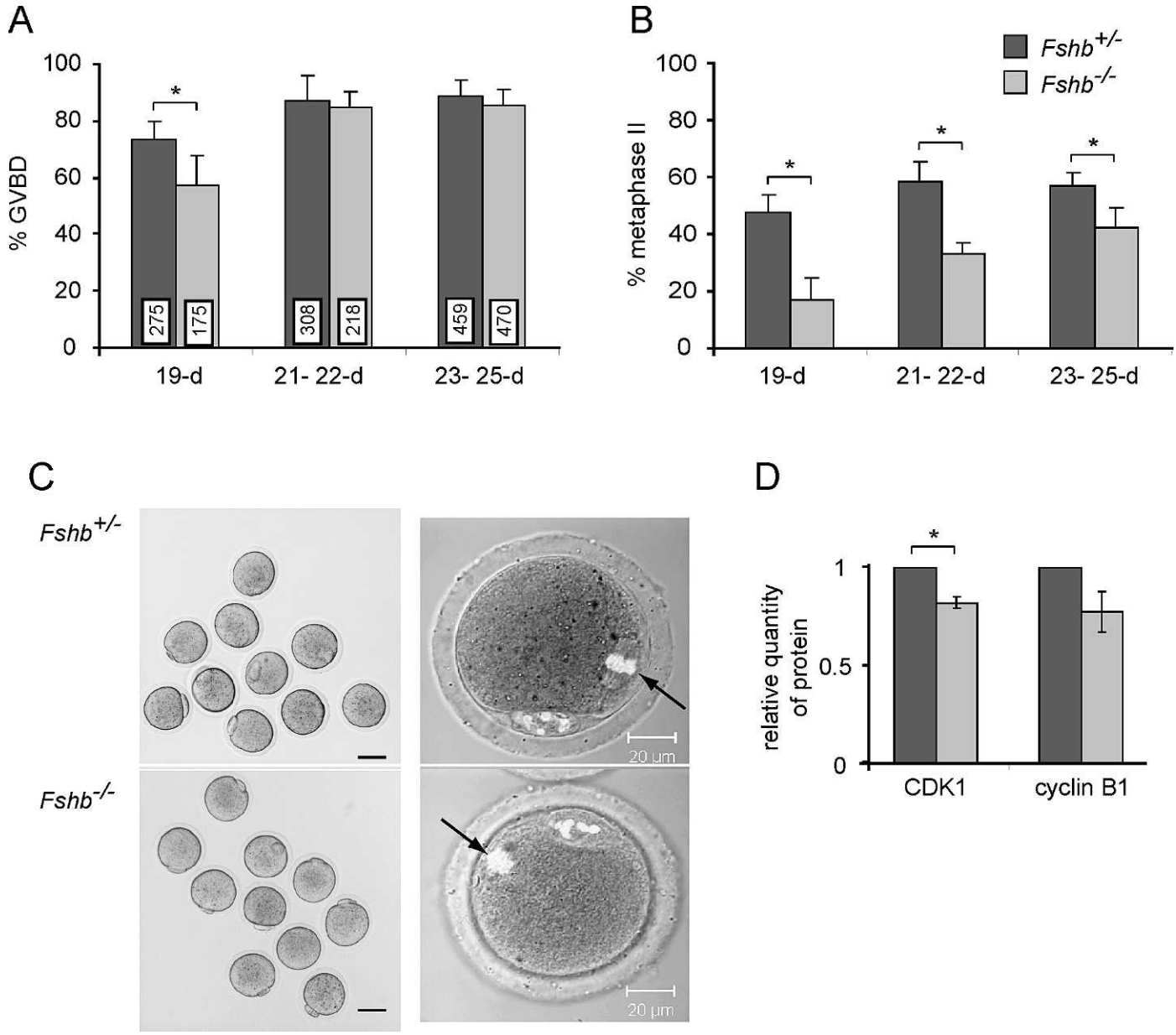

FIG. 5. Meiotic competence of oocytes of *Fshb*$^{+/-}$ and *Fshb*$^{-/-}$ mice. COCs were collected by puncture of large follicles of mice at the indicated ages and incubated overnight. The next morning, the cumulus cells were removed, and the fraction of oocytes that had undergone germinal vesicle breakdown (GVBD) and emitted a polar body (metaphase II) was recorded. **A**) Mean ± SEM percentage of GVBD. **B**) Mean ± SEM percentage of metaphase II. The experiment was performed four times, and the number of oocytes analyzed is indicated at the base of each bar in **A**. Mean values between genotypes at each age were compared using the *t*-test. *Values are statistically different ($P < 0.05$). **C**) Representative photomicrographs of oocytes that have been fixed (left) and stained to reveal the chromosomes aligned on the metaphase II plate (right, arrow). Bars = 20 µm. **D**) Pools of 40 oocytes were subjected to immunoblotting, and the relative quantities of CDK1 and cyclin B1 were determined densitometrically and normalized to those of tubulin in the same sample. Values for the wild-type oocytes were set to 1. Means ± SEM are shown for three experiments. Mean values between genotypes at each age were compared using the single-sample *t*-test. *Values are statistically different ($P < 0.05$).

TABLE 1. Relationship between oocyte size and meiotic competence.*

	Oocyte diameter (µm)†					
	19 Days		21 Days		25 Days	
Genotype	GV (n)	GVBD (n)	GV (n)	GVBD (n)	GV (n)	GVBD (n)
Fshb$^{+/-}$	67.4 ± 0.6[a] (34)	73.3 ± 0.3 (84)	66.9 ± 1.0 (15)	73.2 ± 0.5 (41)	67.6 ± 0.6 (23)	73.9 ± 0.3 (65)
Fshb$^{-/-}$	64.6 ± 0.7[b] (36)	73.0 ± 0.4 (52)	67.3 ± 0.8 (7)	72.5 ± 0.4 (42)	68.6 ± 0.8 (21)	75.0 ± 0.3 (60)

* GV, germinal vesicle; GVBD, GV breakdown; n, number of oocytes examined.
† Data are expressed as means ± SEM, and each pair of means was compared using the *t*-test.
[a,b] Different superscript letters indicate a significant difference ($P < 0.05$).

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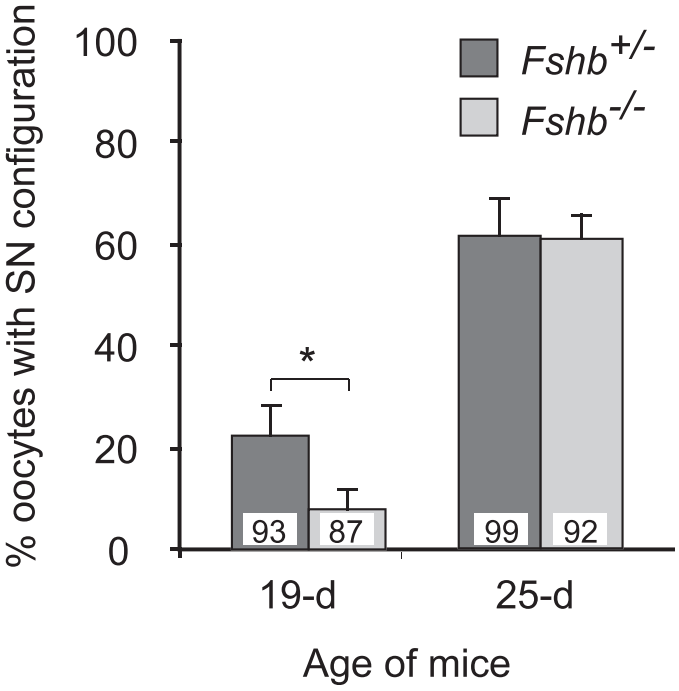

FIG. 6. Chromatin configuration of GV stage oocytes obtained by puncture of large follicles of 19-day-old and 25-day-old *Fshb*$^{+/-}$ and *Fshb*$^{-/-}$ mice. Oocytes were stained using Hoechst 33342 stain and examined using a Zeiss LSM model confocal microscope. Bars are means ± SEM percentages of oocytes showing SN chromatin configuration. The experiment was performed four times, and the total number of oocytes examined is shown at base of each bar. Mean values between genotypes at each age were compared using the *t*-test. *Values are statistically different ($P < 0.05$).

correlated with oocyte size during oogenesis in vivo, we compared the diameters of oocytes that remained GV-arrested with those that underwent GVBD during overnight incubation. Consistent with earlier reports, GV-arrested oocytes were smaller than those that underwent GVBD. Although at 19 days the GV-arrested oocytes of *Fshb*$^{-/-}$ mice were slightly smaller than those of *Fshb*$^{+/-}$ mice, there were no differences in the size of the GVBD-competent oocytes between genotypes at any age (Table 1). Therefore, the impaired ability of the oocytes of *Fshb*$^{-/-}$ mice to progress to metaphase II cannot be attributed to a difference in size, compared to those of wild-type mice. These results indicate that, although FSH is not required for oocytes to become meiotically competent in vivo, this process occurs more rapidly or efficiently when it is present.

To identify a potential molecular basis for the impaired meiotic competence of oocytes of *Fshb*$^{-/-}$ animals, we collected immature oocytes from 21- to 23-day-old *Fshb*$^{+/-}$ and *Fshb*$^{-/-}$ mice and compared the amounts of CDK1 and cyclin B1 by immunoblotting. *Fshb*$^{-/-}$ oocytes contained slightly less CDK1 and showed a trend toward less cyclin B1 (Fig. 5D). This suggests that these proteins may accumulate more slowly in oocytes that grow in the absence of FSH.

The results described so far indicate that events occurring relatively early during early oocyte development, such as increase in cell size and accumulation of mtDNA, proceed independently of FSH, whereas those occurring relatively late, such as acquisition of meiotic competence, are delayed in its absence. To further evaluate this notion, we examined another late event of oocyte development, namely, the change in chromatin configuration from the NSN, when the chromatin is dispersed throughout the nucleus, to the SN stage, where much of the chromatin is condensed around the nucleolus [53–55]. We examined the timing of this transition in oocytes of *Fshb*$^{+/-}$ and *Fshb*$^{-/-}$ mice. Follicles were punctured, oocytes were recovered and stained using Hoechst 33342 and examined using a confocal microscope. At 19 days, approximately 20% of the oocytes of *Fshb*$^{+/-}$ mice had reached the SN stage, whereas only ~5% of the *Fshb*$^{-/-}$ had done so (Fig. 6). By 25 days, however, ~60% of the oocytes of each genotype had reached the SN stage. Thus, the NSN-to-SN transition also occurred independently of FSH in vivo but was accelerated when FSH was present.

Embryonic Development in Fshb$^{-/-}$ *Animals*

To determine whether oocytes of *Fshb*$^{-/-}$ animals could develop as embryos, we first attempted to induce ovulation in adults (5–8 weeks old) by sequential administration of eCG followed 44 h later by hCG. Although production of LH (hCG) receptors would not be expected to occur in the absence of FSH, we anticipated that the FSH activity of eCG might be sufficient to induce their expression. In these and all studies of embryonic development, we used only *Fshb*$^{+/-}$ and *Fshb*$^{-/-}$ littermates in each experiment. COCs were recovered from the oviduct at 14 h post-hCG administration. We recovered a mean ± SEM of 31.3 ± 5.7 eggs per *Fshb*$^{+/-}$ female but only 5.3 ± 2.4 eggs per *Fshb*$^{-/-}$ female. Following in vitro fertilization, ~75% of the *Fshb*$^{+/-}$ eggs cleaved to the 2-cell stage compared to only ~25% of the *Fshb*$^{-/-}$ eggs. The very small number of ovulated eggs that we obtained from the hormonally primed *Fshb*$^{-/-}$ mice precluded further studies using this approach. We therefore turned to in vitro maturation. Cumulus-enclosed oocytes were recovered from 21- to 23-day-old mice by using follicular puncture, incubated to allow meiotic maturation, and then inseminated. As observed using ovulated eggs, very few oocytes from the *Fshb*$^{-/-}$ mice reached the 2-cell stage (data not shown).

TABLE 2. Embryonic development of oocytes from *Fshb*$^{+/-}$ and *Fshb*$^{-/-}$ mice following IVM and ICSI.

	Fshb$^{+/-}$		*Fshb*$^{-/-}$	
Parameter	No eCG	eCG	No eCG	eCG
No. of metaphase II oocytes*	106	128	84	153
No. of 1-cell embryos post-ICSI	94	114	71	137
No. of 2-cell embryos (% of 1-cell)†	64 (68.1)[a]	97 (85.1)[b]	39 (54.9)[a]	100 (73.0)[b]
No. of blastocysts (% of 2-cell)†	7 (10.9)[a]	28 (28.9)[b]	1 (2.6)[a]	21 (21.0)[b]

* Data are the sum of four independent experiments.
† Effect of eCG on the fraction of eggs that reached the 2-cell stage and fraction of 2-cell embryos that reached the blastocyst stage for each genotype was tested using the Fisher exact test.
[a,b] Different superscript letters within each genotype indicate a significant difference ($P < 0.05$).

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In view of the well-established effects of FSH on GC differentiation, we suspected that the poor development at the 2-cell stage might reflect an abnormality in the GCs that inhibited fertilization. To bypass this potential block, we collected and matured oocytes as described above and fertilized them using ICSI. Fertilization by ICSI dramatically increased the fraction of oocytes from *Fshb*$^{-/-}$ animals that reached the 2-cell stage (Table 2). Very few of these embryos, however, were able to reach the blastocyst stage. Because development to the blastocyst stage was low even using *Fshb*$^{+/-}$ animals, we then tested whether this could be improved by administering eCG 48 h before collection of immature oocytes. This procedure provided FSH activity during the 2 days of oocyte development preceding meiotic maturation. eCG administration significantly increased the percentage of eggs that reached the 2-cell stage and the blastocyst stage. Moreover, this effect was observed using both *Fshb*$^{+/-}$ animals and *Fshb*$^{-/-}$ animals. Taken together, these results imply that FSH promotes events in the developing oocyte that increase its ability to undergo embryonic development and that these FSH-dependent changes occur during the late stages of oocyte development preceding meiotic maturation.

DISCUSSION

Our results clearly establish the fact that morphologically normal oocytes can develop in the absence of FSH, thus establishing that many hallmarks of oocyte development can be achieved independently of FSH in vivo. Certain of these characteristics, including the increase in oocyte size, accumulation of mtDNA and acquisition of meiotic competence, normally occur during preantral growth. Previous studies have shown that when GOCs at mid-growth phase were removed from the ovary and placed in culture, oocyte development could progress in the absence of FSH [36, 37, 44]; however, these studies could not exclude an earlier or in vivo role for FSH. Our results support findings from these in vitro studies and extend the concept that the early development of the follicle does not require gonadotropins to include the germ cell compartment. In contrast, the NSN-SN transition normally occurs during the FSH-dependent period of follicular somatic-cell development [53–55]; hence, our results indicate that oocyte development can continue independently of FSH during late folliculogenesis in vivo.

Although FSH is not required, it nonetheless appears to accelerate aspects of oocyte development, specifically the NSN-SN transition and the acquisition of meiotic competence. The mechanism of this acceleration is unknown but presumably is mediated through the GCs. Previous results have shown that in mice lacking the oocyte-secreted growth factor GDF9, the ovarian follicles contain a reduced number of GCs and oocytes do not acquire meiotic competence [56, 57]. This correlation raises the possibility that the rate at which oocytes progress through development is influenced by the number of GCs that surround them. Hence, FSH might accelerate oocyte development in part through its activity to promote granulosa cell proliferation.

FSH might also stimulate the expression of specific genes required for oocyte development. KITL has been proposed to promote oocyte growth (for reviews, see refs. 3 and 58; but see ref. 59), and it has previously been reported that FSH stimulates *Kitl* expression in GCs [34, 49] and that *Kitl* is reduced in RNA purified from whole ovaries of *Fshr*$^{-/-}$ animals [28]. We observed, however, that *Kitl* levels were not detectably altered in the GCs of *Fshb*$^{-/-}$ animals. These apparently conflicting results, however, can be reconciled. First, although FSH increases *Kitl* expression when GOC shells from which the oocyte has been removed are cultured, this effect does not occur when the GOC shells are cocultured with fully grown oocytes [49]. This implies that FSH does not regulate *Kitl* expression in intact follicles containing an oocyte, consistent with our results. Second, we found that *Fshb*$^{-/-}$ ovaries contained fewer growing follicles than wild-type ovaries. If the same is true for *Fshr*$^{-/-}$ ovaries, then the reduced *Kitl* expression in whole-ovary extracts may reflect the possibility that these contain fewer growing follicles. In support of this possibility, the quantity of *Zp3*, which is expressed by growing oocytes, was reduced to the same extent as *Kitl* in *Fshr*$^{-/-}$ ovaries [28]. Based on these considerations, we propose that FSH is not required for *Kitl* expression in vivo.

Despite the morphologically normal appearance of the oocytes of *Fshb*$^{-/-}$ mice, they developed very poorly as embryos. Their developmental competence—specifically, the fraction of embryos that could reach the blastocyst stage—was improved by providing FSH activity via injection of eCG during the final 2 days of development preceding meiotic maturation. The same beneficial effect of injected eCG was also observed in wild-type mice and has been reported by others [60]. Thus, increasing the amount of FSH activity during the final period of growth in vivo increases the number of oocytes that are capable of developing as embryos. These results are consistent with the observations in many species that oocytes acquire developmental competence during late antral folliculogenesis and imply that FSH plays some role in this process [8]. Two observations suggest that this role of FSH, however, is not indispensable. First, in the absence of eCG priming, a small number of oocytes in our experiments could reach the blastocyst stage. Second, healthy pups have been obtained from oocytes that were grown in vitro (beginning at mid-growth) in the absence of FSH. Thus, the acquisition of developmental competence is not absolutely dependent on FSH. Taken together with our observation that hallmarks of oocyte development were achieved more rapidly in the presence of FSH, these observations suggest that oocytes may acquire developmental competence more rapidly or more efficiently when FSH signaling is active. This FSH effect might be mediated directly, such as by regulating oocyte-granulosa cell adhesion or gap junctional communication [26], or indirectly through its well-known activity to protect antral GCs from undergoing apoptosis [61].

Our results together with those of others indicate that many and possibly all events of oocyte development can occur independently of FSH. However, these events occur more rapidly or more efficiently in its presence. We suggest that FSH may serve to regulate the rate of oocyte development in vivo, integrating it with development of the surrounding somatic cells of the follicle. This activity of FSH would ensure that the developing oocyte has completed its growth and become sufficiently competent to give rise to a healthy embryo at the same time as the follicle has completed its development and is able to ovulate the egg in response to appropriate hormonal signals. Alternatively, some oocytes may become developmentally arrested rather than simply delayed in the absence of FSH, depending on whether they achieve sufficient interaction with and support from the GCs. As discussed previously, we interpret our results favor a delay; however, it is plausible that oocytes lacking sufficient granulosa support never acquire full developmental competence.

Our results also have potential implications for the treatment of human infertility. In order to avoid the potential risks of ovarian hyperstimulation, there has been an increasing interest in performing in vitro maturation (IVM) of oocytes in an

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unstimulated menstrual cycle [62–65]. However, the clinical pregnancy and live-birth rates per treatment cycle are much lower using IVM than using conventional stimulated IVF [66–68]. Our results suggest that the targeted administration of FSH just before immature oocyte retrieval may help to increase the success rates of IVM-based treatments.

ACKNOWLEDGMENT

We thank Dr. D. Dankort for suggestions for the manuscript.

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