Subfertility Caused by Altered Follicular Development and Oocyte Growth in Female Mice Lacking PKBalpha/Akt1

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ABSTRACT

Mammalian females are endowed with a finite number of primordial follicles at birth. Immediately following formation of the primordial follicle pool, cohorts of follicles are either culled from the ovary or are recruited to grow until the primordial follicle population is depleted. The majority of ovarian follicles, including the oocytes, undergo atresia through apoptotic cell death. As PKBalpha/Akt1 is known to regulate apoptosis, we asked whether Akt1 functioned in the regulation of folliculogenesis in the ovary. Akt1−/− females display reduced fertility and abnormal estrous cyclicity. At Postnatal Day (PND) 25, Akt1−/− ovaries possessed a reduced number of growing antral follicles, significantly larger primary and secondary oocytes, and an increase in the number of degenerate oocytes. By PND90, there was a significant decrease in the number of primordial follicles in Akt1−/− ovaries relative to Akt1+/+. In vivo granulosa cell proliferation was reduced, as were expression levels of Kitl and Bcl2l1, two factors associated with granulosa cell proliferation/survival. No compensation was observed by Akt2 or Akt3 at the mRNA/protein level. Significantly higher serum LH and trends for lower FSH and higher inhibin A concentrations during folliculogenesis, and spikes to promote ovulation [4]. In contrast, the primordial to primary follicle transition is independent of these hormones [5].

Recent evidence implicates the PTEN/PIK3/PDK1/AKT signaling pathway in the regulation of primordial follicle activation [6]. Mice deleted for the oocyte-specific expression of PTEN, the phosphatase and tensin homolog tumor suppressor gene, exhibit premature ovarian failure (POF) due to the rapid activation and subsequent depletion of the primordial follicle pool [6]. In addition to PTEN-deficient mice, female mice deficient for 3-phosphoinositide-dependent protein kinase (PDK) 1 also exhibit female infertility, with significant primordial follicle loss around the onset of sexual maturity causing POF during early adulthood [7]. Another downstream target of Akt1, Foxo3a null mice exhibit increased primordial follicle activation resulting in complete follicle loss by 15 wk of age [8]. Therefore, the evidence indicates that the PTEN/PIK3/PDK1/AKT signaling pathway is a key pathway in the regulation of female fertility.

The phosphoinositide 3-kinase signaling pathway is a critical regulator of cellular processes, such as cell growth, proliferation, differentiation, and survival. These actions are achieved by phosphorylation and the subsequent activation of effector proteins, one of which is the prosurvival protein kinase, AKT. AKT and PDK1 are recruited to the membrane via their pleckstrin homology domains, and PDK1 subsequently phosphorylates the kinase domain of AKT1 at Thr308, which is required for AKT activation. A second serine residue, Ser473, is contained in the regulatory domain, and must be phosphorylated in order for AKT to exhibit full kinase activity [9].

Targeted disruption of the PKB/Akt gene family has provided valuable insights into the role of these proteins in both development and disease. Homozygous disruption of Akt1 results in growth retardation and increased apoptosis in several tissue types [10, 11]. Mice that harbor a deletion of the Akt2 gene demonstrate insulin resistance and a diabetes mellitus-like syndrome [12, 13]. Homozygous deletion of both Akt1 and Akt2 in mice leads to dwarfism, impaired skin development,
skeletal muscle atrophy, delayed bone development, and impeded adipogenesis [14]; in addition, dosage-dependent effects are observed in the thymus and skin, and in the cardiovascular and nervous systems, in mice with homozygous disruptions in both Akt1 and Akt3 [15]. In addition, Akt3 has been shown to be instrumental in the attainment of normal brain size [16, 17].

Despite intense research on the biological functions of the PKB/Akt gene family, relatively little is known about its role in female reproductive physiology. Akt1+/− males are fertile, but are more sensitive to both genotoxic and nongenotoxic insults [18, 19]. Akt1+/− female mice have been reported to have reduced fertility, whereas Akt3 females are reported to have normal fertility [20]. It is unknown as to whether Akt2 female mice have reduced fertility. In the ovary, Akt1 has been localized in porcine granulosa cells of primordial follicles and in the basal layers of the granulosa cells of preantral and antral follicles, but not in atretic follicles or corpora lutea [21]. In rodents, Akt1 is found in both granulosa cells of the ovary and in oocytes [22, 23]. In the human ovary, Akt1 expression is found in oocytes, in granulosa cells, and in the thecal cells of primordial follicles, in follicles at each growing stage, and in the luteal cells [24].

In the present study, we investigate the effect of Akt1 deletion on female reproductive physiology in the mouse. We found that Akt1+/− females display reduced fertility, a delay in the onset of estrus, an increase in maternal age at first litter, and a reduction in average litter size. At Postnatal Day (PND) the onset of estrus, an increase in maternal age at first litter, growing early antral and antral follicles. The oocytes of

In approximately every 10th ovarian section, the numbers of primordial, primary, early antral, and healthy antral follicles were counted. Only follicles involved in porcine granulosa cells of primordial follicles and in the basal layers of the granulosa cells of preantral and antral follicles, but not in atretic follicles or corpora lutea [21]. In rodents, Akt1 is found in both granulosa cells of the ovary and in oocytes [22, 23]. In the human ovary, Akt1 expression is found in oocytes, in granulosa cells, and in the thecal cells of primordial follicles, in follicles at each growing stage, and in the luteal cells [24].

In the present study, we investigate the effect of Akt1 deletion on female reproductive physiology in the mouse. We found that Akt1+/− females display reduced fertility, a delay in the onset of estrus, an increase in maternal age at first litter, and a reduction in average litter size. At Postnatal Day (PND) 25, Akt1+/− ovaries contain a significantly reduced number of growing early antral and antral follicles. The oocytes of primary follicles of Akt1+/− females are larger than Akt1+/+ animals with some follicles exhibiting multiple oocytes, suggesting a defect in cyst breakdown. Akt1+/− ovaries possessed a reduced number of mature follicles, decreased in vivo granulosa cell proliferation in secondary follicles, and an increase in the number of degenerate oocytes. By PND90, there was a significant decrease in the number of primordial follicles in Akt1+/− females relative to their Akt1+/+ counterparts. At the molecular level, decreased expression of the cell cycle regulator, Ccnd3, and a trend for decreased Ccnd1 was observed. The KITL survival factor, which is expressed primarily by the granulosa cells, was significantly reduced in the Akt1+/− ovary, and decreased expression of the antiapoptotic factor, Bcl2l1, was also observed. Taken together, our data reveal that Akt1 deletion produces changes in the ovary and oocyte, which lead to infertility and the onset of POF.

**MATERIALS AND METHODS**

**Mice**

Akt1 heterozygous breeding pairs in a C57BL/6 background were obtained from the laboratory of Dr. Morris Birnbaum (University of Pennsylvania, Philadelphia, PA). Heterozygous pairs were mated to obtain Akt1−/−, Akt1+/+, and Akt1+/− females for experimental procedures. The frequency of obtaining Akt1−/− mice is approximately 17.3 percent, which is less than the expected Mendelian frequency of 25%. Experiments were conducted at PNDs 25, 50, 56, and 90. The animal room climate was kept at a constant temperature (23.3 ± 2°C) at 30%–70% humidity, with an alternating 12L:12D cycle. All procedures involving animals were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of Brown University in compliance with the guidelines established by the National Institutes of Health.

**Primers**

For genotyping by PCR, the following primers were used in a single reaction: 853, 5′-GGTGATGGAAATGGTGCCGAG-3′; 854, 5′-GCTCAGTGACAGGCGACCAG-3′; 855, 5′-CACCCCCAACAGCTCT- TCTTCCA-3′. The PCR was run with an initial denaturing step of 94°C for 5 min, 39 cycles of 94°C for 30 sec, 63°C for 30 sec, 72°C for 45 sec, followed by a final extension at 72°C for 5 min. For PCR genotyping of progeny, the wild-type and targeted bands were 310 and 194 bp, respectively.

**Fertility Analysis**

To evaluate the reproductive performance of Akt1−/− mice, seven individually housed Akt1−/− females were bred to Akt1+/+ males of proven fertility. Akt1−/− females were used as controls. The females were introduced to the males at 7 to 8 wk of age, and the number of litters and the number of pups were recorded over a minimum 6-mo period.

**Assessment of Pubertal Onset and Estrous Cyclicity**

To assess the onset of puberty in juvenile females, mice were observed daily for signs of vaginal opening. The onset of estrus was also monitored by daily vaginal lavage of females in the morning, for a period of at least 1 mo after vaginal opening based on vaginal cytology, according to the classification of Pedersen [25].

**Classification of the Estrous Cycle and Tissue Collection**

To examine the influence of genotype on the length of the estrous cycle, vaginal smears from wild-type and Akt1−/− deficient female mice at 12 wk of age were monitored daily between 0800 and 1000 h. The mouse’s tail was raised and a 20-µl pipette tip filled with sterile PBS1X was inserted into the vagina and washed up and down. When the tip was removed, it was dropped onto a microscope slide. The smears were classified into one of four phases of estrus: elongated nucleated epithelium indicated proestrous; large cornified epithelial cells were exclusively found in estrus; metestrus was marked by a thick smear composed of equal numbers of nucleated epithelial cells and leukocytes; and a smear consisting almost exclusively of leukocytes depicted diestrus [26]. Each cycle length was determined as the length of time between two consecutive occurrences of estrus. After the mice had progressed through at least three consecutive estrous cycles, the length of the estrous cycle and the number of days spent at each stage of the cycle were evaluated.

**Ovarian Histology**

To determine whether lack of Akt1 influences ovarian development, ovaries from a minimum of three mice per genotype were collected and examined at PNDs 25 and 90. Both ovaries were removed, cleaned of extraneous tissue, weighed, and fixed in Bouins. Tissues were then embedded in paraffin and serial sections (7-µm thick) of ovaries were stained with periodic acid-Schiff (PAS).

**Assessment of Follicle Development**

In approximately every 10th ovarian section, the numbers of primordial, primary, early antral, and healthy antral follicles were counted. Only follicles containing an oocyte with a visible nucleus were counted to avoid double counting, and all counting was done without the knowledge of genotype. Follicles were counted as primordial if they contained an oocyte surrounded by flattened granulosa cells, or a mixture of less than seven flattened and cuboidal granulosa cells. Follicles were counted as primary if they contained an oocyte surrounded by a single layer of seven or more cuboidal granulosa cells. Secondary follicles were those containing an oocyte surrounded by two or more complete layers of granulosa cells. Early antral follicles were those with more than four complete layers of granulosa cells and an antrum <20 µm in diameter. Antral follicles were considered as those that had a visible antrum without a stalk. Graafian follicles were those with a large antrum, cumulus oophorus, and a corona radiata. Antral follicles were considered to be healthy if they had an intact oocyte and <10% pyknotic granulosa cells. Antral follicles were considered to be atretic if there were more than 10% pyknotic granulosa cells.

**Superovulation**

Female mice at 24–26 days of age were injected i.p. with 5.0 IU of ECG followed by 5.0 IU of hCG i.p. 48 h later. Oviducts and ovaries were excised from mice 18 h after hCG administration, and were fixed in 10% neutral formalin. Tissues were then embedded in paraffin and serial sections (7-µm thick) of ovaries were stained with PAS. For analysis of the number of ovarian follicles, every sixth section was mounted and stained with hematoxylin and eosin.
Akt1 cycling levels, blood samples were collected by cardiac puncture from randomly genotyped mice. A total of 87 and 118 primary follicles were measured from Akt1+/+ and Akt1−/− mice, respectively. Follicles were counted as primary if they contained an oocyte surrounded by a single layer of seven or more cuboidal granulosa cells. To prevent counting the same oocyte twice, oocytes were only measured when there was a visible nucleolus. Measurements were then averaged and expressed as the diameter of the oocyte.

Secondary Oocyte Diameter

Ovaries from mice at PND25 were examined (n = 3–4 ovaries per genotype). A total of 55 and 66 secondary follicles were measured from Akt1+/+ and Akt1−/− mice, respectively. Follicles were counted as secondary if they contained an oocyte surrounded by two or more complete layers of granulosa cells and no visible antrum formation. To prevent counting the same oocyte twice, oocytes were only measured when there was a visible nucleolus. Measurements were then averaged and expressed as the diameter of the oocyte.

Measurements of Hormone Levels

To measure serum FSH, LH, inhibin A and B, progesterone, and estradiol levels, blood samples were collected by cardiac puncture from randomly cycling Akt1+/+ and Akt1−/− female mice at specific ages. The serum was stored at −80°C until assayed. FSH, LH, inhibin A, B, progesterone, and estradiol levels were assayed by the University of Virginia Ligand Core Facility as described (http://www.healthsystem.virginia.edu/internet/ocr/lgand.cfm; Specialized Cooperative Center Program in Reproductive Research, National Institute of Child Health and Human Development/National Institutes of Health U54 HD28934).

Bromodeoxyuridine Incorporation

Assessment of granulosa cell proliferation by bromodeoxyuridine (BrdU) incorporation was performed. Hormonally primed mice (5 IU eCG) were injected intraperitoneally with 1 μg/g BrdU and killed after 1 h. The ovaries were dissected, fixed in formalin, and 7-μm serial sections were generated. Primary monoclonal anti-BrdU antibody (Dako) was used at a 1:100 dilution. After counterstaining with methyl green, the number of BrdU-positive cells per follicle was determined to compare in a minimum of five follicles per experimental animal. Secondary follicles were analyzed in the cE7-treated groups.

Immunohistochemistry

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded 7-μm sections using the VectaStain Elite Avidin-Biotin Complex Kit, as directed by the manufacturer (Vector Labs, Burlingame, CA). Sections were probed with primary antibodies against CCND1 and CCND3 both from Cell Signaling (Beverly, MA) and CCND2 from Santa Cruz Research (Santa Cruz, CA). Sections were visualized using a 3,3′-diaminobenzidine peroxidase Substrate Kit (Vector Labs). Digital images were captured using the Aperio Scanscope Imaging System (Vista, CA).

Western Blot Analysis

Ovaries were lysed by homogenization in cold lysis buffer (50 mM Tris 8.0, 250 mM NaCl, 1% NP-40, 0.1% SDS, 5 mM EDTA, 2 mM Na3VO4, 10 mM Na2P2O7, 10 mM NaF) supplemented with 1 mM PMSF and 40 μl/ml Complete Protease Inhibitor Cocktail (Roche, Indianapolis, IN). Lysates were incubated on ice for 20 min with frequent vortexing and cleared by centrifugation (10000 rpm, 10 min, 4°C). Protein concentrations were determined using the DC Protein assay (Bio-Rad, Hercules, CA). Total protein (50 μg) was subjected to SDS-PAGE and transferred onto Immobilon-P polyvinylidene fluoride (Millipore, Billerica, MA). Membranes were blocked for 60 min at room temperature in 5% nonfat milk/Tris-buffered saline/0.1% Tween (TBST). Membranes were incubated for 2 h at room temperature, then overnight at 4°C in the following antibodies: phospho-AKT (Thr308) (#9275, 1:5000 in 5% milk/TBST), phospho-AKT (Ser473) (#9271, 1:1000 in 5% milk/TBST), AKT1 (#2967, 1:1000 in 5% milk/TBST), AKT2 (#2964, 1:1000 in 5% milk/TBST), AKT3 (#4059, 1:1000 in 5% milk/TBST). All antibodies were obtained from Cell Signaling Technology (Danvers, MA). Membranes were washed three times (5 min/wash) in TBST, and incubated for 60 min at room temperature in horseradish peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (Cell Signaling, diluted 1:2000 in 5% milk/TBST). Membranes were washed three times (5 min/wash) in TBST, and once in TBS prior to visualization using enhanced chemiluminescence (GE Healthcare, Piscataway, NJ).

Quantitative RT-PCR

Total RNA (1 μg) was DNase-I (Inviogene, Carlsbad, CA) treated and reverse transcribed using iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer’s protocols, and the cDNA templates were amplified with each of the primer pairs in independent sets of PCR using iQ SYBR Green Supermix (Bio-Rad) on an iCycler iQ Multicolor Real-Time PCR Detection System (Bio-Rad). The concentration of each cDNA sample was determined by gel electrophoresis, and a standard curve of amplification was generated using a serial dilution of each cDNA. Amplification efficiency was determined using the LinRegPCR (version 6.0) software program. The relative levels of Akt1 mRNA were normalized to the level of expression of β-actin, and the relative expression of Akt1 mRNA was calculated using the ΔΔCt method.

Table 1. Effect of Akt1 null mutation on female fertility.

| Genotype | N    | Litter | Total pups | Pups/litter | Litters/month | Days to vaginal opening | Age at first estrous cycle (days) | Age at first litter (days) |
|----------|------|--------|------------|-------------|---------------|------------------------|---------------------------------|--------------------------|
| Akt1+/+  | 5    | 18     | 103        | 5.81 ± 0.6  | 0.644 ± 0.1   | 33 ± 1.5 (N = 4)        | 39.8 ± 1.1 (N = 3)             | 56 ± 5                   |
| Akt1−/−  | 8    | 5      | 20         | 3.56 ± 0.8  | 0.112 ± 0.1*  | 33.2 ± 1.4 (N = 10)     | 44.8 ± 0.6* (N = 3)            | 100 ± 7.2*               |

* Values represent the mean ± SEM; statistical analyses were conducted using Student t-test (P < 0.05).

* Significant differences observed between Akt1+/+ and Akt1−/− animals.

FIG. 1. Akt1 influences estrus cycle phase in female mice at PND90. A) Graph depicts the mean estrous cycle length of Akt1+/+ (black bar), Akt1+/− (light gray bar), and Akt1−/− (dark gray bar) female mice. B) Graph shows the average number of days that each genotype stayed within individual stages of the estrous cycle (P < 0.05). Asterisk indicates significant differences observed between Akt1+/+ and Akt1−/− animals.
Mouse-specific primers were designed using Molecular Beacon Design 4.0 Software (Bio-Rad). Specific primer sequences for each gene for quantitative RT-PCR were as follows: 

**Kitl**
- forward, 5'-ACAGCAGTAGAATATAGG-3'
- reverse, 5'-GACTTGACTGTTTCTTCTTCC-3';

**Bcl2l1**
- forward, 5'-CCGCTGTGTCTCTGGGTCTC-3'
- reverse, 5'-GGTTCTGGTCCTTGTCTCATTATCC-3';

**Ccnd1**
- forward, 5'-ATGTTGTACCAGAAGAGGAAG-3'
- reverse, 5'-TCAGATAGAAAGFIG. 2. Effect of Akt1 null mutation on ovarian follicle development at PND25. A) Akt1+/+ ovary at PND25. B) Akt1-/- ovary at PND25. C) Follicle counts of Akt1+/+ and Akt1-/- females at PND25 (n = 3). The number of primordial (PF), primary (10), secondary (20), early antral (EAF), antral (AF), and mature Graafian follicles (GF) were determined in Akt1+/+ and Akt1-/- at PND25. Ovaries were serially sectioned, every 10th section was counted, and the total follicle numbers were determined. The total numbers of primordial, primary, secondary, early antral, antral, and mature follicles were multiplied by a factor of 10 to obtain an estimate of the total number of follicles per ovary. These data represent the mean ± SEM of combined results from the analysis of three mice per age group (P < 0.05). Asterisks indicate significant differences in follicle number observed between Akt1+/+ and Akt1-/- animals; bar = 200 μm.

FEMALE SUBFERTILITY IN MICE LACKING PKBα/Akt1

FIG. 3. Responsiveness of Akt1 null mutant mice to exogenous gonadotropins at PND25. Histology of ovaries from Akt1+/+(A) and Akt1-/- (B) animals exposed to exogenous gonadotropins. Female mice at 24–26 days of age were injected i.p. with 5.0 IU of ECG followed by 5.0 IU of hCG i.p. 48 h later. Oviducts and ovaries were excised from mice 18 h after hCG. C) The number of primordial (PF), primary (10), secondary (20), early antral (EAF), antral (AF), and mature Graafian follicles (GF) were determined in Akt1+/+ and Akt1-/- at PND25 following exposure to exogenous gonadotropins. Ovaries were serially sectioned, every sixth section was counted, and the total follicle numbers were determined. The total numbers of primordial, primary, secondary, early antral, antral, and mature follicles were multiplied by a factor of six to obtain an estimate of the total number of follicles per ovary. These data represent the mean ± SEM of combined results from the analysis of three mice per age group (P < 0.05). Asterisks indicate significant differences in follicle number observed between Akt1+/+ and Akt1-/- animals; bar = 200 μm.
Inhibin B (pg/ml) 57.4
Inhibin A (pg/ml) 99.8
LH (ng/ml) 0.25
Progesterone (ng/ml) 3.0
Estradiol (pg/ml) 7.0

Akt1

The average maternal age for production of the first litter for Akt1 females bred to Akt1 males was 0.64 days, respectively (Table 1). The number of pups per litter was significantly less fertile relative to Akt1/+ female mice, with 3.6 males. At PND90, the average number of litters per month was 0.1 (n = 8). However, sexual maturity, as determined by first proestrus, was significantly delayed in Akt1 females relative to Akt1/+ females (PND33.0 ± 1.5; n = 4). The maternal age at first litter was significantly higher in Akt1 females relative to Akt1/+ counterparts (100 ± 7 vs. 56 ± 5 days, respectively).

Effect of Akt1 Null Mutation on Female Fertility

When Akt1−/− female mice were mated to Akt1+/+ males of proven fertility, we observed the Akt1−/− females to be significantly less fertile relative to their Akt1+/+ female counterparts (P < 0.05) (Table 1). This subfertility included both a reduction in the overall number of litters and the number of pups per litter in the Akt1−/− females (Table 1). The average number of litters per month for Akt1+/+ females bred with Akt1+/− males was 0.64 ± 0.1 (n = 5), and for Akt1−/− females bred to Akt1+/− males, the average number of litters per month was 0.11 ± 0.1 (n = 8). The number of pups per litter was significantly reduced in Akt1−/− female mice compared to Akt1+/+ female mice, with 3.6 ± 0.8 pups per litter (n = 8) and 5.8 ± 0.5 pups per litter (n = 5), respectively (Table 1). The average maternal age for production of the first litter for Akt1+/+ and Akt1−/− females was 56 ± 5 days and 100 ± 7 days, respectively (P < 0.001).

Comparison of Akt1+/+, Akt1−/−, and Akt1−/− Female Body and Ovarian Weights

To characterize the female reproductive physiology of the Akt1−/− female mice, body weights and ovarian weights were compared with those of Akt1+/+ females at PNDs 25, 56, and 90 (Table 2). At all time points examined, significant decreases in the body weights of the Akt1−/− mice were observed compared with that of Akt1+/+ female mice. At PND56, Akt1−/− female mice exhibited an approximate 20% decrease in body weight and, at PND90, there was a 12% decrease in body weight relative to Akt1+/+ counterparts. Similar to body weight, there was a significant decrease in ovarian weights in the Akt1−/− mice at both PND56 and PND90 (Table 2). Ovarian weights at PND25 were reduced by 27.4% relative to Akt1+/+ mice, and, at PND90, ovarian weights were reduced by 20.6% in Akt1−/− mice relative to the Akt1+/+ littersmates. However, when adjusted for ovarian weight relative to body size (i.e., absolute ovarian weight), no differences were observed in Akt1+/+ and Akt1−/− females (Table 2).

Effect of Akt1 Null Mutation on Sexual Maturation

Due to the significant decrease in body size of the Akt1−/− mice, we determined if there was a delay in sexual maturity in the Akt1−/− females by assessing the effect of Akt1 deletion on the age at pubertal onset and estrous cyclicity. As shown in Table 1, vaginal opening was not significantly delayed in Akt1−/− females (PND33.2 ± 1.4; n = 10) when compared with Akt1+/+ females (PND33.0 ± 1.5; n = 4). However, sexual maturity, as determined by first proestrus, was significantly delayed in Akt1−/− females (PND44.8 ± 0.6) compared with Akt1+/+ females (PND39.8 ± 1.1; n = 3), and the maternal age at first litter was significantly higher in Akt1−/− females relative to Akt1+/+ counterparts (100 ± 7 vs. 56 ± 5 days, respectively).

Extension of the Diestrous Cycle in Mature Akt1−/− Female Mice

Due to the later age at first proestrus and the later maternal age for first litter in the Akt1−/− mice, we examined the length of the estrous cycle of both Akt1+/+ female mice and Akt1−/− female mice at PND90. This age was examined because live litters are observed at this time in Akt1−/− females. At PND90,
Akt1−/− females displayed abnormal cycling patterns, although the average cycle length was similar in Akt1+/+, Akt1+/−, and Akt1−/− mice (4.5 ± 0.2 days). Analysis of the estrous cycle profiles in Akt1−/− female mice and Akt1+/− control mice did reveal a significantly longer diestrus phase in Akt1−/− females of approximately 1.5 days longer in duration relative to Akt1+/− female mice (P < 0.05) (Fig. 1).

**Effect of Akt1 Null Mutation on Follicle Development in Mice at PND25**

To determine the effect of Akt1 deletion on folliculogenesis, we performed histological and morphometric analyses of follicle numbers at PND25, and compared them between Akt1+/+ and Akt1−/− mice. As expected, Akt1−/− females revealed follicles at all developmental stages (primordial, primary, secondary, and antral follicles) and corpora lutea (Fig. 2A). In contrast, the ovaries of primary, secondary, and antral follicles) and corpora lutea revealed follicles at all developmental stages (primordial, preantral, and antral follicles) and corpora lutea (Fig. 2A). In contrast, the ovaries of Akt1+/+ mice contained fewer antral follicles compared with Akt1+/− controls. In Akt1−/− ovaries, we observed follicles with multiple oocytes, as depicted in Figure 2B (dashed circle). Only once did we observe Akt1+/+ females with more than one oocyte per follicle. The increase in multiple oocytes per follicle may indicate a defect in cyst breakdown in the Akt1−/− ovary. Figure 2C shows follicle numbers at PND25 for both Akt1+/+ and Akt1−/− animals. The number of primordial and primary follicles was not significantly different in Akt1−/− ovaries when compared to Akt1+/+ ovaries; however, the number of preantral follicles in Akt1−/− mice was significantly lower than in Akt1+/+ ovaries, as was the number of antral and mature Graafian follicles (Fig. 2C), with a 2-fold increase in the number of atretic follicles with degenerate oocytes (Fig. 2C).

**Effect of Akt1 Null Mutation on Oocyte Size**

Unexpectedly, we found the diameters of both primary and secondary oocytes to be larger in Akt1−/− mice. Primary oocytes were found to have an average diameter of 27.9 ± 1.0 μm versus 22.4 ± 1.0 μm in Akt1+/+ female primary oocytes. Secondary oocyte diameters were found to be 50.0 ± 1.0 μm in Akt1+/− and 53.0 ± 1.0 μm in Akt1−/− ovaries.

**Responsiveness of PND25 Akt1 Null Mutant Mice to Exogenous Gonadotropins**

Due to the decrease in the number of mature follicles in Akt1−/− mice, we examined whether Akt1 is involved in the maturation process of follicles by comparing the number of total mature follicles in immature mice after eCG/hCG treatment. As shown in Figure 3C, the number of secondary follicles in the ovaries of Akt1−/− mice after eCG/hCG treatment was 2-fold higher than that of Akt1+/+ mice; however, fewer mature follicles were observed, suggesting that follicular maturation is indeed impaired in Akt1−/− mice.

**Effect of Akt1 Null Mutation on Ovarian Steroid Synthesis in Female Mice at PND50**

Both the inability to obtain a significant number of fertile Akt1−/− females and the decrease in the number of preantral and antral follicles in the Akt1−/− females led us to examine the levels of FSH, estradiol, progesterone, and LH in the serum of these mice at PND50. As depicted in Table 3 at PND50, Akt1+/− mice showed a trend for lower levels of FSH. FSH levels in Akt1+/− mice were 8.0 ± 1.6 ng/ml (n = 6) and 7.32 ± 1.3 ng/ml (n = 6) in Akt1+/− mice versus 4.8 ± 1.1 ng/ml (n = 8) in Akt1−/− females. Estradiol levels were found to be elevated in Akt1−/− mice (12.2 ± 3.0 pg/ml) relative to Akt1+/+ mice (7.0 ± 3.2 pg/ml). Significantly higher levels of LH were found in Akt1−/− females at PND50, with serum levels of 0.91 ± 0.3 ng/ml (n = 8) versus 0.25 ± 0.04 ng/ml (n = 11) in Akt1+/− female mice and 0.38 ± 0.15 ng/ml (n = 6) in Akt1+/− female mice. Progesterone levels were found to be lower in Akt1−/− females.
(1.1 ± 0.1 ng/ml) (n = 18) relative to Akt1+/+ females (3.0 ± 0.8 ng/ml) (n = 12). Interestingly, there was a trend toward higher inhibin A levels at PND50 in the Akt1–/– mice than in the Akt1+/+ mice (114 pg/ml ± 19.5 [n = 8] versus 99.8 ± 21.8 pg/ml [n = 4], respectively), and there was a trend toward reduced inhibin B serum levels in the Akt1–/– mice relative to Akt1+/+ mice (33.5 ± 11.0 pg/ml [n = 8] versus 57.4 ± 16.6 pg/ml [n = 8], respectively).

Analysis of Estradiol and Progesterone in Akt1–/– Females at PND90

Because Akt1–/– females are capable of delivering pups to term, we examined the levels of estradiol and progesterone at PND90. We found both estradiol and progesterone levels to be similar in both Akt1–/– and Akt1+/+ ovaries at this time point (Table 4). It should be noted that, on several occasions, we observed that Akt1–/– females exhibit weight gain as if pregnant with no pups delivered, suggesting that Akt1 may serve other functions in female reproduction, including placentation, parturition, and/or lactation.

Folliculogenesis in Akt1–/– Females at PND90

To determine the effect of Akt1 deletion on folliculogenesis at PND90, we performed histological and morphometric analyses of follicle numbers and compared them between Akt1+/+ mice (Fig. 4A) and Akt1–/– mice (Fig. 4B). Both Akt1+/+ and Akt1–/– females revealed follicles at all developmental stages (primordial, primary, secondary, and antral follicles) and corpora lutea (Fig. 4, A and C). In contrast, the ovaries of Akt1–/– mice contained significantly fewer primordial follicles compared with Akt1+/+ controls (Fig. 4, B and C). The number of primary and secondary follicles was not significantly different in Akt1–/– ovaries when compared to Akt1+/+ ovaries. Mature Graafian follicles were observed in both genotypes; however, fewer Graafian follicles were observed in the Akt1–/– ovaries.

Effect of Akt1 Null Mutation on Granulosa Cell Proliferation In Vivo

To better understand the cellular basis of the observed folliculogenesis defects in the Akt1–/– ovaries, we assessed the proliferation of granulosa cells in the presence versus absence of Akt1. BrdU incorporation in vivo was utilized to directly label granulosa cells in the S phase of the cell cycle and measure granulosa cell proliferation. At 24 h prior to BrdU labeling, matched 21-day-old Akt1+/+ and Akt1–/– female mice were stimulated with a single injection of eCG to stimulate proliferation of follicular granulosa cells. Ovaries were harvested the next day following 1 h in vivo BrdU treatment, and ovary sections generated from these mice were labeled with an anti-BrdU antibody. BrdU incorporation was readily detectable in granulosa cells derived from both Akt1+/+ mice (Fig. 5A) and Akt1–/– mice (Fig. 5B). However, there was a reduction in the number of positively stained granulosa cells labeled with BrdU in the Akt1–/– ovaries in response to hormonal treatment (Fig. 5C).

Neither Akt2 nor Akt3 Compensate for Akt1 Deficiency at the mRNA or Protein Level

Although the Akt gene family is implicated in a survival response to genotoxic injury in multiple cell types, it is not known to what degree there is functional overlap between these family members in the ovary. We first examined the activation of Akt in the ovary at PND50, and found decreased phosphorylation at the threonine 308 site in Akt1–/– ovaries (Fig. 6A), but not at Ser473 (Fig. 6A). Next, we examined both the level of Akt2 and Akt3 mRNA and protein in Akt1+/+ and Akt1–/– ovaries at PND50. We found similar levels of expression for Akt2 mRNA (Fig. 6B) and protein (Fig. 6A) in both Akt1+/+ and Akt1–/– mice. There was a trend toward reduced Akt3 mRNA expression in the Akt1–/– ovaries (Fig. 6C) relative to Akt1+/+ ovaries, suggesting that Akt3 may contribute to the Akt1–/– phenotype. However, we did not observe a decrease in Akt3 at the protein level (Fig. 6A).

TABLE 4. Serum hormone profiles of Akt1+/+ and Akt1–/– females at PND90.6

| Hormone               | Akt1+/+   | Akt1–/–   |
|-----------------------|-----------|-----------|
| Progesterone (ng/ml)  | 3.1 ± 0.8 (N = 5) | 3.8 ± 1.0 (N = 4) |
| Estradiol (pg/ml)     | 6.2 ± 1.2 (N = 4) | 7.0 ± 1.2 (N = 5) |

6 Values represent the mean ± SEM; statistical analyses were conducted using Student t-test (P < 0.05).
Kitl and Bcl2l1 Are Significantly Reduced in Akt1−/− Ovaries

Kitl plays a role in oocyte growth, survival, and maturation [27]. Therefore, we examined the level of Kitl mRNA in both Akt1+/+ and Akt1−/− ovaries. There was a 2-fold reduction in the levels of Kitl mRNA in Akt1−/− ovaries relative to Akt1+/+ ovaries (Fig. 7A). Because of the role of Kitl in granulosa cell survival and protection of the oocyte, we examined Bcl2l1 (Fig. 7B), an antiapoptotic factor and well-known downstream target of the AKT1 signaling pathway [28]. Again, we found significantly reduced levels of Bcl2l1 in the Akt1−/− ovaries, suggesting that the PI3/AKT1 signaling pathway plays a role in folliculogenesis.

Localization Patterns and Decreased mRNA Expression of Members of the Cyclin D Family in Akt1+/+ and Akt1−/− Ovaries

Due to the decreased proliferative rates of the granulosa cells, as measured by BrdU labeling in the secondary follicles of Akt1−/− ovaries, we examined the expression levels of Ccnd1, Ccnd2, and Ccnd3 (Fig. 7, C–E). Members of the cyclin D family are implicated in the regulation of proliferation of granulosa cells [29, 30]. We found that Ccnd3 mRNA expression levels (Fig. 7E) were reduced 3-fold in the Akt1−/− ovary relative to the Akt1+/+ ovary, and that there was a 2-fold reduction in Ccnd1 mRNA expression levels (Fig. 7C) as well, although it did not reach statistical significance. Importantly, Ccnd2 mRNA expression levels (Fig. 7D) remained the same in both Akt1+/+ and Akt1−/− animals.

To localize Ccnd1, Ccnd2, and Ccnd3 to particular cells within the ovary, ovaries were examined at PND50 by immunohistochemistry. Ccnd1 staining was found to be relatively weak in the ovary, with minimal staining detected in thecal cells of both Akt1+/+ (Fig. 8A, arrows) and Akt1−/− ovaries (Fig. 8D, arrow). Staining of Ccnd1 did appear less intense in the Akt1−/− ovary. We also observed weak cytoplasmic staining of Ccnd1 in the primordial follicles (Fig. 8D, bracket). As a positive control, oviduct stained intensely in the ovary, with minimal staining detected in both Akt1+/+ (Fig. 8B) and Akt1−/− (Fig. 8E) ovaries. Ccnd3 localized to the nuclei of primordial (Fig. 8, C and F, asterisks) and primary follicles (Fig. 8, C and F, arrows) in both Akt1+/+ (Fig. 8C) and Akt1−/− ovaries (Fig. 8F).

DISCUSSION

Numerous studies have shown a role for the deletion of Akt1 in mice to lead to decreases in both cell proliferation and apoptosis [9–19]. However, the role of Akt1 in female reproductive tissues remains uncharacterized. Our results show that Akt1 deletion leads to POF. The extreme subfertility that Akt1−/− female mice exhibit appears to arise from altered hormonal signals, as exemplified by a delay in the first estrous cycle and an extension of the diestrous phase in Akt1−/− females. The ovaries of Akt1−/− female mice also exhibit abnormal gene expression of two of the cyclin D family members, Ccnd1 and Ccnd3, and reduced levels of expression of the survival factors, Kitl and Bcl2l1, resulting in decreased granulosa cell proliferation in vivo. Moreover, by PND90, there is a dramatic decrease in the number of primordial follicles in Akt1−/− female mice, indicating POF.

Previous studies have demonstrated that an adequate mass of adipose tissue is required for proper onset of puberty and fertility, suggesting an important link between energy homeostasis and reproductive function [31]. Because Akt1−/− mice exhibit a smaller body size throughout their lifetime, we examined the onset of puberty in Akt1+/+ and Akt1−/− mice. We found no difference in the timing of vaginal opening, but we did observe a difference in the timing of the first proestrus, with a delay of approximately 5 days in Akt1−/− females. No difference in the absolute ovarian weights of Akt1+/+ or Akt1−/− mice was found at PND25, suggesting that the small

![Figure 6](https://www.biolreprod.org)
body size phenotype of the *Akt1<sup>−/−</sup>* female is not the sole reason for the extreme female subfertility in these mice.

At PND50, *Akt1<sup>−/−</sup>* animals exhibit higher LH levels. This finding suggests that the trigger to signal ovulation is present in the *Akt1<sup>−/−</sup>* mice; however, the significant decrease in preantral and antral follicle formation in the *Akt1<sup>−/−</sup>* animals and the increased number of degenerate oocytes indicates an aberrant response to this LH signal. Chronically elevated LH levels have been reported to deplete the primordial follicle pool, which hastens the onset of reproductive senescence [32]. In agreement with this, by PND90, we observed a significant reduction in the number of primordial follicles in the *Akt1<sup>−/−</sup>* ovaries, suggesting accelerated depletion of the primordial follicle pool. In addition to the higher LH levels, we also observed a trend toward higher inhibin A serum levels and a reduction in inhibin B serum levels in the *Akt1<sup>−/−</sup>* ovaries. In women, inhibin A is a marker of dominant follicle and corpus

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**FIG. 7.** Reduced levels of *Kitl, Ccnd3*, and *Bcl2l1* in *Akt1<sup>−/−</sup>* mouse ovaries at the mRNA level at PND50. Changes in the relative mRNA expression of *Kitl* (A), *Bcl2l1* (B), *Ccnd1* (C), *Ccnd2* (D), and *Ccnd3* (E) are depicted in graphic form in *Akt1<sup>+/−</sup>* (black bars) and *Akt1<sup>−/−</sup>* (gray bars) ovaries. Expression was determined by using the Pfaffl method. The data are shown as means ± the SEM. Significance was determined by ANOVA; *P* < 0.05.

**FIG. 8.** Localization of *Ccnd1, Ccnd2*, and *Ccnd3* Family members in *Akt1<sup>+/−</sup>* and *Akt1<sup>−/−</sup>* ovaries at PND50. CCND1, CCND2, and CCND3 protein were analyzed by immunohistochemistry. Immunodetection of CCND1 in *Akt1<sup>+/−</sup>* (A) and *Akt1<sup>−/−</sup>* (D) ovaries, and immunodetection of CCND1 in the oviduct (I). Weak positive staining for CCND1 is observed in the thecal cells of both genotypes (A and D, arrows). Immunodetection of CCND2 in *Akt1<sup>+/−</sup>* (B) and *Akt1<sup>−/−</sup>* (E) ovaries. Positive staining, as indicated by diaminobenzidine signal (brown staining), for CCND2 is observed in granulosa cells (GC) of both genotypes (B and E). Immunodetection of CCND3 in *Akt1<sup>+/−</sup>* (C) and *Akt1<sup>−/−</sup>* (F) ovaries. Positive staining is observed in the nuclei of oocytes from primordial (C and F, asterisks) and primary follicles (C and F, arrows). G) Higher magnification of nuclear staining for CCND1 in *Akt1<sup>−/−</sup>* follicle (A), which can be observed around the perimeter of the follicle (arrows). H) Higher magnification of nuclear staining for CCND1 in *Akt1<sup>−/−</sup>* follicle (D), which can be observed around the perimeter of follicle (arrows). Cytoplasmic staining for CCND1 is also observed in primordial follicles (bracket). I) Strong nuclear staining for CCND1 is observed in the oviduct; bar = 200 μm.
luteum activity, and decreases in inhibin A are associated with polycystic ovary syndrome [33].

Besides changes in gonadotropin levels, the reduced numbers of growing follicles in Akt1−/− ovaries prompted us to examine the proliferative potential of the granulosa cells in the Akt1−/− mice. We reasoned that the decreased fertility may be due, in part, to defective follicle growth due to reduced granulosa cell proliferation and/or an increase in atretic follicles. Indeed, we observed decreased granulosa cell proliferation as evidenced by decreased incorporation of BrdU in the follicles of Akt1−/− mice. Moreover, we observed decreased expression levels of Cdk1, which is expressed primarily in granulosa cells. Kit, along with c-Kit, protects preantral follicles from apoptosis [34, 35]. Ccnd3 expression, which regulates G1/S-phase progression, was also reduced in the Akt1−/− ovaries; however, Ccnd2 levels remained fairly constant in both Akt1+/+ and Akt1−/− ovaries. This is in contrast to a recent report in which Ccnd1 levels were regulated by an Akt1-dependent pathway [36]. Bcl2l1 expression, an established downstream target of the PIK3/AKT1 signaling pathway, was also reduced. Importantly, Bcl2l1 has been shown to play a protective role in rat primordial oocyte survival in vitro by regulating BAX expression levels [37]. It should be noted, however, that mRNA expression was measured in whole-ovary extract, and not isolated cell types. Use of the whole-ovary extract in our studies may mask changes in a specific cell type.

Several features of our current study with respect to oocyte development also merit attention. The incidence of multi-oocyte follicles appeared to be unique to the Akt1−/− ovary, because they were extremely rare or not present in the Akt1+/+ or Akt1+/− ovaries. This type of abnormal follicle has also been noted in ovaries of mutant mice lacking Gdf9 or Bmp15, both of which are oocyte-secreted growth factors [38, 39]. Several other deletions of genes expressed in granulosa cells also exhibit this aberration, as in ovaries of mice lacking the Nr0b1 (previous symbol, Ahrh) gene [40], which encodes the transcription factor, DAX1, involved in sex determination, or the Ca++/calmodulin-dependent protein kinase IV knockout females that show reduced fertility [41], and in mice that overexpress the inhibin alpha gene [42]. Although the mechanisms underlying such abnormalities are complex, it is possible that early developmental events that lead to follicle organization are aberrant and/or incomplete in the Akt1−/− ovary. Moreover, evidence indicates that estrogen signals through multiple pathways to regulate oocyte cyst breakdown and primordial follicle assembly in the neonatal mouse ovary [43]. Indeed, we did observe both follicles with multiple oocytes per follicle and higher estradiol levels in the Akt1−/− ovary.

Another intriguing observation was the increase in the size (diameter) of the primary and secondary oocytes in the Akt1−/− mice relative to Akt1+/+ mice and the increase in the number of degenerate oocytes. Although not statistically significant, we did observe reduced serum levels of FSH in PND50 Akt1−/− females compared with Akt1+/+ and Akt1+/− animals. Previous work has indicated that microinjection of a myristilated form of Akt in mouse oocytes leads to in vitro meiotic maturation, and this effect requires cGMP-inhibited CAMP phosphodiestarase 3A [44]. Moreover, the activation of Akt stimulates the metaphase I-to-metaphase II transition in bovine oocytes [45]; and the PIK3/AKT pathway has been shown to participate in the FSH-induced meiotic maturation of both mouse and Xenopus oocytes [46, 47]. Perhaps, the majority of oocytes of the Akt1−/− female fail to undergo FSH-induced meiotic maturation, resulting in degenerate oocytes and subsequent infertility.

In conclusion, we propose a role for the PTEN/PI3K/AKT1 signaling pathway that promotes healthy oocyte development and folliculogenesis. We propose that the communication between granulosa cells and oocyte is altered, leading to an increase in the size of primary and secondary oocytes in the Akt1−/− animals. Therefore, Akt1 is critical for the proper growth and maturation of both the oocyte and granulosa cells. The loss of Akt1 leads to miscommunication between oocyte and granulosa cells, resulting in degenerate oocytes and POF. This significant but incomplete disruption in female fertility is not likely due to compensation by other PKB/AKT family members, but factors yet to be identified. PKBα/akt1 is instrumental in the precise timing of the female reproductive lifespan. We believe that these phenomena may have relevance to POF and lower reproductive success in middle-aged women. Further studies on the functions of Akt1 and its family members in the ovary can be expected to advance the understanding of the molecular mechanisms of female infertility.

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