The Ability of the Aryl Hydrocarbon Receptor to Regulate Ovarian Follicle Growth and Estradiol Biosynthesis in Mice Depends on Stage of Sexual Maturity[1]

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ABSTRACT

The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor that mediates the toxicity of environmental chemicals and regulates many physiological functions, including processes in female reproduction. Previous studies demonstrated that *Ahr* deletion leads to slow ovarian follicle growth because of impaired estradiol production and reduced gonadotropin responsiveness in prepubertal mice. These studies, however, did not determine how *Ahr* deletion impairs estradiol production or whether the effects of *Ahr* deletion on follicle growth and estradiol production persist in adulthood. Thus, the present study evaluated the effect of *Ahr* deletion on steroid precursors in the estradiol biosynthesis pathway. Furthermore, this study evaluated follicle growth and estradiol biosynthesis in wild-type (WT) and *Ahr* knockout (*Ahr*KO) antral follicles at different stages of sexual maturity. *Ahr*KO antral follicles from prepubertal mice had slower growth, produced lower estradiol levels, and had reduced cyclin D2 (*Ccnd2*) expression compared to WT follicles. *Ahr*KO follicles from adult mice, however, produced higher androgen levels and expressed higher levels of *Ccnd2* compared to WT follicles. Furthermore, *Ahr*KO follicles from adult mice had growth to that of WT follicles. These findings suggest that the AHR regulates follicle growth by altering factors involved in the estradiol biosynthesis pathway as well as key regulators of follicle growth and that this role of AHR depends on stage of sexual maturity.

androgens, aryl hydrocarbon receptor, estradiol, follicle, ovary

INTRODUCTION

The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor located in the cytosol of cells [1]. For several years, the AHR was only recognized as a mediator of toxicity caused by polychlorinated dioxins and related compounds, because binding of these toxicants to the AHR initiates a cascade of events that often leads to toxicity [2–4]. Recent studies, however, have revealed that the lack of a functional *Ahr*

in mice results in phenotypic changes that suggest participation of the AHR in physiological functions, including female reproduction [5–9]. Female reproductive functions that may require the AHR signaling pathway include fertility, uterine epithelium cyclic changes, embryo nourishment, maintenance of pregnancy, and normal ovarian function [9].

In the ovary, primordial follicles develop into primary, preantral, and early antral follicles [10]. Early antral follicles become highly responsive to follicle-stimulating hormone (FSH) and luteinizing hormone (LH) to initiate biosynthesis of steroid hormones, such as androgens and estrogens [11]. Steroid hormones are paracrine/autocrine regulators necessary for follicle growth and development to the preovulatory stage [11–13]. Thus, antral follicles are required for fertility because they are the only follicles that are capable of releasing eggs for fertilization and producing steroid hormones that regulate menstrual/estrous cyclicity [10].

The presence of the AHR has been described in female reproductive tissues and all cell types in the ovarian follicle (oocyte, granulosa, and theca cells) [14–16]. Deletion of the *Ahr* produces alterations in follicle growth [17–19]. Specifically, previous studies using *Ahr* knockout (*Ahr*KO) mice have shown that *Ahr*KO ovaries on Postnatal Days (PDs) 45–53, a time when mice are cycling, contain approximately 50% fewer antral follicles than wild-type (WT) ovaries [17]. A later study by Barnett et al. [19] investigated why *Ahr*KO ovaries on PDs 45–53 have fewer antral follicles than WT ovaries, and those authors found that *Ahr*KO antral follicles isolated from PD 30 ovaries grow more slowly than WT antral follicles. Thus, the slow *Ahr*KO follicle growth at PD 30 likely leads to fewer antral follicles being present in *Ahr*KO ovaries at PDs 45–53. Barnett et al. [19, 20] further elucidated that *Ahr*KO antral follicles have an impaired ability to grow because of reduced abilities to respond to gonadotropins and to produce estradiol.

Although these previous studies suggest that the AHR regulates estradiol production and follicle growth [17–20], the mechanisms by which the AHR regulates estradiol production are unknown. Thus, the present study evaluated the effect of *Ahr* deletion on the levels of sex steroid precursors and sex steroids. Furthermore, the present study evaluated the effect of *Ahr* deletion on the expression of key hormone receptors involved in steroid biosynthesis.

Because previous studies only examined the effects of *Ahr* deletion on follicle growth and estradiol levels in prepubertal mice, it was not known if effects of *Ahr* deletion on the ovary persist in adulthood. Thus, the present study also evaluated follicle growth and estradiol biosynthesis in WT and *Ahr*KO antral follicles at different stages of sexual maturity. The expression of cyclin D2 (*Ccnd2*), a gene that plays a fundamental role in follicle growth and development [21],

TABLE 1. Primers used in quantitative real-time polymerase chain reaction (qPCR).

Gene name	Gene symbol	Primer sequence (5′-3′)[a]	Annealing temp. (°C)	Band (bp)	GenBank accession no.
Actin, beta	Actb	F: ctggcaccacaccttctac	55.0	238	NM_007393
		R: gggcacagtgtgggtgac			
Luteinizing hormone/ choriogonadotropin receptor	Lhcgr	F: aacccggtgcttttttacaaacc	56.4	246	NM_013582
		R: tcccattgaatgcatggctt			
Cyclin D2	Ccnd2	F: agctgtccctgatccgcaag	55.0	350	NM_009829
		R: gtcaacatcccgcacgtctg			
Androgen receptor	Ar	F: ggcggtccttcactaatgtcaact	56.0	84	NM_013476
		R: gagacttgtgcatgcggtactcat			
Follicle stimulating hormone receptor	Fshr	F: gcagatgtgttctccaacctacc	61.0	172	NM_013523
		R: ggagagactggatcttgtgaaagg			

[a] F, forward; R, reverse.

was also compared in WT and *Ahr*KO antral follicles at different stages of sexual maturity.

MATERIALS AND METHODS

Animals

The *Ahr*KO mice (official symbol *Ahr^{tm1Bra}*) were generated by Schmidt et al. [6] and are in a C57BL/6J background, along with their WT littermates. All mice used were from breeding colonies currently maintained by our laboratory at the University of Illinois, Urbana-Champaign, Veterinary Medicine Animal Facility. Mouse colonies were provided with food and water for ad libitum consumption and were maintained in a temperature- and light-controlled room (24 ± 1°C, 12L:12D photoperiod) with 35% ± 4% relative humidity. Cohorts of homozygous mice (WT [*Ahr^{+/+}*] and *Ahr*KO [*Ahr^{-/-}*]) used in each experiment were generated by intercrossing either heterozygous (*Ahr^{+/-}*) female and male mice or heterozygous *Ahr^{+/-}* female mice with *Ahr*KO male mice. Mouse genetic screening was performed using ear tissue punches as previously described [17, 18]. All animal care, euthanasia, and tissue collection were approved by the Institutional Animal Use and Care Committee at the University of Illinois.

Antral Follicle Isolation

Female WT and *Ahr*KO mice were euthanized by CO_2 inhalation on PDs 30–32, 50–54, and 90–94, and ovaries were removed and placed in α-minimal essential media (α-MEM). WT and *Ahr*KO mice on PDs 30–32 were considered to be of a prepubertal age, as evidenced by lack of a vaginal opening and lack of regular estrous cyclicity. WT and *Ahr*KO mice on PDs 50–54 and 90–94, considered to be adult mice, were monitored daily for estrous cycles via vaginal swabs and were euthanized on estrus. An estrous stage was indicated if the vaginal swab contained predominantly keratinized cells.

Follicles with a diameter of 260–400 μm were mechanically isolated from WT and *Ahr*KO ovaries and cleaned of both interstitial tissue and small follicles using fine watchmaker forceps under a dissecting microscope [22]. Follicles of 260–400 μm were used because their size correlates with the histological appearance of early antral follicles containing multiple granulosa cells layers, a theca cell layer, and an antral space [22]. Upon isolation, early antral follicles were subjected to follicle culture for in vitro follicle growth evaluation or were snap-frozen and stored at −80°C until quantitative PCR (qPCR) analyses as described below.

Measurement of In Vitro Follicle Growth

At least 20 early antral follicles from two to three WT and two to three *Ahr*KO mice on PDs 30–32, 50–54, and 90–94 per experiment were isolated as described above and cultured individually in wells of a 96-well culture plate for 7 days as described previously [19, 22]. Follicle growth patterns were examined by measuring follicle diameters in perpendicular axes every 24 h using an inverted microscope equipped with a calibrator ocular micrometer [22]. Three separate cultures per age-matched WT and *Ahr*KO mice were performed. Culture media were collected on Days 3 and 7 and subjected to measurements of steroid hormone levels as described below.

Measurement of In Vivo Follicle Growth

Ovaries were harvested from four to five WT and four to five *Ahr*KO mice on PDs 90–94. This age range was selected because it is when mice are considered to be cycling adults [10] and when general health problems are not present [23]. Ovaries (one per mouse) were collected and preserved by immersion in Dietrich fixative and were stored at room temperature. Ovaries were then dehydrated in ethanol series, cleared, infiltrated with paraffin wax, and serially sectioned (thickness, 8 μm) throughout the entire ovary. Sections were mounted on slides and stained in hematoxylin with eosin using standard procedures.

Every 10th section of the entire ovary was used to count follicle populations as described previously [17, 18]. Follicles were considered to be primordial if they contained an oocyte surrounded by a single layer of fusiform granulosa cells or by an incomplete layer of cuboidal cells. Follicles were considered to be primary if they contained an oocyte surrounded by a fully formed, single layer of cuboidal granulosa cells. Follicles were considered to be preantral if they contained an oocyte surrounded by two to five layers of granulosa cells and no antral space. Follicles were considered to be antral if they contained an oocyte surrounded by more than five layers of granulosa cells and an antral space. To avoid double-counting follicles, only follicles containing an oocyte with a visible nucleus were counted. To avoid bias, all ovaries were analyzed without knowledge of genotype. Numbers of follicles counted per ovary were multiplied by 10 to obtain an estimate of total follicle number per ovary.

Measurement of Steroid Hormone Levels

Culture media samples were subjected to ELISA using kits for measurement of dehydroepiandrosterone (DHEA; Alpco Diagnostics), androstenedione (DRG International, Inc.), testosterone (Alpco Diagnostics), and estradiol (Calbiotech). Samples were diluted 1:4 (v/v) for androstenedione and 1:10 (v/v) for testosterone analyses. Samples collected on Days 3 and 7 were diluted 1:10 and 1:100, respectively, for estradiol assays. All ELISA procedures were performed according to the manufacturer's protocol. α-MEM was used as background control. Positive controls containing known amounts of specific hormones (provided by the manufacturer) were included in every assay. All samples were run in duplicate, and values were calculated by multiplying by the corresponding dilution factor. Final values were obtained by the sum of hormone levels on Days 3 and 7 of follicle culture. The minimum detection limits, as determined by the ELISA kit manufacturers, were 0.005 ng/ml for DHEA, 0.019 ng/ml for androstenedione, 0.022 ng/ml for testosterone, and 10 pg/ml for estradiol. No samples were below the limit of detection. Intra- and interassay coefficients of variation for all assays were less than 5%.

Topical Autoradiography

Topical autoradiography was performed according to the method described by Oxberry and Greenwald [24] and by Roby [25]. Ovaries were harvested from three WT and three *Ahr*KO mice on PDs 30–32, 50–54, and 90–94 and then frozen in liquid nitrogen, embedded in optimal cutting temperature medium, and cut in a cryostat. The frozen sections (thickness, 10 μm) were placed on Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA), air-dried, fixed in picric acid-formaldehyde, and stored in a desiccator at −80°C until subjected to topical autoradiography of human chorionic gonadotropin (hCG) or FSH receptors. After bringing the slides to room temperature, ^{125}I-labeled hormone (hCG CR-127 or rat FSH-I-9; 10 000 cpm of 20 μCi/mg) was added to the tissue section in 0.2 ml of PBS containing 0.1% bovine serum albumin and incubated for 2 h at 37°C. The hCG and recombinant FSH obtained from the National Hormone and Pituitary Program were iodinated using the lactoperoxidase method [26, 27]. Control, nonspecific binding sections had 10 IU of unlabeled homologous hormone in addition to the radiolabeled
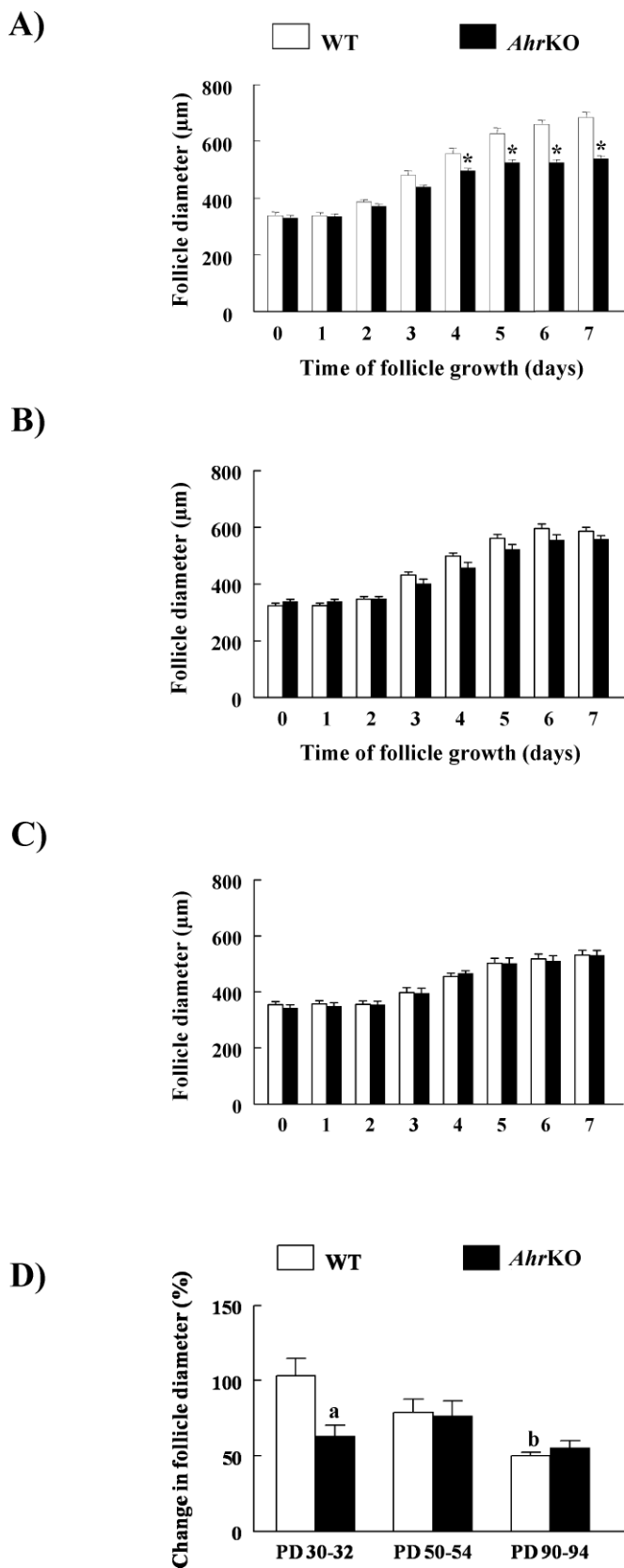
A)

B)

C)

D)

FIG. 1. In vitro growth of WT and *Ahr*KO antral follicles. Early antral follicles were isolated from WT and *Ahr*KO ovaries on PDs 30–32 (before vaginal opening; **A**), PDs 50–54 (estrus; **B**), and PDs 90–94 (estrus; **C**), and were cultured in supplemented media. WT and *Ahr*KO follicle growth was evaluated by measuring follicle diameter in perpendicular axes every 24 h for 7 days. Total percentage change in WT and *Ahr*KO follicle diameters
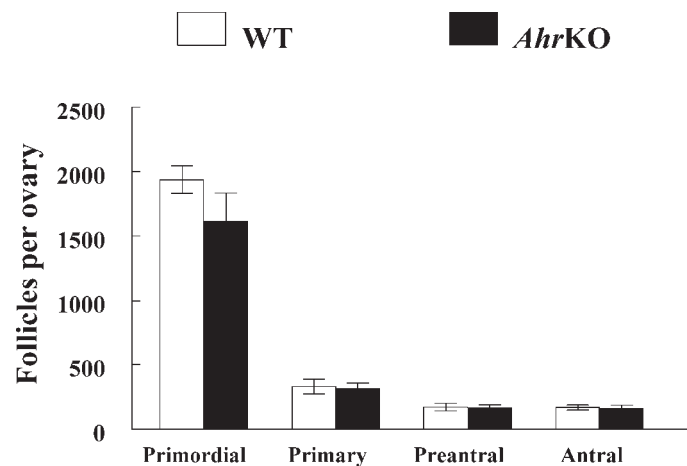

FIG. 2. Follicle numbers in WT and *Ahr*KO ovaries from adult ovaries. Ovaries were collected from WT and *Ahr*KO mice on PDs 90–94 during estrus, and complete serial sections were prepared for histological examination of the total numbers of primordial, primary, preantral, and antral follicles as described in *Materials and Methods*. Each bar represents the mean ± SEM (n = 4–5 ovaries per genotype).

hormone. Following incubation, the tissue sections were postfixed in 4% paraformaldehyde and rinsed in PBS. The slides were dipped in autoradiographic emulsion, dried, stored at 4°C for 5 days when using ^{125}I-labeled rat FSH or for 12 days when using ^{125}I-labeled hCG CR-127, and then developed through routine photographic steps of Dektol, Stop, and Fix. Next, the slides were washed and stained with nuclear fast red and picric acid. Tissue sections were photographed on an Olympus 1X71 inverted microscope with an Olympus DP71 digital camera and software. Percentage area occupied by grains in antral follicles was analyzed using ImageJ 1.41o (www.nih.gov/). Data were analyzed using GraphPad Prism software (Version 5.0C; GraphPad Software, La Jolla, CA), and differences in antral follicle grain density/binding between WT and *Ahr*KO ovaries of the same age were evaluated.

Real-Time qPCR

Between 15 and 18 early antral follicles from three to six WT and three to six *Ahr*KO mice on PDs 30–32, 50–54, and 90–94 were isolated as described above and subjected to qPCR. Total RNA (1–2 µg) was extracted from the follicles using the RNeasy Mini Kit (Qiagen, Inc.) and then converted to cDNA using an Omniscript RT kit (Qiagen, Inc.) and random primers (Invitrogen) according to the manufacturers' protocols. The cDNA was amplified by qPCR as described previously [19, 28] using an MJ Research Chromo4 Real Time PCR machine (MJ Research, Inc.) and accompanying software according to the manufacturer's instructions. To allow analysis of the amount of cDNA in the exponential phase, a standard curve from five serial dilutions was generated using cDNA from a pool of WT and *Ahr*KO antral follicles. Specific qPCR primers for the genes of interest and annealing temperatures are listed in Table 1. SYBR Green (Finnzymes, c/o New England) was used as dye for all qPCR analyses. A melting curve was generated at 55–90°C to confirm the generation of a single product, and PCR products were loaded in 3% agarose gel to confirm the product size according to Table 1. Actin, beta (*Actb*) was used for each sample as an internal control. Relative numbers were generated by the qPCR software per sample, and final values were calculated as the ratio of the gene of interest to *Actb* for each sample.

Statistical Analysis

The data were analyzed using SAS 9.2 (Statistical Analysis System Institute, Inc.). Independent *t*-tests were used to compare the numbers of

from Day 0 to Day 7 of culture is shown (**D**). Each bar represents the mean ± SEM from three separate cultures, each with at least 20 follicles per genotype. In **A**, asterisks above the bars indicate statistically significant differences between WT and *Ahr*KO follicles ($P \leq 0.05$). In **D**, the letter "a" above the bar indicates a significant difference between WT and *Ahr*KO follicles on PDs 30–32 ($P \leq 0.05$). The letter "b" indicates significant difference between WT follicles on PDs 30–32 and WT follicles on PDs 90–94 ($P \leq 0.05$).
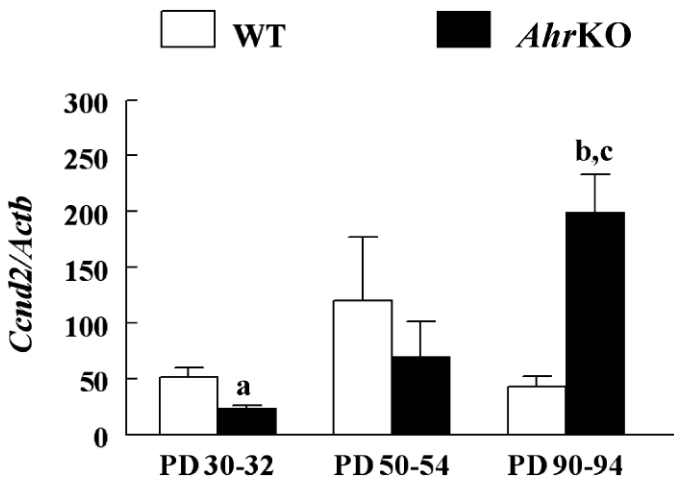
FIG. 3. Expression of *Ccnd2* in WT and *Ahr*KO early antral follicles. Ovaries were removed from WT and *Ahr*KO mice on PDs 30–32 (before vaginal opening), PDs 50–54 (estrus), and PDs 90–94 (estrus). The early antral follicles were immediately isolated from the ovaries and subjected to extraction of RNA. The RNA was subjected to qPCR analysis for *Ccnd2* as described in *Materials and Methods*. All values represent relative expression units of *Ccnd2* normalized to *Actb* as a loading control. Each bar represents the mean ± SEM (n = 3–6 mice/genotype and 15–18 follicles/mouse at each selected age). The letter "a" above the bar indicates a significant difference between WT and *Ahr*KO follicles on PDs 30–32 ($P \leq 0.05$). The letter "b" above the bar indicates a significant difference between WT and *Ahr*KO follicles on PDs 90–94 ($P \leq 0.05$). The letter "c" above the bar indicates a significant difference between *Ahr*KO follicles on PDs 90–94 and *Ahr*KO follicles on PDs 30–32 ($P \leq 0.05$).

follicles per ovary between WT and *Ahr*KO samples. General linear model (GLM) with repeated measures was used for comparisons over time in follicle cultures. GLM was also used to investigate the effects of age, genotype, and their interaction on the total change in follicle diameter, *Ccnd2*, hormone levels, steroid hormone receptors, and gonadotropin binding receptors. If the global tests from GLM were significant, Tukey tests were used for pairwise comparisons. Data were expressed as the mean ± SEM. Statistical significance was assigned at $P \leq 0.05$.

RESULTS

Effect of Ahr Deletion on Ovarian Follicle Growth and Ccnd2

Previous studies demonstrated that follicles isolated from *Ahr*KO ovaries on PD 30 grow more slowly than WT follicles in vitro [19]. In the present study, follicle growth was assessed at different ages of sexual maturity to evaluate whether the effect of the *Ahr* deletion on follicle growth persists in adulthood (Fig. 1). Similar to previously reported work [19], *Ahr*KO follicles on PDs 30–32 had smaller diameters than WT follicles beginning on Day 4 of culture and persisting until the end of culture (Fig. 1A). *Ahr*KO follicles from both PD 50–54 (Fig. 1B) and PD 90–94 (Fig. 1C) ovaries, however, had diameters similar to those of WT follicles at all time points.
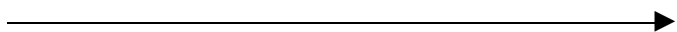
FIG. 4. Hormone levels in media samples from WT and *Ahr*KO follicle cultures. Early antral follicles from WT and *Ahr*KO ovaries on PDs 30–32 (before vaginal opening), PDs 50–54 (estrus), and PDs 90–94 (estrus) were placed in culture for 7 days. Media samples were collected from follicle cultures and subjected to ELISA to measure levels of DHEA (**A**), androstenedione (**B**), testosterone (**C**), and estradiol (**D**). Each bar
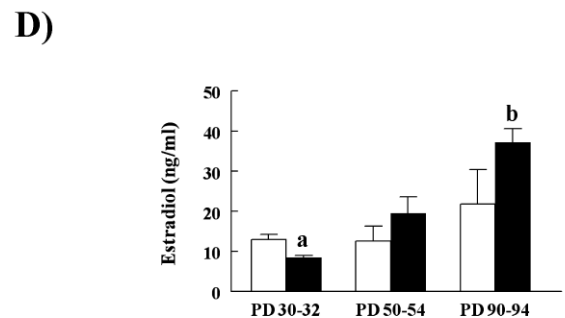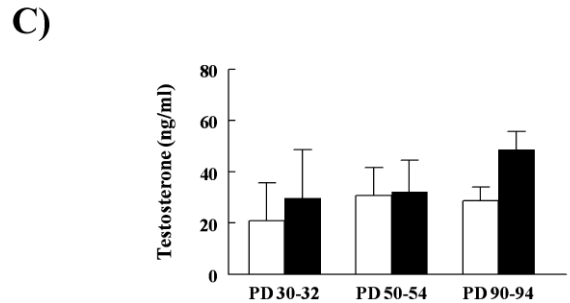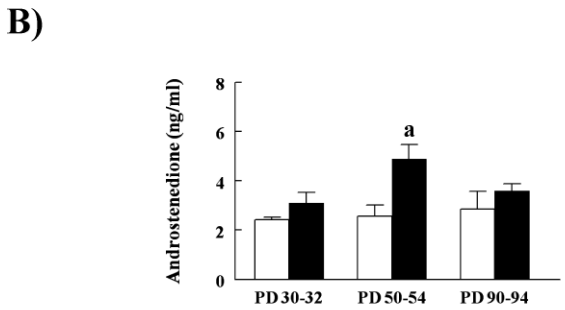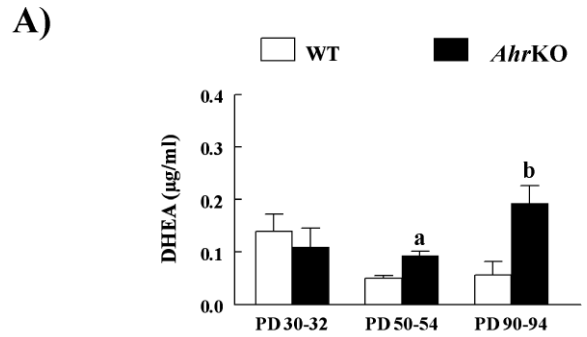
represents the mean ± SEM from three separate cultures, each with at least three separate cultured follicles per genotype at each selected age. In **A**, the letter "a" above the bar indicates a significant difference between WT and *Ahr*KO samples on PDs 50–54 ($P \leq 0.05$), and the letter "b" above the bar indicates a significant difference between WT and *Ahr*KO samples on PDs 90–94 ($P \leq 0.05$). In **B**, the letter "a" above the bar indicates a significant difference between WT and *Ahr*KO samples on PDs 50–54 ($P \leq 0.05$). In **D**, the letter "a" indicates a significant difference between WT and *Ahr*KO samples on PDs 30–32 ($P \leq 0.05$), and the letter "b" indicates a significant difference between *Ahr*KO samples on PDs 90–94 and *Ahr*KO samples on PDs 30–32 ($P \leq 0.05$).
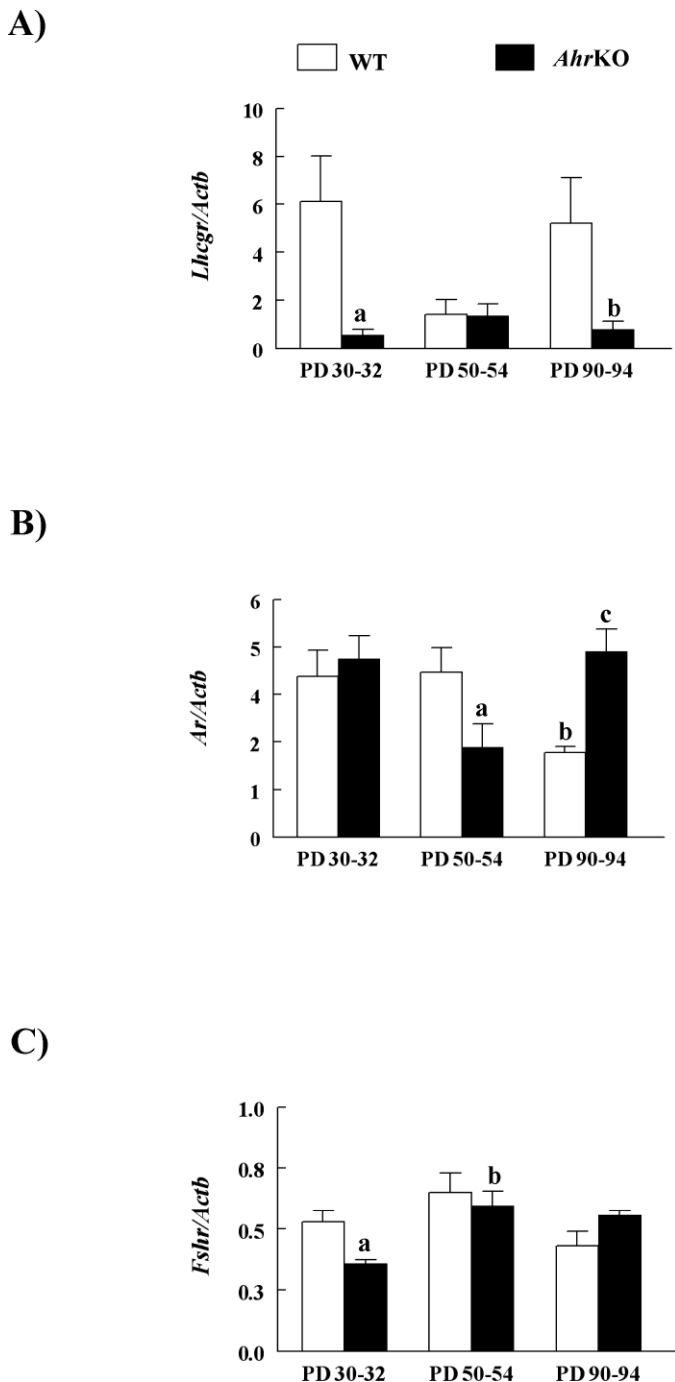
A)

B)

C)

FIG. 5. Expression of mRNA encoding follicular receptors involved in estradiol biosynthesis. Ovaries were removed from WT and *Ahr*KO mice on PDs 30–32 (before vaginal opening), PDs 50–54 (estrus), and PDs 90–94 (estrus). The early antral follicles were immediately isolated from the ovaries and subjected to extraction of RNA. The RNA was subjected to qPCR analysis for *Lhcgr* (**A**), *Ar* (**B**), and *Fshr* (**C**) as described in *Materials and Methods*. All values represent relative expression units of the gene of interest normalized to *Actb* as a loading control. Each bar represents the mean ± SEM relative units (n = 3–6 mice/genotype and at least 15–18 follicles/mouse at each selected age). In **A**, the letter "a" above the bar indicates a significant difference between WT and *Ahr*KO samples on PDs 30–32 ($P \leq 0.05$), and the letter "b" above the bar indicates a significant difference between WT and *Ahr*KO follicles on PDs 90–94 ($P \leq 0.05$). In **B**, the letter "a" above the bar indicates a significant difference between WT and *Ahr*KO follicles on PDs 50–54 ($P \leq 0.05$), the letter "b" above the bar indicates a significant difference between WT follicles on PDs 90–94 and WT follicles on PDs 30–32 ($P \leq 0.05$), as well as a significant difference between WT follicles on PDs 90–94 and WT follicles on PDs 50–54 ($P \leq 0.05$). The letter "c" above the bar indicates a significant

To compare the total change in growth of WT and *Ahr*KO follicles with age, the percentage change in follicle diameter from Day 0 to Day 7 of culture was compared in WT and *Ahr*KO follicles at PDs 30–32, 50–54, and 90–94 (Fig. 1D). The data indicate that the total percentage change in follicle diameter differs between WT and *Ahr*KO follicles with age. Specifically, the total change in follicle diameter in *Ahr*KO follicles was less than that of WT follicles on PDs 30–32, but not on PDs 50–54 and PDs 90–94 (Fig. 1D). Interestingly, the percentage change in growth of WT follicles declined over time (i.e., the percentage change in growth of WT follicles at PDs 90–94 was less than the percentage change in growth of WT follicles at PDs 30–32), whereas it remained stable over time in *Ahr*KO follicles (i.e., the percentage change in growth was similar in *Ahr*KO follicles at all time points).

To confirm whether follicle growth was similar in WT and *Ahr*KO ovaries on PDs 90–94, in vivo follicle growth was evaluated in ovarian sections by counting the relative numbers of follicles at each stage of development (Fig. 2). Similar numbers of primordial, primary, preantral, and antral follicles were observed in WT and *Ahr*KO ovaries on PDs 90–94 (Fig. 2).

Because in vitro follicle growth differed between WT and *Ahr*KO follicles on PDs 30–32, but at not older ages, the levels of *Ccnd2* mRNA, a cell-cycle regulator that promotes the granulosa cell proliferation necessary for follicle growth [21], was compared in WT and *Ahr*KO early antral follicles collected at different ages of sexual maturity (Fig. 3). The data indicate that the levels of *Ccnd2* mRNA significantly differed between WT and *Ahr*KO follicles with age. Specifically, on PDs 30–32, *Ahr*KO antral follicles had 2.2-fold lower levels of *Ccnd2* mRNA compared to WT follicles, but this difference did not exist on PDs 50–54. On PDs 90–94, *Ahr*KO antral follicles had 4.6-fold higher levels of *Ccnd2* mRNA compared to WT follicles. Furthermore, *Ahr*KO follicles on PDs 90–94 had increased levels of *Ccnd2* mRNA compared to *Ahr*KO follicles on PDs 30–32 (Fig. 3).

Effect of Ahr *Deletion on Follicle-Produced Hormones*

Because granulosa cell proliferation and follicle growth are stimulated by estradiol [21], the abilities of WT and *Ahr*KO follicles to produce estradiol and estradiol precursors were compared by measuring levels of DHEA, androstenedione, testosterone, and estradiol in media from cultured follicles on PDs 30–32, 50–54, and 90–94 (Fig. 4). The levels of DHEA (Fig. 4A), androstenedione (Fig. 4B), and estradiol (Fig. 4D) significantly differed between media samples from WT and *Ahr*KO follicle cultures with age. Specifically, DHEA levels were similar in media samples from WT and *Ahr*KO follicle cultures on PDs 30–32 but were 1.8-fold and 3.4-fold higher in media samples from *Ahr*KO follicle cultures compared to WT follicle cultures on PDs 50–54 and 90–94, respectively (Fig. 4A). Androstenedione levels were similar in media samples from WT and *Ahr*KO follicle cultures on PDs 30–32 and 90–94 but were 1.9-fold higher in media samples from *Ahr*KO follicle cultures compared to WT follicle cultures on PDs 50–54 (Fig. 4B). Estradiol levels were 1.5-fold lower in media samples from *Ahr*KO follicle cultures compared to media

difference between WT and *Ahr*KO follicles on PDs 90–94 ($P \leq 0.05$). In **C**, the letter "a" indicates a significant difference between WT and *Ahr*KO follicles on PDs 30–32 ($P \leq 0.05$), and the letter "b" indicates a significant difference between *Ahr*KO follicles on PDs 50–54 and *Ahr*KO follicles on PDs 30–32 ($P \leq 0.05$).
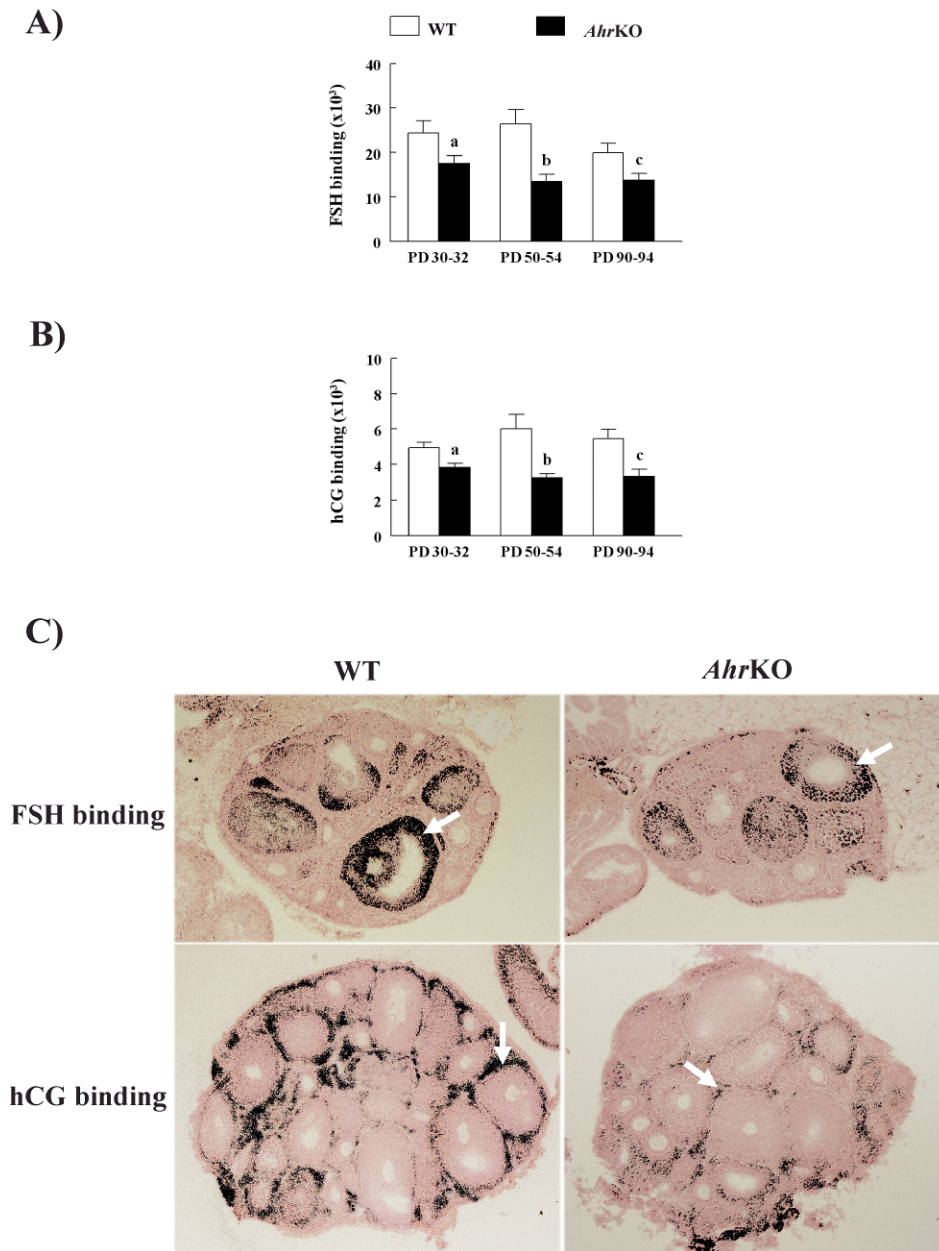
A)

B)

C)

FIG. 6. Effect of *Ahr* deletion on FSHR and LHCGR in antral follicles. Ovaries were removed from WT and *Ahr*KO mice on PDs 30–32 (before vaginal opening), PDs 50–54 (estrus), and PDs 90–94 (estrus) and subjected to topical autoradiography as described in *Materials and Methods*. **A)** ^{125}I-labeled rat FSH was used to localize FSHR binding sites. **B)** ^{125}I-labeled hCG CR-127 (LH analog) was used to localize LHCGR binding sites. Arrows point to antral follicle grain/density binding for FSHR (**top**) and LHCGR (**bottom**). The letter "a" above bars indicates a significant difference between WT and *Ahr*KO ovaries on PDs 30–32 ($P \leq 0.05$). The letter "b" above bars indicates a significant difference between WT and *Ahr*KO ovaries on PDs 50–54 ($P \leq 0.05$). The letter "c" above bars indicates a significant difference between WT and *Ahr*KO ovaries on PDs 90–94 ($P \leq 0.05$). **C)** Representative micrographs of ovaries on PDs 30–32 collected from each genotype (original magnification ×50) and incubated with ^{125}I-labeled rat FSH (**top**) or ^{125}I-labeled hCG CR-127 (**bottom**).

samples from WT follicle cultures on PDs 30–32. On PDs 50–54 and 90–94, however, media samples had similar levels of estradiol in WT and *Ahr*KO follicle cultures. Furthermore, media samples from *Ahr*KO follicle cultures on PDs 90–94 had increased estradiol levels compared to media samples from *Ahr*KO follicle cultures on PDs 30–32 (Fig. 4D). Testosterone levels in media samples from WT and *Ahr*KO follicle cultures were similar and did not differ with age (Fig. 4C).

Effect of Ahr *Deletion on Genes Encoding Receptors Involved in Follicle Steroidogenesis*

The mRNA expression of genes encoding receptors involved in the synthesis of sex steroid hormone precursors and sex steroids was compared in WT and *Ahr*KO early antral follicles isolated from mice on PDs 30–32, 50–54 and 90–94 (Fig. 5). The levels of luteinizing hormone/choriogonadotropin receptor (*Lhcgr*) mRNA (Fig. 5A), androgen receptor (*Ar*) mRNA (Fig. 5B), and *Fshr* mRNA (Fig. 5C) were significantly different between WT and *Ahr*KO follicles with age. Specifically, the levels of *Lhcgr* mRNA were 10.8-fold lower in *Ahr*KO follicles compared to WT follicles on PDs 30–32, but this difference did not exist on PDs 50–54. On PDs 90–94, the levels of *Lhcgr* mRNA were 6.6-fold lower in *Ahr*KO follicles compared to WT follicles (Fig. 5A). The levels of *Ar* mRNA were similar in WT and *Ahr*KO antral follicles on PDs 30–32 but were 1.8-fold lower in *Ahr*KO follicles compared to WT follicles on PDs 50–54. In contrast, the levels of *Ar* mRNA were 2.2-fold higher in *Ahr*KO follicles compared to WT follicles on PDs 90–94. Furthermore, the levels of *Ar* mRNA were lower in WT follicles on PDs 90–94 compared to WT follicles on PDs 30–32 and 50–54 (Fig. 5B). The levels of *Fshr* mRNA were 1.5-fold lower in *Ahr*KO follicles compared to WT follicles on PDs 30–32 but were similar in WT and *Ahr*KO follicles on both PDs 50–54 and PDs 90–94. In addition, *Ahr*KO follicles on PDs 50–54 had increased levels of *Fshr* mRNA compared to *Ahr*KO follicles on PDs 30–32 (Fig. 5C).

FIG. 7. The impact of the AHR on follicle growth and estradiol biosynthesis. **A**) The lack of the *Ahr* reduces antral follicle growth in prepubertal ovaries (PDs 30–32), but follicle growth returns to WT levels in adulthood (PDs 50–94). **B**) Decreased follicle growth in *Ahr*KO antral follicles on PDs 30–32 may be caused by decreased estradiol levels and decreased *Ccnd2*, a main regulator of granulosa cell proliferation. Between PD 32 and PD 50, sex steroid biosynthesis becomes modified in *Ahr*KO ovaries, resulting in higher levels of androstenedione and DHEA. This may help restore estradiol levels and *Ccnd2* expression in *Ahr*KO follicles and, thus, return *Ahr*KO follicle growth to WT levels. Even though follicle growth is normal in *Ahr*KO follicles on PDs 90–94, sex steroid biosynthesis in *Ahr*KO follicles also varies from PD 53 to PD 90, because *Ahr*KO follicles on PDs 90–94 produce high levels of DHEA. As a result, estradiol production by *Ahr*KO follicles increases at this time compared to estradiol production by *Ahr*KO follicles at PDs 30–32, which may promote increases in the levels of *Ccnd2*.
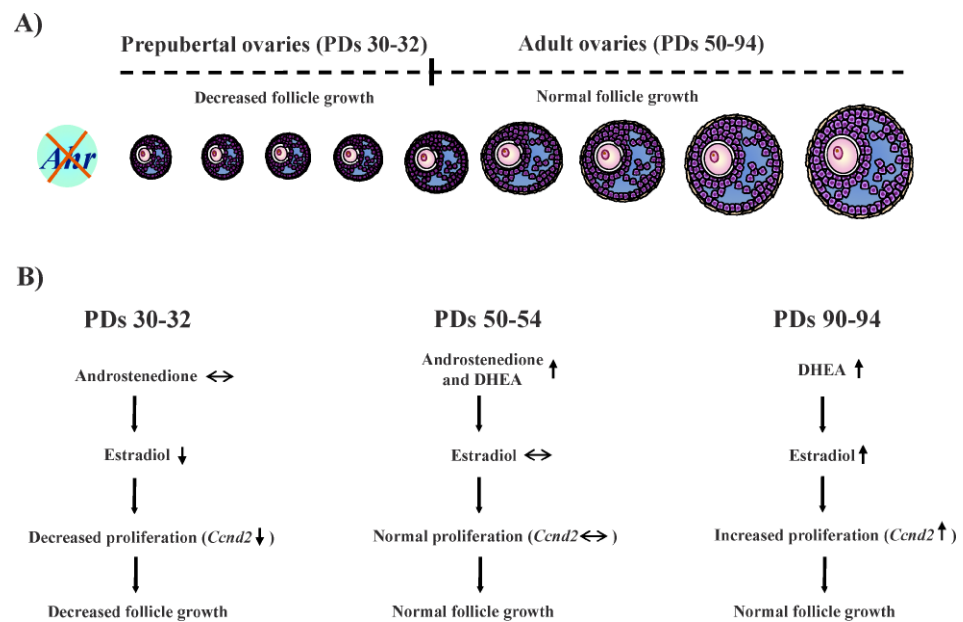
Effect of *Ahr* Deletion on Functional Binding Ability of FSHR and LHCGR in Follicles

Because the synthesis of sex steroid hormone precursors and sex steroids in antral follicles are promoted by FSH and LH binding to their corresponding receptors in the follicle, the functional binding-abilities of both FSHR and LHCGR were compared in WT and *Ahr*KO early antral follicles from ovaries on PDs 30–32, 50–54, and 90–94 (Fig. 6). The abilities of WT and *Ahr*KO follicles to bind both ^{125}I-radiolabeled FSH (Fig. 6A) and ^{125}I-radiolabeled hCG (Fig. 6B) differed by genotype but not by age. Specifically, *Ahr*KO early antral follicles showed reduced ability to bind both ^{125}I-radiolabeled FSH (Fig. 6A) and ^{125}I-radiolabeled hCG (Fig. 6B) compared to WT early antral follicles at all evaluated ages. Furthermore, ^{125}I-radiolabeled FSH was predominately localized in the granulosa cells of both WT and *Ahr*KO follicles (Fig. 6C, top), whereas ^{125}I-radiolabeled hCG was mainly in theca cells of WT and *Ahr*KO follicles (Fig. 6C, bottom).

DISCUSSION

The present study describes the dynamics of follicle growth in *Ahr*KO mice and demonstrates differences in follicle growth and estradiol biosynthesis between sexually immature and sexually mature *Ahr*KO mice (Fig. 7). Previous studies demonstrated that *Ahr*KO ovaries on PD 30 have similar numbers of antral follicles compared to WT ovaries [17, 18]. When follicle growth patterns were compared in WT and *Ahr*KO antral follicles from ovaries on PD 30 using in vitro follicle cultures, the present study and that by Barnett et al. [19] found that *Ahr*KO antral follicles grow more slowly than WT follicles. This pattern of slow follicle growth in *Ahr*KO follicles may explain why almost half as many follicles reach the antral stage by PDs 45–53 in *Ahr*KO ovaries as in WT ovaries [17, 18]. This slow growth of *Ahr*KO antral follicles, however, seems to occur only in the early reproductive ages, because the present study further shows that WT and *Ahr*KO antral follicles have similar growth patterns on PDs 50–54 and 90–94 in vitro, as well as similar numbers of antral follicles in WT and *Ahr*KO ovaries on PD 90 in vivo.

Whereas all the reasons for the slow antral follicle growth in *Ahr*KO ovaries compared to WT ovaries are unknown, Barnett et al. [19] showed that the slow *Ahr*KO follicle growth may be caused by reduced estradiol biosynthesis and that estradiol replacement restores *Ahr*KO follicle growth to WT levels. The present study expands these previous findings, because it examined whether the reduced estradiol biosynthesis by *Ahr*KO follicles was caused by reduced steroid precursor production by *Ahr*KO follicles compared to WT follicles. The present study also expands previous studies by determining whether any differences in the production of steroid hormone precursors and estradiol between WT and *Ahr*KO follicles from prepubertal mice persist in adulthood. Collectively, the data indicate that the levels of the selected steroid hormone precursors are not significantly lower in *Ahr*KO mice compared to WT mice (Fig. 7). Thus, it seems unlikely that the low levels of estradiol produced by *Ahr*KO follicles at PDs 30–32 are caused by low levels of steroid precursors produced by *Ahr*KO follicles compared to WT follicles at this time. Interestingly, the levels of androgens (DHEA and androstenedione) produced by *Ahr*KO follicles from adult mice are significantly higher than those produced by WT follicles from adult mice. Because levels of estradiol produced by *Ahr*KO follicles are restored by WT levels around this same time, it is possible one mechanism that leads to restoration of estradiol levels involves an increase in androgen production followed by conversion of the androgens to estradiol. Estradiol may then up-regulate *Ccnd2* to maintain normal follicle growth (Fig. 7). This idea is supported by studies showing the proliferative effects of estrogens via regulating *Ccnd2* in granulosa cells [21, 29, 30].

The reasons for differences in growth and estradiol biosynthesis between WT and *Ahr*KO follicles with stage of sexual maturity are unknown. Interestingly, the percentage change in diameter in WT follicles, but not in *Ahr*KO follicles, declines with age, suggesting that WT follicles are perhaps losing some capacity to grow over time compared to *Ahr*KO follicles. This in turn could lead to WT follicles and *Ahr*KO follicles eventually having similar growth rates. Several studies indicate that immature follicles are more responsive than mature follicles to gonadotropin stimulation [31–33]. Thus, it is

possible that WT follicles from PD 30–32 ovaries are more responsive to gonadotropins than WT follicles from PD 50–94 ovaries, and that this results in increased growth of WT follicles from PD 30–32 ovaries compared to WT follicles from PD 50–94 ovaries. In addition, previous studies indicate that whereas circulating levels of both FSH and LH are normal in adult *Ahr*KO mice [8, 20], *Ahr*KO ovaries have reduced gonadotropin responsiveness compared to WT ovaries [20]. Furthermore, in the present study, we observed a reduced binding capability of both FSHR and LHCGR in *Ahr*KO ovaries compared to that in WT ovaries. Therefore, it is possible that the reduced FSHR and LHCGR binding capacity and gonadotropin responsiveness of *Ahr*KO follicles leads to decreased expression of genes required for normal follicle growth and estradiol biosynthesis. This hypothesis is supported by studies indicating that FSH plays a role in the regulation of some steroidogenic genes, such as *Cyp19a1* [34, 35], *Cyp11a1* [36], as well as *Lhcgr* and *Fshr* [35]. It is also supported by data from Baba et al. [8], who showed that the AHR is required for the proper expression of *Cyp19a1* in immature ovaries primed with external hormones.

Although we observed reduced binding capacity both FSHR and LHCGR in *Ahr*KO ovaries compared to WT ovaries, expression of *Lhcgr* and *Fshr* was not always lower in *Ahr*KO ovaries compared to WT ovaries. These data suggest that that the protein and mRNA expression of both receptors are not correlated completely. Whereas mRNA expression of both *Lhcgr* and *Fshr* in *Ahr*KO follicles returns to WT levels by PDs 50–54, corresponding binding capacity remains diminished in the *Ahr*KO follicles. These findings suggest that the transcription of both receptors may be more directly modulated by the hormonal changes observed in the *Ahr*KO follicles from PDs 50–54 than protein translation. However, further studies are needed to test this hypothesis. Similar uncoupled transcription and translation have also been described for estrogen receptors in *Ahr*KO follicles [19].

The normal levels of *Fshr* mRNA observed in *Ahr*KO follicles on PDs 50–54 in the present study seem to be in contrast to previous findings, which showed via chromatin immunoprecipitation (ChIP) assays that the AHR directly binds to *Fshr* promoter in adult ovaries [20]. ChIP assays, however, only measure binding of proteins to a promoter region of a target gene; they do not measure transcription directly [37]. Thus, taken together, the data suggest that the AHR binds to the *Fshr*, but that the binding does not alter transcription of the *Fshr* at all ages.

In our study, we observed lower levels of *Ar* mRNA on PDs 50–54 in *Ahr*KO follicles compared to WT follicles. It is possible that increases in DHEA and androstenedione on PDs 50–54 in *Ahr*KO follicles lead to down-regulation of the *Ar* at PDs 50–54. At PDs 90–94, however, *Ahr*KO follicles showed an increase in the levels of *Ar* mRNA. The reasons for this are unknown, but studies have demonstrated both an up-regulation of *Ar* mRNA in ventral prostate from castrated rats (testosterone deprivation) and a down-regulation of *Ar* mRNA in ventral prostate from castrated rats after treatment with testosterone propionate [38]. Thus, it is possible that factors in the *Ahr*KO ovaries cause either an up-regulation or a down-regulation of *Ar* mRNA, depending on the age of the animal.

The restoration of growth and estradiol production in *Ahr*KO antral follicles to WT levels by PDs 50–54 is consistent with data obtained from in vivo studies of follicle numbers and estradiol production. Specifically, our in vitro data indicate that growth of early antral follicles is slow in *Ahr*KO ovaries compared to WT ovaries on PDs 30–32. This slow growth of *Ahr*KO follicles likely results in the reduced number of antral follicles observed in *Ahr*KO ovaries compared to WT ovaries by PDs 45–53 [17]. Because antral follicles produce estradiol, the reduced number of antral follicles in *Ahr*KO mice may lead to the low levels of circulating estradiol observed in *Ahr*KO mice compared to WT mice on PDs 30–90 [19]. Furthermore, our in vitro data indicate that the growth of *Ahr*KO follicles is similar to WT levels by PDs 50–54. This growth pattern likely leads to the similar number of antral follicles observed in WT and *Ahr*KO ovaries by PD 90. It also likely means that after antral follicle numbers in *Ahr*KO ovaries are restored to WT levels, circulating estradiol levels in *Ahr*KO mice are restored to WT levels at some point. In fact, our data indicate that WT and *Ahr*KO mice have similar levels of circulating estradiol on PD 180 (data not shown). Taken together, the data obtained in the present study suggest that the AHR may play a role in regulating follicle growth and estradiol biosynthesis in prepubertal mice but not in adult mice. Future studies should address the molecular and biochemical mechanisms by which the AHR regulates follicle growth and estradiol biosynthesis in prepubertal mice. Such studies will lead to a better understanding of the physiological role of the AHR.

ACKNOWLEDGMENTS

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