Premature ovarian failure in androgen receptor-deficient mice

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Premature ovarian failure (POF) syndrome, an early decline of ovarian function in women, is frequently associated with X chromosome abnormalities ranging from various Xq deletions to complete loss of one of the X chromosomes. However, the genetic locus responsible for the POF remains unknown, and no candidate gene has been identified. Using the Cre/LoxP system, we have disrupted the mouse X chromosome androgen receptor (Ar) gene. Female $AR^{-/-}$ mice appeared normal but developed the POF phenotype with aberrant ovarian gene expression. Eight-week-old female $AR^{-/-}$ mice are fertile, but they have lower follicle numbers and impaired mammary development, and they produce only half of the normal number of pups per litter. Forty-week-old $AR^{-/-}$ mice are infertile because of complete loss of follicles. Genome-wide microarray analysis of mRNA from $AR^{-/-}$ ovaries revealed that a number of major regulators of folliculogenesis were under transcriptional control by AR. Our findings suggest that AR function is required for normal female reproduction, particularly folliculogenesis, and that AR is a potential therapeutic target in POF syndrome.

male hormone | nuclear receptor | female physiology | folliculogenesis | kit ligand

Premature ovarian failure (POF) is defined as an early decline of ovarian function after seemingly normal folliculogenesis (1). Genetic causes of POF have been frequently associated with X chromosome abnormalities (1, 2). Complete loss of one of the X chromosomes, as in Turner syndrome, and various Xq deletions are commonly identified as a cause of POF. However, responsible X-linked genes and their downstream targets have not been identified so far.

The androgen receptor (Ar) gene, which is the only sex hormone receptor gene on the X chromosome, is well known to be essential not only for the male reproductive system, but also for male physiology. In contrast, androgens are considered as male hormones; therefore, little is known about androgens' actions in female physiology, although AR expression in growing follicles has been described (3). However, because excessive androgen production in polycystic ovary syndrome causes infertility with abnormal menstrual cycles (4, 5), it is possible that AR-mediated androgen signaling also plays an important physiological role in the female reproductive system. Recently, using Cre/LoxP system, we generated an AR-null mutant mouse line (6) and demonstrated that inactivation of AR resulted in arrest of testicular development and spermatogenesis, impaired brain masculinization, high-turnover osteopenia, and late onset of obesity in males (7–9). At the same time, no overt physical or growth abnormalities were observed in female $AR^{-/-}$ mice. Therefore, to further examine potential role of AR in female physiology, we characterized female reproductive system in $AR^{-/-}$ females. Herein we show that female $AR^{-/-}$ mice develop the POF phenotype. At 3 weeks of age, $AR^{-/-}$ females had

apparently normal ovaries with numbers of follicles similar to those in the wild-type females. However, thereafter the number of healthy follicles in the $AR^{-/-}$ ovary gradually declined, with a marked increase of atretic follicles, and by 40 weeks $AR^{-/-}$ mice became infertile, with no follicle detectable in the ovary. Reflecting this age-dependent progression in ovarian abnormality, several genes known to be involved in the oocyte–granulosa cell regulatory loop were identified by microarray analysis as AR downstream target genes. These findings clearly demonstrate that AR-mediated androgen signaling is indispensable for the maintenance of folliculogenesis and implicate impaired androgen signaling as a potential cause of the POF syndrome.

Materials and Methods

Generation of AR Knockout Mice. AR genomic clones were isolated from a TT2 embryonic stem cell genomic library by using human AR A/B domain cDNA as a probe (6). The targeting vector consisted of a 7.6-kb 5′ region containing exon 1, a 1.3-kb 3′ homologous region, a single loxP site, and a neo cassette with two loxP sites (10). Targeted clones (FB-18 and FC-61) were aggregated with single eight-cell embryos from CD-1 mice (11, 12). Floxed AR mice (C57BL/6) were then crossed with CMV-Cre transgenic mice (6). The two lines exhibited the same phenotypic abnormalities. The chromosomal sex of each pup was determined by genomic PCR amplification of the Y chromosome Sry gene (13).

Western Blot Analysis. To detect AR protein expression, ovarian cell lysates were separated by SDS/PAGE and transferred onto nitrocellulose membranes (14). Membranes were probed with polyclonal AR antibodies (N-20; Santa Cruz Biotechnology), and blots were visualized by using peroxidase-conjugated second antibody and an ECL detection kit (Amersham Pharmacia Biosciences).

Morphologic Classification of Growing Follicles. Sections were taken at intervals of 30 μm, and 6-μm paraffin-embedded sections were mounted on slides. Routine hematoxylin and eosin staining was performed for histologic examination by light microscopy. Follicle numbers in 12 sections per ovary were evaluated as primary follicles (oocyte surrounded by a single layer of cuboidal granulosa cells), preantral follicles (oocyte surrounded by two or

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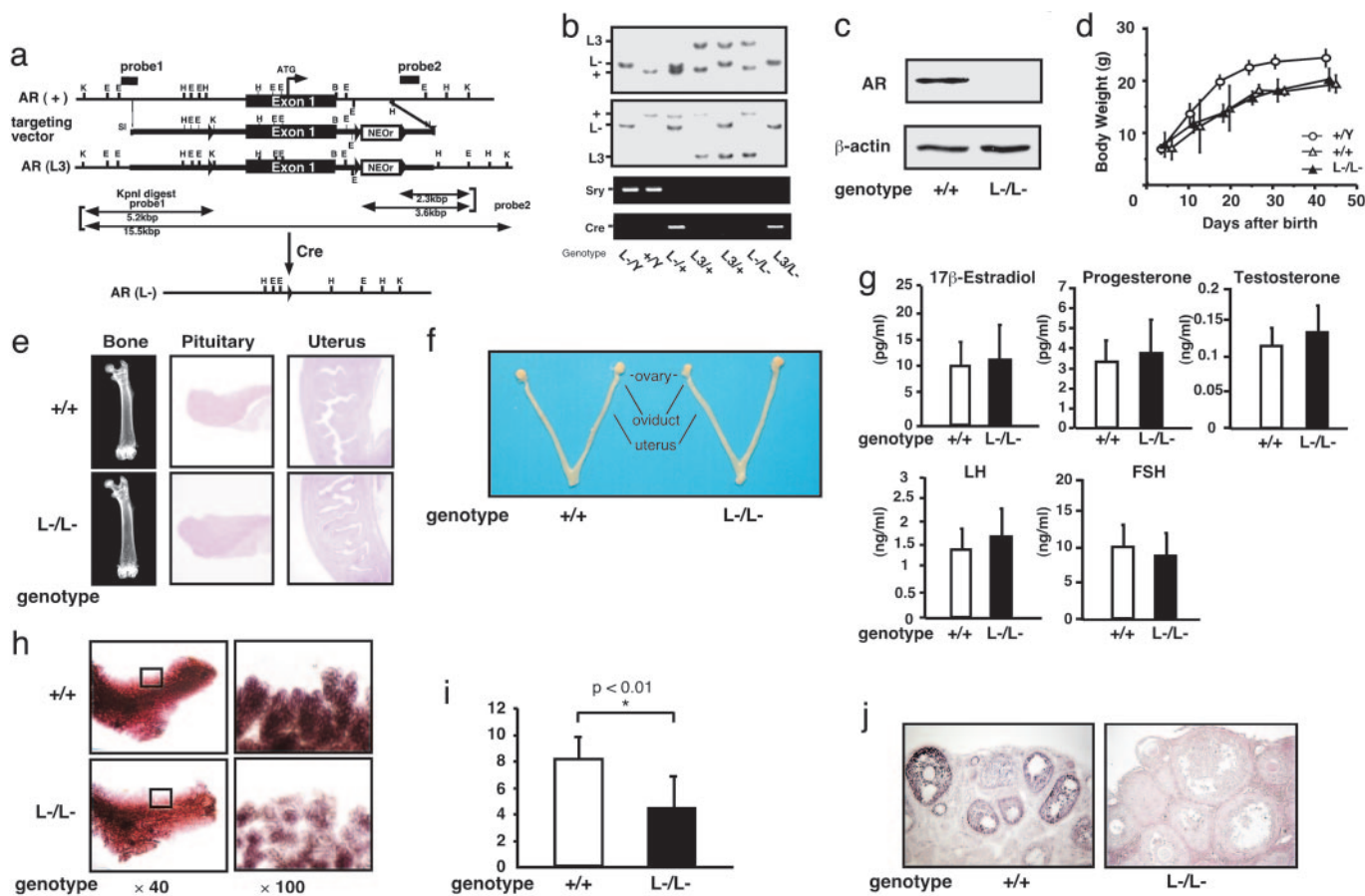
Fig. 1. Phenotypic characterization of AR knockout female mice. (*a*) Diagram of the wild-type *Ar* genomic locus (+), floxed *AR* L3 allele (L3), and *AR* allele (L-) obtained after Cre-mediated excision of exon 1. K, KpnI; E, EcoRI; H, HindIII; B, BamHI. LoxP sites are indicated by arrowheads. The targeting vector consisted of a 7.6-kb 5′ homologous region containing exon 1, a 1.3-kb 3′ homologous region, a single loxP site, and the neo cassette with two loxP sites. (*b*) Detection of the Y chromosome-specific *Sry* gene in $AR^{-/Y}$ mice by PCR. (*c*) Absence of AR protein in $AR^{-/-}$ mice ovaries by Western blot analysis using a specific C-terminal antibody. (*d*) Normal weight gain in $AR^{-/-}$ females. (*e*) Histology of pituitary, uterus, and bone tissues in $AR^{+/+}$ and $AR^{-/-}$ females at 8 weeks of age. (*f*) Female reproductive organs were macroscopically normal in $AR^{-/-}$ mice. (*g*) Serum hormone levels at the proestrus stage in $AR^{-/-}$ mice were not significantly altered. Serum 17β-estradiol, progesterone, testosterone, luteinizing hormone (LH), and follicle-stimulating hormone (FSH) levels in $AR^{+/+}$ ($n = 13$) and $AR^{-/-}$ ($n = 10$) females at 8–10 weeks of age are shown. (*h*) Lobuloalveolar development is impaired in $AR^{-/-}$ mammary glands. Whole mount of inguinal mammary glands (*Left*) and its higher magnification (*Right*) were prepared on day 3 of lactation. (*i*) Average number of pups per litter is markedly reduced in $AR^{-/-}$ mice at 8 weeks of age. Data are shown as mean ± SEM and analyzed by using Student's *t* test. (*j*) AR immunocytochemistry in $AR^{+/+}$ and $AR^{-/-}$ ovaries. Sections were counterstained with eosin.

more layers of granulosa cells with no antrum), or antral follicles (antrum within the granulosa cell layers enclosing the oocyte). Follicles were determined to be atretic if they displayed two or more of the following criteria within a single cross section: more than two pyknotic nuclei, granulosa cells within the antral cavity, granulosa cells pulling away from the basement membrane, or uneven granulosa cell layers (15).

Immunohistochemistry. Sections were subjected to a microwave antigen retrieval technique by boiling in 10 mM citrate buffer (pH 6.0) in a microwave oven for 30 min (16). The cooled sections were incubated in 1% H_2O_2 for 30 min to quench endogenous peroxidase and then incubated with 1% Triton X-100 in PBS for 10 min. To block nonspecific antibody binding, sections were incubated in normal goat serum for 1 h at 4°C. Sections were then incubated with anti-AR (1:100) or anti-cleaved caspase-3 (1:100) in 3% BSA overnight at 4°C. Negative controls were incubated in 3% BSA without primary antibody. The ABC method was used to visualize signals according to the manufacturer's instructions. Sections were incubated in biotinylated goat anti-rabbit IgG (1:200 dilution) for 2 h at room temperature, washed with PBS, and incubated in avidin–biotin–horseradish peroxidase for 1 h. After thorough washing in PBS, sections were developed with 3,3′-diaminobendizine tetra-hydrochloride substrate, slightly counterstained with eosin, de-hydrated through an ethanol series and xylene, and mounted.

Estrus Cycles and Fertility Test. To determine the stage of the estrus cycle (proestrus, estrus, and diestrus), vaginal smears were taken every morning and stained with Giemsa solution. For evaluation of female fertility for 15 weeks, an 8- or 24-week-old wild-type or $AR^{-/-}$ female was mated with a wild-type fertile male, replaced every 2 weeks with the other fertile male. Cages were monitored daily and for an additional 23 days, and the presence of seminal plugs and number of litters were recorded.

RNA Extraction and Quantitative Competitive RT-PCR. Total ovarian RNA was extracted by using TRIzol (Invitrogen) (16). Oligo-dT-primed cDNA was synthesized from 1 μg of ovarian RNA by using SuperScript reverse transcriptase (Gibco BRL, Gaithersburg, MD) in a 20-μl reaction volume, 1 μl of which was then diluted serially (2- to 128-fold) and used to PCR-amplify an internal control gene, *cycA*, to allow concentration estimation.
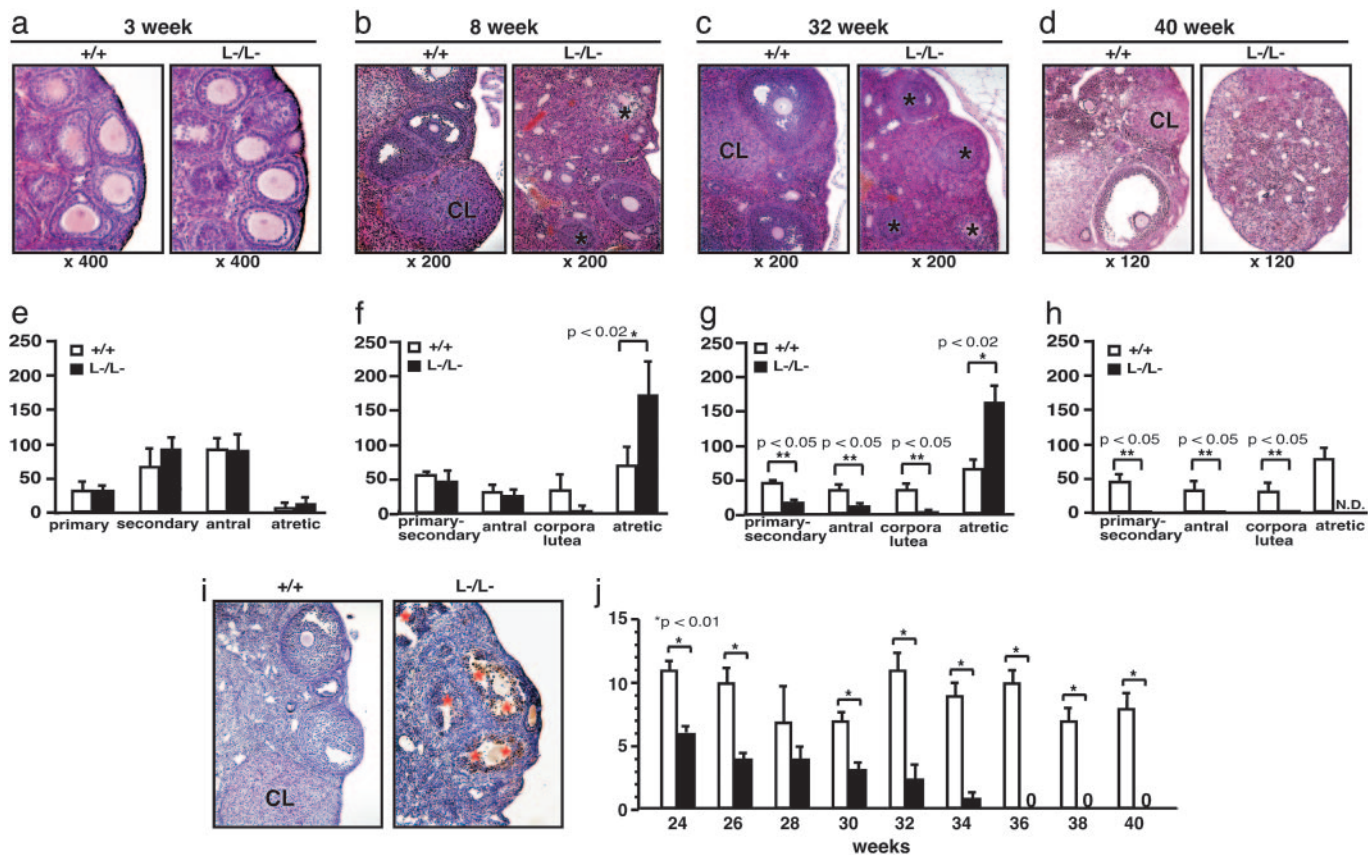
Fig. 2. POF in $AR^{-/-}$ female mice. (*a–d*) Histology of $AR^{+/+}$ and $AR^{-/-}$ ovaries at 3 weeks, 8 weeks, 32 weeks, and 40 weeks of age. All sections were stained with hematoxylin and eosin. An asterisk marks the atretic follicle. CL, corpus luteum. (*e–h*) Relative follicle counts at 3 weeks (*e*), 8 weeks (*f*), 32 weeks (*g*), and 40 weeks (*h*) of age. Numbers represent total counts of every fifth section from serially sectioned ovaries ($n = 4$ animals per genotype). (*i*) Immunohistochemical study for activated, cleaved caspase-3 revealed increased positive cells (apoptotic cells) in $AR^{-/-}$ ovaries. Sections were counterstained with hematoxylin. An asterisk marks the caspase-3-positive cell. CL, corpus luteum. (*j*) Age-dependent reduction in the number of pups per litter in $AR^{-/-}$ female mice. A continuous breeding assay was started at 24 weeks of age ($n = 6$–10 animals per genotype). For all panels, data are shown as mean $\pm$ SEM and were analyzed by using Student's t test.

Primers were designed from cDNA sequences of *Kitl* (M57647; nucleotides 1099–1751), Gdf9 (NM008110; nucleotides 720-1532), *Bmp15* (NM009757; nucleotides 146–973), *Ers2* (NM010157; nucleotides 1139–1921), *Pgr* (NM008829; nucleotides 1587–2425), *Cyp11a1* (NM019779; nucleotides 761-1697), *Cyp17al* (M64863; nucleotides 522–932), *Cyp19* (D00659; nucleotides 699-1049), *Fshr* (AF095642; nucleotides 625-1427), *Lhr* (M81310; nucleotides 592-1331), *Ptgs2* (AF338730; nucleotides 3–605), and *Ccnd2* (NM009829; nucleotides 150-1065) and chosen from different exons to avoid amplification from genomic DNA.

GeneChip Analysis. Ovaries were isolated and stabilized in RNA-*later* RNA Stabilization Reagent (Ambion, Austin, TX) before RNA purification (17). Total RNA was purified by using an RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. First-strand cDNA was synthesized from 5 μg of RNA by using 200 units of SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA), 100 pmol T7-(dT)$_{24}$ primer [5′-GGCCAGTGAATTGTAATACGACTCAC-TATAGGGAGGCGG-(dT)$_{24}$-3′], 1$\times$ first-strand buffer, and 0.5 mM dNTPs at 42°C for 1 h. Second-strand synthesis was performed by incubating first-strand cDNA with 10 units of *Escherichia coli* ligase (Invitrogen), 40 units of DNA polymerase I (Invitrogen), 2 units of RNase H (Invitrogen), 1$\times$ reaction buffer, and 0.2 mM dNTPs at 16°C for 2 h, followed by 10 units of T4 DNA polymerase (Invitrogen) and incubation for another

5 min at 16°C. Double-stranded cDNA was purified by using GeneChip Sample Cleanup Module (Affymetrix, Santa Clara, CA) according to the manufacturer's instructions and labeled by *in vitro* transcription by using a BioArray HighYield RNA transcript labeling kit (Enzo Diagnostics, Farmingdale, NY). Briefly, dsDNA was mixed with 1$\times$ HY reaction buffer, 1$\times$ biotin-labeled ribonucleotides (NTPs with Bio-UTP and Bio-CTP), 1$\times$ DTT, 1$\times$ RNase inhibitor mix, and 1$\times$ T7 RNA polymerase and incubated at 37°C for 4 h. Labeled cRNA was then purified by using GeneChip Sample Cleanup Module and fragmented in 1$\times$ fragmentation buffer at 94°C for 35 min. For hybridization to the GeneChip Mouse Expression Array 430A or 430B or Mouse Genome 430 2.0 Array (Affymetrix), 15 μg of fragmented cRNA probe was incubated with 50 pM control oligonucleotide B2, 1$\times$ eukaryotic hybridization control, 0.1 mg/ml herring sperm DNA, 0.5 mg/ml acetylated BSA, and 1$\times$ hybridization buffer in a 45°C rotisserie oven for 16 h. Washing and staining were performed by using a GeneChip Fluidic Station (Affymetrix) according to the manufacturer's protocol. Phycoerythrin-stained arrays were scanned as digital image files and analyzed with GENECHIP OPERATING SOFTWARE (Affymetrix) (17).

Luciferase Assay. The *Kitl* promoter region (-2866 to -1 bp) was inserted into the pGL3-basic vector (Promega) for assay using the Luciferase Assay System (Promega) (14, 16). Cells at 40–50% confluence were transfected with a reference pRL-CMV
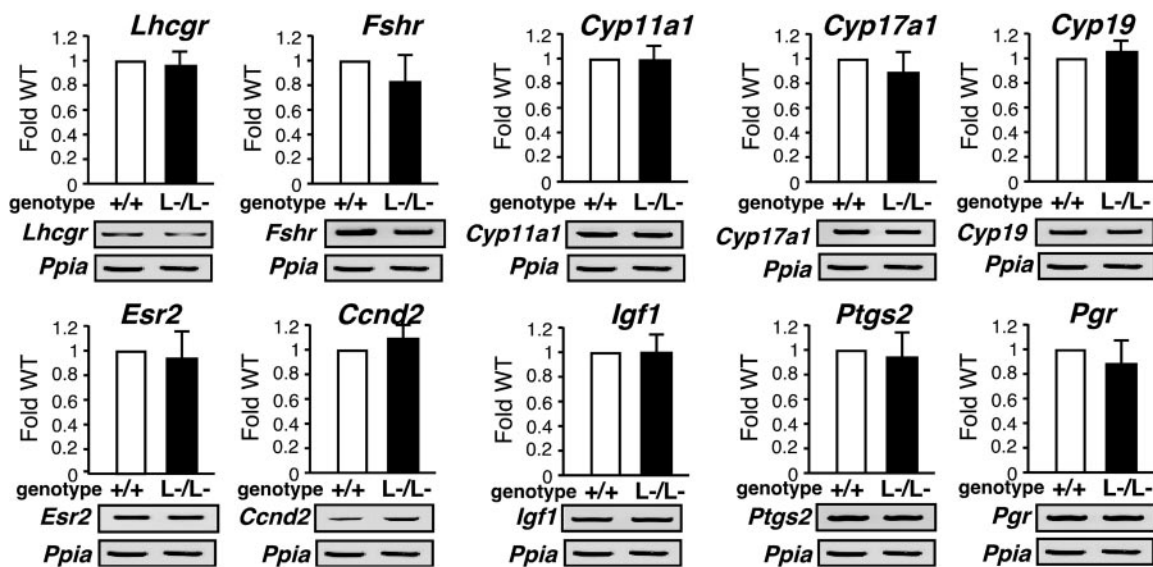
Fig. 3. No significant alterations in mRNA levels of several major regulators in folliculogenesis. Shown is semiquantitative RT-PCR of LH receptor (*Lhr*), FSH receptor (*Fshr*), p450 side chain cleavage enzyme (*Cyp11a1*), 17-α-hydroxylase (*Cyp17a1*), Aromatase (*Cyp19*), estrogen receptor-β (*Esr2*), cyclin D2 (*Ccnd2*), insulin-like growth factor 1 (*Igf1*), cyclooxygenase 2 (*Ptgs2*), or progesterone receptor (*Pgr*) gene expression in $AR^{+/+}$ and $AR^{-/-}$ ovaries. Results shown were representative (using one ovary per genotype in each experiment) of five independent experiments.

plasmid (Promega) using Lipofectamine reagent (GIBCO/BRL, Grand Island, NY) to normalize transfection. Results shown are representative of five independent experiments.

Results and Discussion

Subfertility of $AR^{-/-}$ Female Mice at 8 Weeks of Age. The *Ar* gene located on the X chromosome was disrupted in mice by using the Cre/Lox P system (6) (Fig. 1 *a–c*). Female $AR^{-/-}$ mice showed normal growth compared with the wild-type littermates (Fig. 1*d*), with no detectable bone loss (Fig. 1*e*) or obesity common for male $AR^{-/Y}$ mice (8, 9). Young (8-week-old) $AR^{-/-}$ females appeared indistinguishable from the wild-type littermates, displayed normal sexual behavior (7), and produced the first offspring of normal body size at the expected age. Macroscopic appearance of their reproductive organs, including uteri, oviducts, and ovaries, also appeared normal (Fig. 1*f*). Histological analysis showed no significant abnormality in the uterus or pituitary (Fig. 1*e*), whereas mammary ductal branching and elongation were substantially reduced, as revealed by whole-mount analysis (Fig. 1*h*). Serum levels of 17β-estradiol, progesterone, testosterone, luteinizing hormone, and follicle-stimulating hormone were also within normal range in 8-week-old mutant females at the proestrus stage (Fig. 1*g*), suggesting that the two-cell two-gonadotrophin system in female reproductive and endocrine organs (18) was intact in $AR^{-/-}$ mice at 8 weeks of age. The most obvious early sign of abnormal reproductive function in the $AR^{-/-}$ females was that their average numbers of pups per litter were only about half of those of the wild-type littermates, ($AR^{+/+}$, 8.3 ± 0.4 pups per litter; $AR^{-/-}$, 4.5 ± 0.5 pups per litter) (Fig. 1*i*).

$AR^{-/-}$ Female Mice Developed POF Phenotypes. Histological analysis of 8-week-old $AR^{-/-}$ ovaries clearly showed that numbers of atretic follicles were significantly increased, with decreased numbers of corpora lutea (Fig. 2 *b* and *f*). This finding suggests that the reduced pup numbers were due to impaired folliculogenesis in AR-deficient ovaries. Indeed, AR protein expression was readily detectable in the wild-type 8-week-old ovaries (Fig. 1*j*), with AR expressed at the highest levels in growing follicle granulosa cells at all developmental stages and at relatively low

levels in corpora lutea. Thus, AR appears to play a regulatory role in granulosa cells during their maturation to the luteal phase.

To investigate this possibility, we examined the ovarian phenotype of female $AR^{-/-}$ mice at different ages. At 3 weeks, ovaries contain various stages of follicles, including primary, secondary, and antral follicles in wild-type animals (Fig. 2*a*) (19). In $AR^{-/-}$ ovaries at 3 weeks of age, the folliculogenesis appeared to be unaltered, with normal numbers and localization of primary and secondary follicles (Fig. 2 *a* and *e*). However, degenerated folliculogenesis became evident with further aging. Although follicles and corpora lutea at all developmental stages were still present, corpora lutea numbers were clearly reduced in 8-week-old $AR^{-/-}$ mutants (Fig. 2 *b* and *f*), similar to that observed in another mouse line (20). Expected apoptosis was seen in atretic follicles by activated caspase-3 immunohistochemistry assays (Fig. 2*i*). But, by 32 weeks of age, defects in folliculogenesis in $AR^{-/-}$ ovaries became profound, with fewer follicles observed and increased atretic follicles (Fig. 2 *c* and *g*), and >40% (5 of 12 mice) of the $AR^{-/-}$ females were already infertile. By 40 weeks, all $AR^{-/-}$ females became infertile, with no follicles remaining (Fig. 2 *d* and *h*); at the same age, $AR^{+/+}$ females were fertile and had normal follicle numbers. Consistent with progressive deficiency in folliculogenesis, the pup number per litter steadily decreased in aging $AR^{-/-}$ females (Fig. 2*i*). These data indicate that AR plays an important physiological role at the preluteal phase of folliculogenesis.

Alteration in Gene Expressions of Several Major Regulators Involved in the Oocyte–Granulosa Cell Regulatory Loop. To explore the molecular basis underlying the impaired folliculogenesis in $AR^{-/-}$ ovaries, we analyzed expression of several major known regulators and markers of folliculogenesis (21–23). Surprisingly, no significant alterations in mRNA levels of LH receptor (*Lhr*), FSH receptor (*Fshr*), p450 side chain cleavage enzyme (*Cyp11a1*), 17-α-hydroxylase (*Cyp17a1*), aromatase (*Cyp19*), estrogen receptor-β (*Esr2*), cyclin D2 (*Ccnd2*), or insulin-like growth factor 1 (*Igf1*) of 8-week-old $AR^{-/-}$ ovaries at the proestrus stage, and further cyclooxygenase 2 (*Ptgs2*) or progesterone receptor (*Pgr*) at the estrus stage, were detected by

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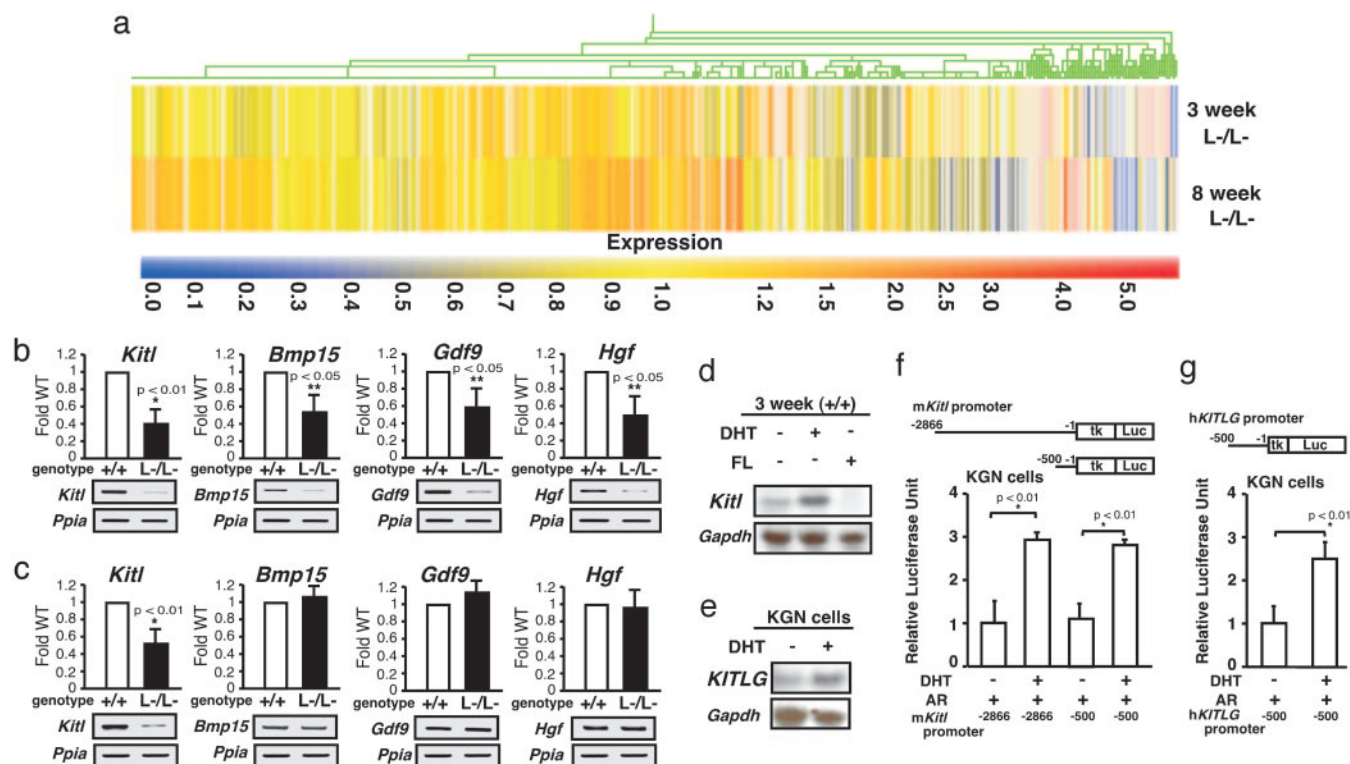
Fig. 4. Genome-wide microarray analysis and semiquantitative RT-PCR revealed that expression of the oocyte–granulosa cell regulator loop was down-regulated in $AR^{-/-}$ ovaries. (*a*) Microarray analysis of $AR^{-/-}$ compared with $AR^{+/+}$ ovaries at 3 and 8 weeks of age. Data obtained from microarray analysis as described in *Materials and Methods* were used to generate a cluster analysis. Each vertical line represents a single gene. The ratios of gene expression levels in $AR^{-/-}$ ovaries compared with wild type are presented. (*b* and *c*) Semiquantitative RT-PCR analysis of AR-regulated genes identified from the microarray study. Results shown are representative (using one ovary per genotype in each experiment) of five independent experiments. Data are shown as mean ± SEM and were analyzed by using Student's *t* test. (*d*) Comparison of *Kitl* gene expression by Northern blot analysis among placebo-, DHT-, and flutamide (FL)-treated $AR^{+/+}$ mouse ovaries. (*e*) Induction of *KITLG* gene expression by DHT treatment in KGN cells. (*f* and *g*) Androgen responsiveness in the mouse and human *kit ligand* promoters by a luciferase assay performed by using KGN cells. Data are shown as mean ± SEM and were analyzed by using Student's *t* test.

semiquantitative RT-PCR analysis (Fig. 3). Genome-wide microarray analysis (17) of RNA from 8-week-old $AR^{-/-}$ ovaries at the proestrus stage has been undertaken to identify AR-regulated genes. In comparison with $AR^{+/+}$ ovaries, expressions of 772 genes were down-regulated, whereas 351 genes were up-regulated in $AR^{-/-}$ ovaries (Fig. 4*a*; see also Tables 1 and 2, which are published as supporting information on the PNAS web site). Several genes known to be involved in the oocyte–granulosa cell regulatory loop (24) were identified as candidate AR target genes, including KIT ligand (*Kitl*) (25), morphogenetic protein 15 (*Bmp15*) (26), growth differentiation factor-9 (*Gdf9*) (27), and hepatocyte growth factor (*Hgf*) (28). Impaired folliculogenesis had been reported in mice deficient in each of these three regulators (26, 27, 29). To validate the microarray data, we performed semiquantitative RT-PCR analysis of 8-week-old $AR^{-/-}$ ovary RNA and confirmed that expression of these factors was down-regulated (Fig. 4*b*). To identify a regulator downstream of the AR signaling at an earlier stage of folliculogenesis, 3-week-old $AR^{-/-}$ ovaries that, as pointed out earlier, display no apparent phenotypic abnormality were examined. Fewer genes had altered expression levels (519 genes up-regulated; 326 genes down-regulated) (Fig. 4*a*; see also Tables 3 and 4, which are published as supporting information on the PNAS web site), and, of the four regulators tested by RT-PCR, only *Kitl* was found to be down-regulated at this age (Fig. 4*c*). Because *Kitl* is a granulosa cell-derived factor and stimulates oocyte growth and maturation (29–31), down-regulation of the *Kitl* expression in 3-week-old or even younger $AR^{-/-}$ ovaries may trigger impairment in folliculogenesis at a

later age. To test for possible *Kitl* gene regulation by AR, 3-week-old wild-type females were treated with 5α-dihydrotestosterone (DHT). At 4 h after hormone injection, a clear induction of *Kitl* expression was observed in the ovaries, whereas a known antiandrogen flutamide attenuated the induction by DHT (Fig. 4*d*). The induction of endogenous human *kit ligand* (*KITLG*) gene by DHT was also observed in human granulosa-like tumor cells (KGN) in culture (Fig. 4*e*). Furthermore, androgen-induced transactivation of mouse and human *kit ligand* promoters (32) was observed by a luciferase reporter assay (33) in KGN (Fig. 4 *f* and *g*), 293T, and HeLa (data not shown) cells. However, no response to DHT was detected in the similar assay using promoters of the *Bmp15*, *Gdf9*, and *Hgf* genes (data not shown). Thus, we have shown that, in a regulatory cascade controlling folliculogenesis, *Kitl* represents a direct downstream target of androgen signaling.

As an upstream regulator, AR may also be indirectly involved in control of expression of other genes critical for folliculogenesis, because an age-dependent down-regulation of *Bmp15*, *Gdf9*, and *Hgf* gene expression was also observed in $AR^{-/-}$ ovaries. *Bmp15* and *Gdf9* are oocyte-derived factors that promote the development of surrounding granulosa cells in growing follicles (34, 35), whereas *Hgf* is secreted by theca cells and acts as a granulosa cell growth factor (36). Down-regulation of these factors, presumably due to decreased *Kitl* expression, may lead to impaired bidirectional communication between oocyte and granulosa cells (24) and, eventually, to early termination of folliculogenesis, as in POF syndrome.

Thus, we have identified AR as a novel regulator of follicu-

logenesis that apparently acts in the regulatory cascade upstream of the major factors controlling ovarian function, confirming the previous findings of the AR expression in granulose cells of growing follicles (3). Although not immediately relevant to the ovarian physiology, abnormal development of the mammary glands observed in our AR-deficient mice adds further strong evidence of an essential role of the AR not only in male, but also in female, reproductive function.

With increasing age of the first childbirth by women in the modern society, POF syndrome has become an important social and medical problem. Our findings suggest that POF syndrome may be caused by an impairment in androgen signaling and that X chromosomal mutations affecting the *AR* gene function may play a key role in hereditary POF. From clinical perspective, the present study provides evidence that AR can be a beneficial therapeutic target in treatment of POF syndrome patients.

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