

# Anti-Müllerian Hormone Attenuates the Effects of FSH on Follicle Development in the Mouse Ovary

ALEXANDRA L. L. DURLINGER, MARIA J. G. GRUIJTERS, PIET KRAMER, BAS KARELS, T. RAJENDRA KUMAR, MARTIN M. MATZUK, URSULA M. ROSE, FRANK H. DE JONG, JAN TH. J. UILENBROEK, J. ANTON GROOTEGOED, AND AXEL P. N. THEMME

*Departments of Endocrinology and Reproduction (A.L.L.D., M.J.G.G., P.K., B.K., F.H.J., J.T.J.U., J.A.G., A.P.N.T.) and Internal Medicine (F.H.J.), Faculty of Medicine and Health Sciences, Erasmus University Rotterdam, Rotterdam, The Netherlands 3000; Departments of Pathology (T.R.K., M.M.M.), Molecular and Cell Biology (T.R.K., M.M.M.), and Molecular and Human Genetics (M.M.M.), Baylor College of Medicine, Houston, Texas 77030; and N.V. Organon, Department of Pharmacology (U.M.R.), Oss 5340 BH, The Netherlands*

Although ovarian follicle growth is under the influence of many growth factors and hormones of which FSH remains one of the most prominent regulators. Therefore, factors affecting the sensitivity of ovarian follicles to FSH are also important for follicle growth. The aim of the present study was to investigate whether anti-Müllerian hormone (AMH) has an inhibitory effect on follicle growth by decreasing the sensitivity of ovarian follicles to FSH. Furthermore, the combined action of AMH and FSH on ovarian follicle development was examined. Three different experiments were performed. Using an *in vitro* follicle culture system it was shown that FSH-stimulated preantral follicle growth is attenuated in the presence of AMH. This observation was confirmed by an *in vivo* experiment showing that in immature AMH-deficient females, more

follicles start to grow under the influence of exogenous FSH than in their wild-type littermates. In a third experiment, examination of the follicle population of 4-month-old wild-type, FSH $\beta$ -, AMH-, and AMH/FSH $\beta$ -deficient females revealed that loss of FSH expression has no impact on the number of primordial and preantral follicles, but the loss of inhibitory action of AMH on the recruitment of primordial follicles in AMH-deficient mice is increased in the absence of FSH. In conclusion, these studies show that AMH inhibits FSH-stimulated follicle growth in the mouse, suggesting that AMH is one of the factors determining the sensitivity of ovarian follicles for FSH and that AMH is a dominant regulator of early follicle growth. (*Endocrinology* 142: 4891–4899, 2001)

FSH IS REQUIRED FOR follicular growth from the large preantral to the preovulatory stage. This is clearly shown by the phenotype of FSH $\beta$ -deficient mice (1) or the hypogonadal mouse model (2), which are infertile owing to a block in folliculogenesis at the large preantral stage. Although preantral follicle growth can occur in the absence of FSH (1, 2), these follicles are able to respond to FSH. In prepubertal rats, for example, treatment with GnRH antagonist for several days severely suppresses the gonadotropin serum level and decreases the number of preantral follicles (3), whereas treatment with FSH of intact, hypophysectomized, or GnRH antagonist-treated juvenile rats, increased preantral follicle growth (4).

During the estrous cycle, FSH sensitivity of ovarian follicles seems to be important during the process of cyclic recruitment (5), a process by which some large preantral and small antral follicles are selected to grow to the preovulatory follicle stage, and the nonselected follicles will undergo atresia and disappear through apoptosis of the granulosa cells (6). Experiments have shown that the level of serum FSH is very important in this process. Elevated level of serum FSH at estrus prevents some, but not all, follicles to become atretic so that the former follicles are able to continue growth (7). In

this process of selection, the sensitivity of the individual follicle to FSH appears to be of central importance. Studies in bovines show that increased FSH sensitivity could be a result of enhanced FSH receptor expression (8, 9).

The development of ovarian follicles, however, is not regulated by FSH alone. Other intraovarian factors, such as E and growth factors, also are involved. Activins, inhibins, and growth and differentiation factor 9 (GDF9), all members of the TGF $\beta$  family of growth and differentiation factors, can influence follicular development (10–12). Using anti-Müllerian hormone-deficient female mice, we showed recently that anti-Müllerian hormone (AMH), another member of the TGF- $\beta$  family, inhibits the initiation of primordial follicle growth (13). In the same study, we found that AMH-deficient (AMHKO) female mice have an increased number of growing follicles in spite of a lower serum FSH level, compared with their wild-type littermates. Together with previous studies showing inhibitory effects of AMH on FSH-stimulated actions of cultured granulosa cells (14–16), this would suggest that AMH inhibits FSH-stimulated follicle growth. This hypothesis was studied using three different experimental approaches.

In an *in vitro* study, FSH-stimulated growth of preantral follicles was studied in the presence or absence of AMH. Subsequently, immature AMHKO females and their wild-type littermates were treated for several days with GnRH antagonist or with GnRH antagonist in combination with FSH after which follicle growth was examined. Finally, fol-

Abbreviations: AMH, Anti-Müllerian hormone; AMHKO, AMH-deficient; FAKO, AMH-/FSH $\beta$ -deficient; FSH $\beta$ KO, FSH $\beta$ -deficient; GDF9, growth and differentiation factor 9; HEK, human embryonic kidney.

licle growth was studied in 4-month-old AMH-/FSH $\beta$ -deficient (FAKO) females, by comparing the follicle population of the FAKO females with the follicle populations in AMHKO, FSH $\beta$ KO, and wild-type females of the same age.

## Materials and Methods

### Animals

Different strains of mice were used in the three experiments described in this paper. All strains were maintained on a C57Bl/6J background. Animals were kept under standard animal housing conditions in accordance with the NIH Guidelines for the Care and Use of Experimental Animals.

In Exp 1, F<sub>1</sub> female offspring (B6CBA) from C57Bl/6J females and CBA/J males from Harlan Winkelmann GmbH (Bohren, Germany) were used to collect 21- to 23-d-old ovaries. The mice were anesthetized with ether, and blood was collected by eye extraction. After clotting, blood was centrifuged for 15 min at 4,000  $\times$  g, and serum was collected and stored at -20 C until it was used in the follicle culture medium. The ovaries were used to isolate preantral follicles (17).

For Exp 2, wild-type and AMHKO female mice on a C57Bl/6J background were generated as described previously (13). Female mice of 25 d of age were divided into four groups, each group containing six wild-type and six AMHKO animals. The different treatment schedules are shown in Fig. 2A. All injections were given twice daily at 0900 h and at 2100 h. The animals were killed by decapitation 12 h after receiving the last injection. Group A was treated with saline from d 25 to d 28. Group B received during the same period 200  $\mu$ g GnRH antagonist per injection (Org 30276; NV Organon, Oss, The Netherlands). Two additional groups of mice received GnRH antagonist from d 25 to d 31. In addition to GnRH antagonist, one of these two groups received saline (group C), and the other group (group D) received 2.5 IU recombinant human FSH (Purgen; NV Organon) per injection from d 29 to d 31.

Blood samples were collected from all animals directly after decapitation, kept overnight at 4 C, and centrifuged the following day at 3000 rpm for 15 min at 4 C. Serum samples were stored at -20 C until assayed for FSH, inhibin A, or inhibin B.

In Exp 3, female and male mice heterozygous for both FSH $\beta$  and AMH null allele were used to obtain AMH-/FAKO females, FSH $\beta$ -deficient (FSH $\beta$ KO), AMHKO, and wild-type female mice. Male FSH $\beta$ KO mice that were used in the breedings were originally developed by Kumar *et al.* (1). FAKO females 4 months of age and their wild-type, FSH $\beta$ KO and AMHKO littermates were killed by decapitation at 1600 h. Wild-type and AMHKO females were killed on the day of estrus because this day of the cycle can be recognized by female behavior (13). Every group contained five animals.

In Exp 2 and 3, after decapitation ovaries were removed, weighed, and fixed overnight in Bouin's fluid. For histological examination of the follicle population, the fixed ovaries were embedded in paraffin, and after routine histological procedures, 8- $\mu$ m sections were mounted on slides and stained with hematoxylin and eosin. In addition, uteri of these mice were also weighed.

### Determination of mouse AMH and FSH genotype

The genotype of the mice used in Exp 2 and 3, were determined by PCR reactions. The AMH genotyping was described previously (13), but a different method for isolation of genomic DNA was used (18).

Primers FSH $\beta$ -FOR (5'-TTCAGCTTTCAGGAGAAGAG-3') and FSH $\beta$ -REV (5'-CTGCTGACAAAGAGTCTATG-3') were used to determine the presence of the FSH $\beta$  allele. Primer FSH $\beta$ -FOR anneals to nucleotide sequence 32–51 located in exon 1 of the FSH $\beta$  gene (numbering according to GenBank sequence, accession number U12932), whereas primer FSH $\beta$ -REV anneals to antisense sequence 278–259 located in intron 1 of the FSH $\beta$  gene, resulting in a PCR product of 247 bp. In animals carrying the FSH $\beta$  null allele, exons 1 and 2 and most of exon 3 are replaced by the PGK-Hprt expression cassette. Primer HPRT-FOR (5'-CCTGCTGGATTACAT-TAAAGCACT-3') and HPRT-REV (5'-GTCAAGGCA-TATCCAACAACAAA-3') were used to determine the presence of the FSH $\beta$  null allele, *i.e.* the PGK-hprt expression cassette (1). Primer HPRT-FOR anneals to the nucleotide sequence 318–341, whereas

primer HPRT-REV anneals to the antisense sequence 669–646 in the PGK-hprt expression cassette, resulting in a PCR product of 352 bp.

For the PCR reactions, 25 pmol of all primers were used, and the PCR reactions were executed as described previously (18). An annealing temperature of 45 C for the *wild-type* allele and 55 C for the FSH $\beta$  null allele was used. The products were electrophoresed on a 1.5% agarose gel.

### Ovarian histology and follicle counting

Serial 8- $\mu$ m sections of the ovaries were used for follicle counting, which was performed as described previously (13). In Exp 2, both ovaries were used to determine the follicle population. With the exception of primordial follicles (diameter < 20  $\mu$ m), both nonatretic and atretic follicles were included in the study. Atresia was defined according to the description of Osman (6). The follicles were divided into four groups on the basis of their mean diameter, which was determined by measuring two perpendicular diameters in the section in which the nucleolus of the oocyte was present: small preantral follicles (20–170  $\mu$ m), large preantral follicles (171–220  $\mu$ m), small antral follicles (221–310  $\mu$ m), and large antral follicles (311–370  $\mu$ m).

In Exp 3 the right ovary of each animal was used for follicle counting because we did not observe a difference in the composition of the follicle populations of the right and left ovaries of the same female at the day of estrus (unpublished observations). In this study, all follicles, both nonatretic and atretic, were counted, including primordial follicles. The follicles were divided into three groups on the basis of their mean diameter (micrometers); primordial follicles (diameter < 20  $\mu$ m), small follicles (20–310  $\mu$ m), and large follicles (diameter > 310  $\mu$ m). The number of atretic oocytes was also determined (13).

### AMH preparation

In the follicle culture experiment (Exp 1) recombinant rat AMH was used. Human embryonic kidney 293 (HEK293) cells were stably transfected with a cDNA encoding His-tagged rat AMH inserted in the pRc/CMV expression vector. The AMH cDNA contained an optimized cleavage site to ensure maximal amounts of cleaved mature AMH (19, 20). HEK293 cells were cultured in DMEM/F-12 (Life Technologies, Inc., Paisley, Scotland, UK) supplemented with 10% FCS, penicillin (400 IU/ml), streptomycin (0.4 mg/ml), and neomycin G418 (0.4 mg/ml). At a cell confluence of about 80–90%, the medium was replaced by medium without FCS. After 4 d the medium was collected.

His-tagged rat AMH was purified from the concentrated medium in a three-step procedure. First, proteins with a molecular mass above 10 kDa (including AMH) were concentrated approximately 35-fold using an Amicon filter system (Amicon/Millipore Corp., Bedford, MA). Next, the concentrated medium was run over a Ni-column (NiNTA Superflow, QIAGEN GmbH, Hilden, Germany) to separate the His-tagged AMH from other proteins; subsequently the His-tagged rat AMH was eluted from the Ni-column by imidazole (Sigma, St. Louis, MO). Finally, imidazole and AMH were separated using size chromatography (PD10 column, Amersham Pharmacia Biotech, Buckinghamshire, UK).

The amount of AMH was measured by ELISA, using the TMB peroxidase EIA substrate kit (Bio-Rad Laboratories, Inc., Hercules, CA). The primary antibody, pentaHis monoclonal antibody (QIAGEN), was used at 100 ng/ml. The secondary antibody, a goat-antimouse IgG peroxidase conjugate (Sigma), was used in a 1:1000 dilution. The amount of AMH was calibrated using the same standard preparation of His-tagged AMH in every ELISA and was expressed in arbitrary units. The concentrated supernatant of nontransfected HEK293 cells was used as control medium.

The presence of AMH in the media used in the experiments was investigated by Western blotting using a primary polyclonal antibody to AMH (C-20, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in a 1:2000 dilution and a secondary peroxidase-conjugated mouse-anti-goat/sheep antibody (Sigma) in a dilution of 1:10000 (data not shown).

The AMH preparations (800 U/ml) were shown to be bioactive using a Müllerian duct regression assay (19) (data not shown).

### Follicle collection and follicle culture

For each culture, the ovaries of at least four animals were collected aseptically and transferred into L-15 Leibovitz medium (Life Technol-

ogies, Inc.) supplemented with 0.3% BSA (Sigma), 5  $\mu\text{g}/\text{ml}$  insulin (Sigma), 2 mM glutamine (Life Technologies, Inc.), 10  $\mu\text{g}/\text{ml}$  transferrin (Sigma), 50  $\mu\text{g}/\text{ml}$  L-ascorbic acid (Sigma), and 2 ng/ml selenium (Sigma) at 37 C. Preantral follicles with a diameter of 135–210  $\mu\text{m}$  were isolated by needle dissection (29-gauge  $\frac{1}{2}$  in.) and collected in  $\alpha\text{MEM}$  medium (Life Technologies, Inc.) supplemented with 0.3% BSA, 5  $\mu\text{g}/\text{ml}$  insulin, 2 mM glutamine, 10  $\mu\text{g}/\text{ml}$  transferrin, 50  $\mu\text{g}/\text{ml}$  L-ascorbic acid, and 2 ng/ml selenium. Follicles from different ovaries were pooled in the collection medium and incubated in a humidified incubator, gassed with 5%  $\text{CO}_2$  in air at 37 C.

Isolated follicles with a normal morphological appearance (*i.e.*, a central spherical oocyte, high density of granulosa cells, and a theca cell layer enclosing the entire follicle) were divided into two groups with a diameter of 135–165  $\mu\text{m}$  or 165–210  $\mu\text{m}$  and were subsequently individually cultured in Millicel-CM culture plate inserts (Millipore Corp.) with 250  $\mu\text{l}$   $\alpha\text{MEM}$  culture medium supplemented with 5% immature mouse serum in a humidified incubator gassed with 5%  $\text{CO}_2$  in air at 37 C. Half of the number of follicles was cultured in the presence of purified rat AMH (800 U/ml). To induce follicle growth, 100 mIU/ml (10 ng/ml) recombinant human FSH (Puregon; NV Organon) was added to the culture medium after the first 24 h of culture. Culture medium was exchanged on culture d 1 and 4. The diameter of the follicles was measured on d 0, 1, 4, and 5 using 100 $\times$  magnification and a calibrated micrometer. In addition, on the same days, the survival rate of the follicles was checked by evaluation of degeneration (blackening of the follicle) and bursting (loss of oocyte). Three individual culture experiments were performed.

#### Hormone analyses

In Exp 2 serum FSH was determined by RIA using rat FSH as ligand and antibodies against ovine FSH (21). All results are expressed in terms of NIDDK rat FSH RP-2. The intraassay variation was 7.2%, and all samples were measured in one assay.

Inhibin A and B were measured using kits purchased from Serotec Limited (Oxford, UK), using the human standards provided with the kits. Suitability of the Serotec assays for measuring serum mouse inhibin

A and B dimers was investigated previously (22). The intraassay variation of the inhibin A assay was 9% and of inhibin B assay 15%. All samples were measured in one assay.

#### Statistical analysis

Results are presented as the mean  $\pm$  SEM. The data were evaluated for statistical differences either by one-way ANOVA, followed by Duncan's new multiple range test or by independent samples *t* test using SPSS, Inc. 9 (SPSS, Inc., Chicago, IL) computer software. Serum inhibin A and B levels were tested by a nonparametric test (Mann-Whitney test). Differences were considered significant at  $P \leq 0.05$ .

#### Results

##### Exp 1: Effect of AMH on FSH-stimulated preantral follicle growth *in vitro*

To determine the effect of AMH on FSH-stimulated follicle growth, preantral follicles with a mean diameter of 135–165  $\mu\text{m}$  (small preantral) and 165–210  $\mu\text{m}$  (large preantral) were cultured in the presence of FSH alone or in the presence of FSH and AMH.

In both follicle size groups, addition of exogenous AMH caused inhibition of FSH-stimulated preantral follicle growth in a time-dependent manner, indicated by the significantly smaller diameter of follicles cultured in the presence of AMH at d 4 and 5 of culture (Fig. 1). During the follicle culture, the increase in diameter was mainly the result of an increase in the number of granulosa cells (data not shown). The follicles with the larger size at the start of the culture maintained a larger diameter during the culture than the follicles with a smaller starting size.

The survival rates of the follicles with a mean diameter

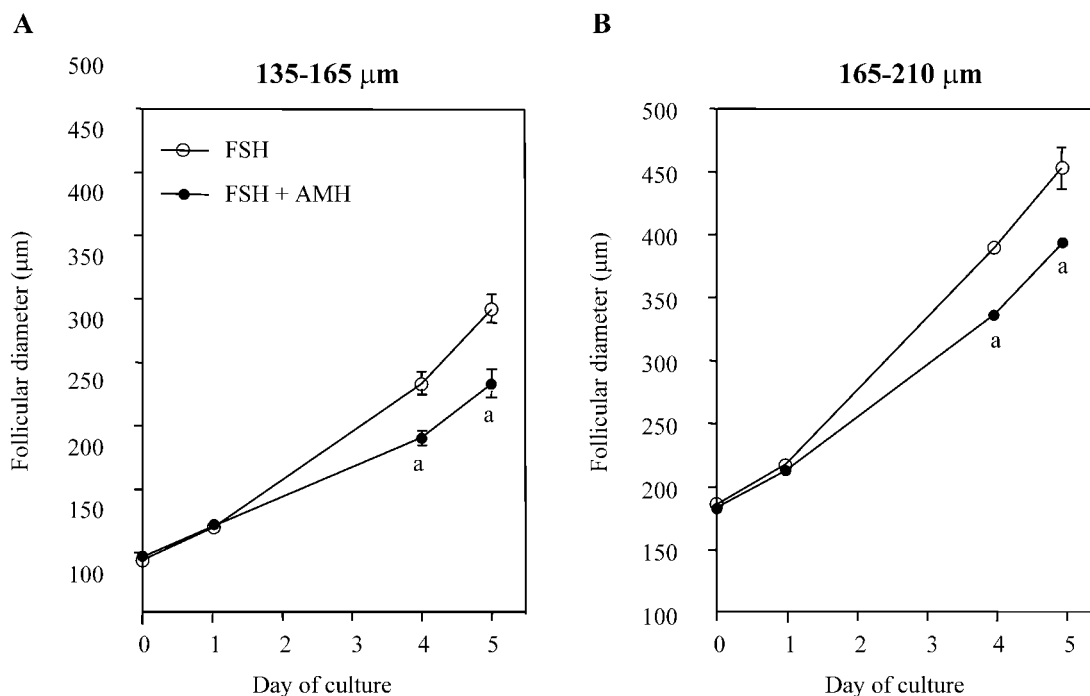


FIG. 1. Effect of AMH on FSH-stimulated preantral follicle growth *in vitro*. Preantral follicles with a diameter between 135 and 165  $\mu\text{m}$  (A) or between 165 and 210  $\mu\text{m}$  (B) at d 0 of culture were cultured for 5 d in the presence of recombinant FSH (100 mIU/ml), with or without AMH (800 U/ml). Follicle diameter was determined after 1, 4, and 5 d of culture. Data represent the mean  $\pm$  SEM (n ranging between 25 and 60). <sup>a</sup>, Significant difference from follicles cultured in the absence of AMH ( $P \leq 0.05$ ). Note that error bars on d 1 and 2 in Fig. 1, A and B and on d 3 in Fig. 1B are too small to be noticed.

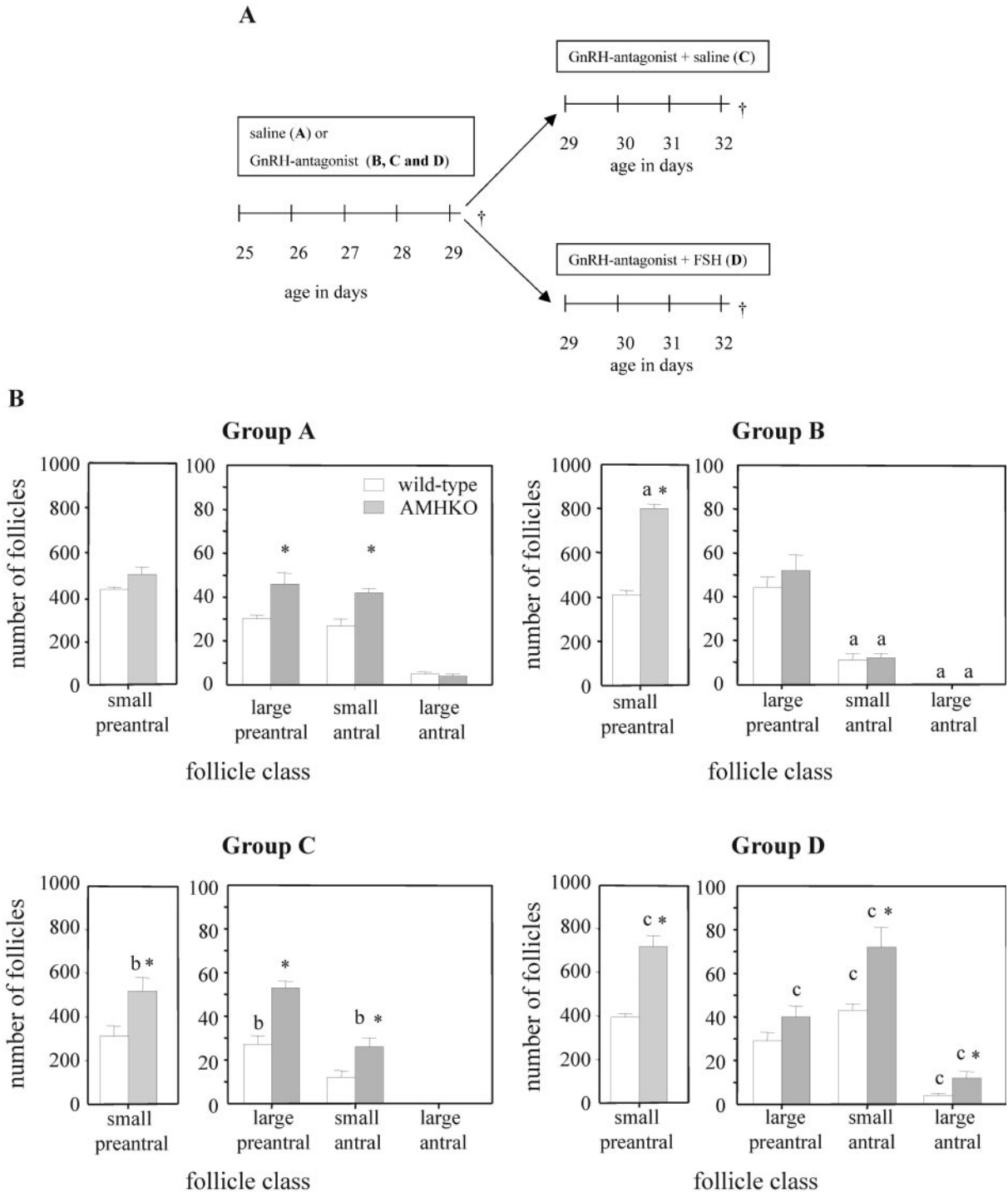


FIG. 2. Treatment of immature wild-type or AMHKO females with saline, GnRH antagonist, or GnRH antagonist in combination with saline or FSH. A, Groups A and B were treated twice daily from d 25 to d 28, with saline or GnRH antagonist (200  $\mu$ g), respectively. The animals were killed on d 29. Groups C and D were treated twice daily from d 25 to d 29 with GnRH antagonist, and from d 29 to d 31 with GnRH antagonist in combination with either saline or FSH (2.5 IU), respectively. The animals were killed on d 32. B, Number of follicles in different follicle classes. Follicles were divided into four groups on basis of their diameter: small preantral follicles (20–170  $\mu$ m), large preantral follicles (171–220  $\mu$ m), small antral follicles (221–310  $\mu$ m), and large antral follicles (311–370  $\mu$ m). Data represent the mean  $\pm$  SEM (n = 6). <sup>a, b, c</sup>, Significant difference from groups A, B, and C, respectively. \*, Significant difference from wild-type females within the same treatment group ( $P \leq 0.05$ ).

between 135 and 165  $\mu$ m or 165 and 210  $\mu$ m on d 4 and 5 were 83% and 56% or 97% and 93%, respectively, when cultured with only FSH, and 68% and 55% or 96% and 87% in the

presence of both FSH and AMH. No statistically significant difference was found for the survival rate between follicles cultured in absence or presence of AMH (results not shown).

### Exp 2: FSH-stimulated follicle growth in AMHKO females vs. wild-type females

The effect of AMH on FSH-stimulated follicle growth was also determined *in vivo*. Therefore, immature wild-type and AMHKO female mice were treated with GnRH antagonist to induce low-serum FSH levels or with GnRH antagonist in combination with FSH to obtain high levels of serum FSH. The treatment schedule is shown in Fig. 2A.

#### Serum FSH level

Serum FSH level was measured in groups A, B, and C to confirm the inhibitory effect of GnRH antagonist treatment on endogenous serum FSH level and to compare this level between wild-type and AMHKO females. The serum FSH level of group D could not be determined because the antibody used in the RIA partially cross-reacts with the exogenous FSH (23).

As expected, GnRH antagonist treatment resulted in a significantly reduced serum FSH level in mice of both genotypes (Table 1). Treatment with GnRH antagonist for 7 d (group C) instead of 4 d (group B) did not significantly lower the serum FSH level further. No significant difference in serum FSH level was found between wild-type and AMHKO females in the three treatment groups.

#### Uterine and ovarian weight

GnRH antagonist treatment suppressed both uterine and ovarian weights in wild-type females but not in AMHKO females (group B vs. group A), but FSH treatment increased ovarian weight in both genotypes (group D vs. group C) (Table 2). Within the treatment groups, a significant difference between the two genotypes was found only for the ovarian weight and only when a low serum level of FSH was present (groups B and C). In these groups the ovarian weight

was significantly higher in AMHKO females, compared with the ovarian weight of their wild-type littermates.

#### Follicle counts

The total number of small preantral, large preantral, small antral, and large antral follicles in both wild-type and AMHKO females of the four different treatment groups were determined and are shown in Fig. 2B.

In mice treated with saline from d 25 to d 28 (group A), the follicles reached the large antral stage in both genotypes, and no significant difference was found in the number of large antral follicles between wild-type and AMHKO females. In contrast, the number of large preantral and small antral follicles was significantly higher in AMHKO females than in wild-type females, whereas no significant difference was found between the two genotypes for the number of small preantral follicles. The decrease in serum FSH level in group B resulted in the expected decrease in the number of antral follicles in both genotypes. However, in AMHKO females a large increase in the number of small preantral follicles was found. An extended period of low-serum FSH level (group C) caused a significant decrease in the number of large preantral follicles in wild-type females, but this number was not affected in AMHKO females. Notwithstanding the low serum FSH level, the number of small antral follicles was increased in AMHKO ovaries, but it was not affected in wild-type females. Administration of FSH (group D) resulted in the presence of large antral follicles and in an increase in the number of small antral follicles in both genotypes. However, this increase in follicle number and size was more pronounced in AMHKO mice.

#### Serum inhibin A and B levels

To determine whether the different treatments affected serum inhibin A and B levels, serum levels of both hormones were determined in all treatment groups.

In all groups, serum inhibin B level was higher than the serum inhibin A level (Table 3). Treatment of the animals with GnRH antagonist resulted in a decrease of both serum inhibin A and B levels in both genotypes, although for inhibin B, this decrease was not significant in AMHKO mice. Treatment with GnRH antagonist together with FSH resulted in an increase of both inhibins.

Within all treatment groups, no significant difference in the concentrations of both inhibins was found between wild-type and AMHKO females, with the exception of the serum

**TABLE 1.** Serum FSH level in immature wild-type and AMHKO females of different treatment groups

Treatment group	FSH (ng/ml)	
	Wild-type	AMHKO
A (saline, d 25–28)	14.8 ± 1.4	14.5 ± 1.3
B (GnRH antagonist, d 25–28)	5.3 ± 0.4 <sup>a</sup>	5.4 ± 1.1 <sup>a</sup>
C (GnRH antagonist, d 25–31)	4.9 ± 0.4	4.7 ± 0.3

Values represent the mean ± SEM (n = 6). Animals were killed on d 29 (groups A and B) or d 32 (group C).

<sup>a</sup> Significant difference from group A. Values were evaluated by independent samples *t* test (*P* ≤ 0.05).

**TABLE 2.** Uterine and ovarian weights in wild-type and AMHKO female mice of different treatment groups

Treatment group	Ovarian weights (mg)		Uterine weight (mg)	
	Wild-type	AMHKO	Wild-type	AMHKO
A (saline, day 25–28)	4.5 ± 0.2	4.5 ± 0.4	13.2 ± 0.4	14.4 ± 2.1
B (GnRH antagonist, day 25–28)	3.2 ± 0.3 <sup>a</sup>	4.0 ± 0.2 <sup>b</sup>	11.9 ± 0.3 <sup>a</sup>	10.4 ± 1.1
C (GnRH antagonist, day 25–31 + saline day 29–31)	2.3 ± 0.2	3.6 ± 0.2 <sup>b</sup>	7.3 ± 1.1	7.7 ± 0.4
D (GnRH antagonist, day 25–31 + FSH, day 29–31)	4.5 ± 0.3 <sup>c</sup>	6.3 ± 0.5 <sup>c</sup>	8.2 ± 0.7	7.6 ± 0.7

Values represent the mean ± SEM (n = 6). Animals were killed on d 29 (groups A and B) or d 32 (groups C and D).

<sup>a,c</sup> Significant difference from groups A and C, respectively. <sup>b</sup> Significant difference from wild-type females. Values were evaluated by independent-samples *t* test (*P* ≤ 0.05).

**TABLE 3.** Serum inhibin A and B levels in immature wild-type and AMHKO females of different treatment groups

Treatment group	Inhibin A (ng/liter)		Inhibin B (ng/liter)	
	Wild-type	AMHKO	Wild-type	AMHKO
A (saline, d 25–28)	24 ± 3	16 ± 2	72 ± 9	52 ± 5
B (GnRH antagonist, d 25–28)	<5 <sup>a</sup>	<5 <sup>a</sup>	13 ± 3 <sup>a</sup>	30 ± 6 <sup>b</sup>
C (GnRH antagonist, d 25–31 + saline, d 29–31)	<5	<5	10 ± 2	14 ± 2
D (GnRH antagonist, d 25–31 + FSH, d 29–31)	59 ± 19 <sup>c</sup>	90 ± 17 <sup>c</sup>	266 ± 85 <sup>c</sup>	317 ± 60 <sup>c</sup>

Values represent the mean ± SEM (n = 6). Animals were killed on d 29 (groups A and B) or d 32 (groups C and D).

<sup>a,c</sup> Significant difference from groups A and C, respectively. <sup>b</sup> Significant difference from wild-type females. Values were evaluated by a nonparametric test (Mann-Whitney test) ( $P \leq 0.05$ ).

inhibin B level in group B, which was slightly but significantly higher in AMHKO females than in wild-type females.

### Exp 3: Comparison of follicle growth in 4-month-old wild-type, AMHKO and FSH $\beta$ KO, and FAKO females

The long-term effects of the absence of FSH and/or AMH on the ovarian follicle population were investigated *in vivo* in 4-month-old AMHKO, FSH $\beta$ KO, and FAKO mice.

#### Ovarian and uterine weight

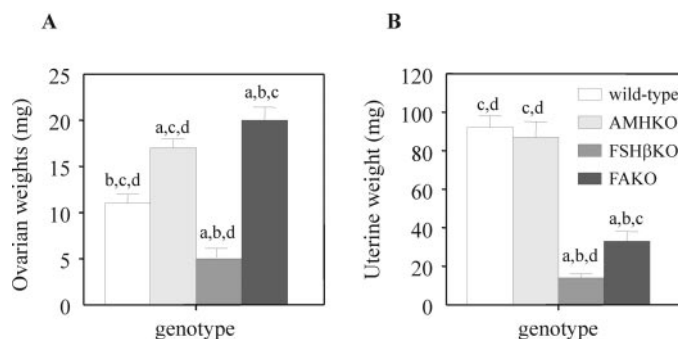
In FSH $\beta$ KO mice ovarian weight was significantly decreased, but ovarian weight was increased in AMHKO mice, compared with wild-type mice (Fig. 3A). In FAKO females, the effect of the absence of AMH on ovarian weight was further augmented, resulting in a slight but significantly larger ovarian weight than in AMHKO females.

Absence of FSH caused a large decrease in uterine weight in both FSH $\beta$ KO and FAKO females. This decrease, however, was less pronounced in FAKO females (Fig. 3B). The absence of only AMH did not affect uterine weight.

#### Ovarian morphology and follicle counts

The ovaries of all genotypes contained primordial, preantral, small antral follicles, both nonatretic and atretic, and atretic oocytes (Fig. 4, A–D). As expected, no large antral follicles were found in FSH $\beta$ KO and FAKO females (Fig. 4, C and D) because of the lack of FSH in these animals, but in wild-type and AMHKO females, large follicles were absent (Fig. 4, A and B) because at estrus these follicles have not yet developed in mice with a normal estrous cycle. In wild-type and AMHKO females, numerous fresh and old corpora lutea were present (Fig. 4A). The ovaries of FSH $\beta$ KO females were small owing to the lack of corpora lutea but contained follicles that had progressed to the small antral stage (Fig. 4C). In contrast, despite the lack of corpora lutea, ovaries of FAKO females were large and contained many small preantral follicles and small antral follicles (Fig. 4D). Furthermore, in these ovaries many remnants of atretic follicles, also referred to as atretic oocytes, were found (Fig. 4, E and F).

Figure 5 shows the effects of the absence of AMH and/or FSH on the number of follicles in the different follicle classes. Two-fold more nonatretic small follicles were found in the absence of AMH (AMHKO), and this effect was strongly increased in the additional absence of FSH (FSH $\beta$ KO) (Fig. 5B). Absence of FSH alone did not affect the number of nonatretic small follicles. In all genotypes the changes in the number of atretic small follicles and atretic oocytes were similar to the changes in the number of nonatretic small



**FIG. 3.** Uterine and ovarian weight in 4-month-old wild-type, AMHKO, FSH $\beta$ KO, and FAKO female mice. The combined weight of both ovaries (A) and uterine weight (B) of wild-type, AMHKO, FSH $\beta$ KO, and FAKO mice was determined. Data represent the mean ± SEM (n = 4). <sup>a, b, c, d</sup>, Significant difference from wild-type, AMHKO, FSH $\beta$ KO, and FAKO females, respectively ( $P \leq 0.05$ ).

follicles (Fig. 5, C and D). The increase in the number of small follicles in AMHKO and FAKO females is reflected by a concomitant decrease in the number of primordial follicles (Fig. 5A).

## Discussion

Previously we have reported a quantitative analysis of the follicle population in AMHKO females, which revealed that AMH inhibited the recruitment of primordial follicles (13). In the same study, more preantral and small antral follicles were found in 4-month-old AMHKO females, compared with wild-type females, despite a relatively low serum FSH level, suggesting an effect of AMH on the sensitivity of growing follicles to FSH.

In the present study, we have studied the effect of AMH on the FSH sensitivity of growing follicles more in detail. In an *in vitro* follicle culture, we found that AMH inhibits FSH-stimulated growth of mouse preantral follicles. However, in another recent *in vitro* study (24), it was reported that AMH enhanced FSH-stimulated growth of rat preantral follicles. Two possible explanations for these contradictory results can be put forward. Animal age (prepubertal *vs.* adult) could be the cause, as was demonstrated by its influence on the effect of activin A and TGF $\beta$ , like AMH members of the TGF $\beta$  superfamily, on preantral follicle growth. Although activin A stimulates growth of preantral follicles from immature mice and not from adult mice, TGF $\beta$  stimulates growth of preantral follicles derived from adult mice and not from immature mice (25, 26). Besides age, the developmental stage (*e.g.*, number of granulosa cell layers) of the follicle can also be of

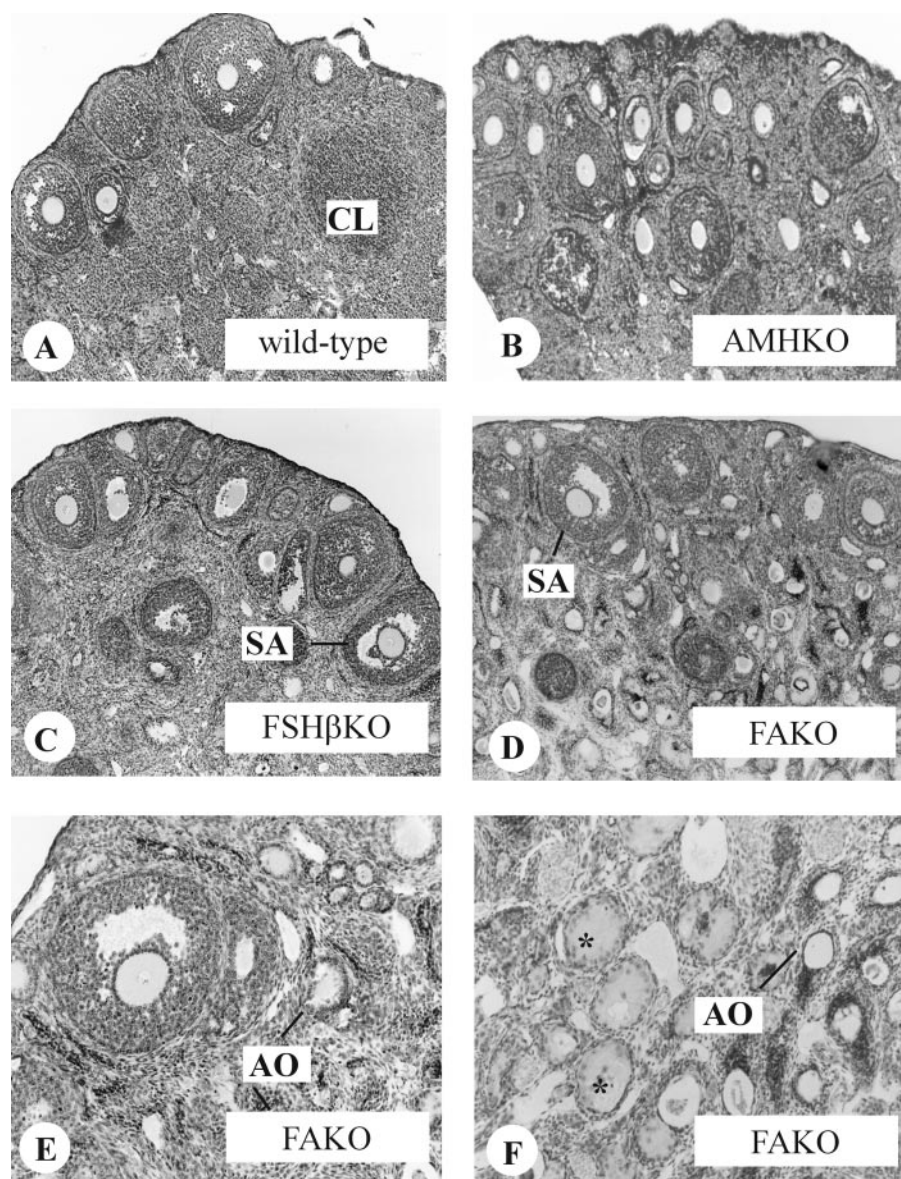


FIG. 4. Photomicrographs of ovarian sections of 4-month-old wild-type, AMHKO, FSH $\beta$ KO, and FAKO female mice. A, B, Section of an ovary of a wild-type mouse (A) and of an AMHKO mouse (B). Follicles of different developmental stages are found in both genotypes. In the wild-type ovary, a fresh corpus luteum (CL) is present. Magnification  $\times 100$ . C, D, Section of an ovary of an FSH $\beta$ KO mouse (C) and a FAKO mouse (D). In both FSH $\beta$ KO and FAKO females, follicle development occurs in the small antral stage (SA). Magnification,  $\times 100$ . E, F, Sections of an ovary of a FAKO mouse. Besides small antral follicles, many remnants of atretic follicles, also referred to as atretic oocytes (AO), are found in the interstitium. In some the degenerating oocyte can be clearly seen (\*). Magnification,  $\times 200$ .

importance for the outcome of follicle culture experiments because we found that growth of preantral follicles with a diameter between 165 and 210  $\mu\text{m}$  derived from immature mice was stimulated by GDF9, but smaller follicles (135–165  $\mu\text{m}$ ) were unaffected by GDF9 (our unpublished results). However, because in both our study and the study of McGee *et al.* (24) preantral follicles with seven to nine layers of granulosa cells from immature animals were used, a difference in age or developmental stage of the follicle cannot readily be used to explain the difference in outcome. The difference, however, could be the result of the use of mouse serum in the culture medium in our study, suggesting that the serum might contain factors influencing the action of AMH.

The inhibitory effect of AMH on preantral follicle growth *in vitro* is in agreement with previous studies in cultured granulosa cells in which AMH was shown to negatively influence FSH-induced processes, such as a decrease of the aromatase activity and LH receptor number and the inhibi-

tion of progesterone synthesis (14–16). In addition, the result of our *in vitro* study is in accordance with the result of our *in vivo* study, in which follicle growth in the presence of high or low serum FSH level was compared between wild-type and AMHKO females. Growth of large preantral and small antral follicles in AMHKO mice is less affected by longtime exposure to low serum FSH level than in wild-type females (group C *vs.* group B). Furthermore, in the presence of high serum FSH level (group D), stimulation of follicle growth was more pronounced in AMHKO females than in wild-type females both in terms of numbers and of developmental stage. These findings indicate that in the absence of AMH, follicle growth is more sensitive to stimulation by FSH.

Both *in vivo* studies show that ovarian as well as uterine weight can indicate changes in the follicle population. Although in adult mice the ovarian weight is mainly determined by the mass of corpora lutea, as is clearly illustrated by the 5-fold decrease in weight of the anovulatory ovaries of FSH $\beta$ KO females, compared with the weight of wild-type

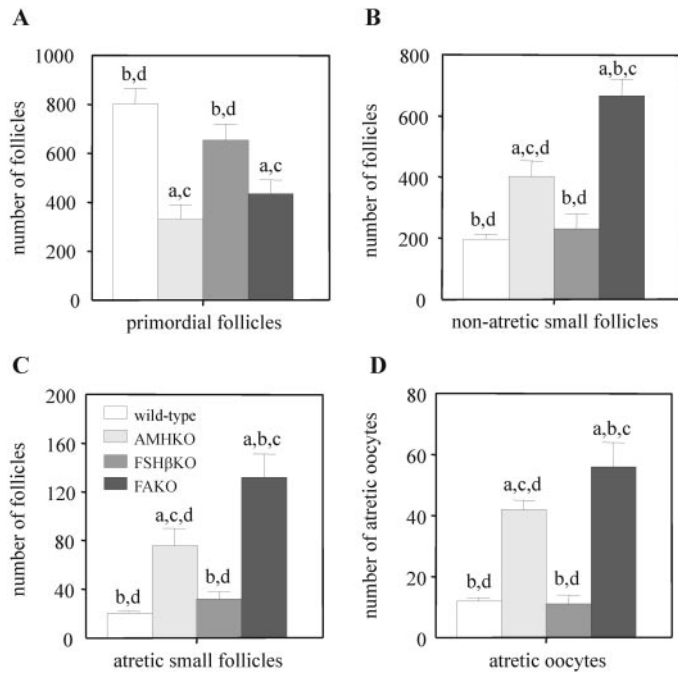


FIG. 5. Follicle population in 4-month-old wild-type, AMHKO, FSH $\beta$ KO, and FAKO female mice. A–D, Number of primordial follicles (A), nonatretic small follicles (B), atretic small follicles (C), and atretic oocytes (D). Data represent the mean  $\pm$  SEM ( $n = 4$ ). a, b, c, d, Significant difference from wild-type, AMHKO, FSH $\beta$ KO, and FAKO females, respectively ( $P \leq 0.05$ ). Note the difference in scale of the y-axis among the different graphs.

ovaries (1) (and present results), a previous study on AMHKO females (13) and this study showed that a high number of preantral follicles can significantly increase ovarian weight. This observation is supported by the ovarian weight of FAKO females, which is even further augmented by the very high number of small follicles. Uterine weight can be informative because the uterine epithelium is strongly stimulated by E2 (27), produced mainly by large antral and preovulatory follicles, although small antral and even preantral follicles are able to produce small amounts of E as well. In both *in vivo* studies, the changes in uterine weight clearly support the histological observations in the ovaries.

In addition to the ovarian and uterine weights, serum inhibin can also be used to register changes in the ovarian follicle population. Inhibin A level was high in mice having large antral follicles in their ovaries, but inhibin B level was high in mice predominantly having preantral and small antral follicles. These results are in accordance with follicle culture experiments using immature mouse follicles showing that inhibin A is predominantly produced by large antral follicles and inhibin B by preantral and small antral follicles (28).

Going back to the respective roles and interaction of AMH and FSH in the ovary, the results of cross-breeding FSH $\beta$ KO and AMHKO animals (Exp 3) clearly indicate that AMH is a dominant regulator of early follicle growth. FSH does not appear to be essential in preantral follicle growth because in both AMHKO and FAKO females, less primordial and more growing follicles were found. Indeed, even more growing follicles are found in ovaries of FAKO females, probably as

a result of accumulation of these follicles because the absence of FSH causes a block of follicular growth at the large preantral/small antral follicle stage. It is known that FSH is not necessary for preantral follicle growth, but because preantral follicles are sensitive to FSH, one might expect that a complete loss of FSH production in mice would lead to some effects on preantral follicle growth. However, comparison of the follicle population between wild-type and FSH $\beta$ KO females revealed that preantral follicle growth occurs to the same extent in both genotypes. In addition, no effect was seen in the absence of FSH on the primordial follicle pool. This is in contrast to several studies in which the pool of primordial and growing follicles was influenced by decreasing or increasing gonadotropin levels, either by hypophysectomy in adult mice or unilateral ovariectomy in aged rats (29, 30). The only difference between these experimental animal models and the model used in this study, the FSH $\beta$ KO mouse model, is that in the present model, only FSH production is absent, but in the other models both FSH and LH and/or other hormones or growth factors are affected. In particular, it has been shown that LH can stimulate primordial follicle recruitment because in mice overexpressing LH increases the outgrowth of primordial follicles (31). Until now, no data are available about other factors from the pituitary gland, which might exert an effect on recruitment of primordial follicles.

The question of by which mechanisms AMH inhibits the stimulatory effect of FSH on follicle growth remains. Unlike other members of the TGF $\beta$  superfamily, little is known about the AMH signaling pathway. Members of this family signal via complexes containing type I and II receptors. So far only an AMH type II receptor has been identified (32). More insight into the signaling pathway of AMH will be gained after identification of the AMH type I receptor. Only recently the activin receptor-like kinase 2 (33, 34) and 6 (35) have been identified as candidate AMH type I receptors.

The inhibitory mechanism could involve an effect of AMH on FSH receptor expression. A change in the expression of the FSH receptor may change the sensitivity of a follicle to FSH, as was demonstrated by studies in the bovine (8, 9). However, several studies have shown that, besides FSH effects on the ovary, AMH can also inhibit similar effects induced by cAMP, which is the second messenger of FSH (14, 36, 37). This would suggest that the molecular target site of AMH action is downstream of the FSH receptor.

This study provides evidence for an inhibitory role of AMH on FSH-stimulated preantral follicle growth. Regulation of the sensitivity of large preantral follicles to FSH by AMH could be important during cyclic recruitment because FSH is a crucial regulator of this process. A possible role of AMH in cyclic recruitment is indicated by the presence at estrus of a group of nonatretic large preantral/small antral follicles with high AMH mRNA expression and a group with much lower AMH mRNA expression, whereas these follicles were otherwise indistinguishable (38). The increased sensitivity to FSH of the follicles with low AMH mRNA expression may allow these follicles to be selected for continued growth and ovulation in the next estrous cycle. The inhibitory action of AMH on growth stimulation by FSH can also be relevant for small preantral follicles, even though these follicles do not depend on FSH for their growth. Besides



factors that stimulate preantral follicle growth, like FSH (3, 4), stem cell factor (39), and GDF9 (40), other inhibitory factors, such as AMH, are important to make preantral follicle growth a well-balanced process.

Taken altogether, ovarian follicle growth is under the influence of many growth regulatory factors, and from the present study, it can be concluded that AMH is one of these regulatory factors. Besides inhibiting the outgrowth of primordial follicles, AMH is also able to inhibit FSH-stimulated follicle growth, by diminishing the sensitivity of the follicle for FSH. Furthermore, AMH proves to be a more dominant regulator of early follicle growth than FSH.

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Address all correspondence and requests for reprints to: Axel P. N. Themmen, Department of Endocrinology and Reproduction, Faculty of Medicine and Health Sciences, Erasmus University Rotterdam, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands. E-mail: themmen@endov.fgg.eur.nl.

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