

Control of Primordial Follicle Recruitment by Anti-Müllerian Hormone in the Mouse Ovary*

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ABSTRACT

The dimeric glycoprotein anti-Müllerian hormone (AMH) is a member of the transforming growth factor- β superfamily of growth and differentiation factors. During male fetal sex differentiation, AMH is produced by Sertoli cells and induces degeneration of the Müllerian ducts, which form the anlagen of part of the internal female genital system. In females, AMH is produced by the ovary, but only postnatally. The function of AMH in the ovary is, however, still unknown. Female AMH *null* mice were reported to be fertile, with normal litter size, but this does not exclude a more subtle function for ovarian AMH.

To investigate the function of AMH in the ovary, the complete follicle population was determined in AMH *null* mice, in mice heterozygous for the AMH *null* mutation, and in wild-type mice of different ages: 25 days, 4 months, and 13 months. In the present study we found that ovaries of 25-day- and 4-month-old AMH *null* females, compared to those of wild-type females, contain more preantral and small antral follicles. In addition, in 4- and 13-month-old AMH *null* females, smaller numbers of primordial follicles were found. Actually, in 13-month-old AMH *null* females, almost no primordial follicles could be detected, coinciding with a reduced number of preantral and

small antral follicles in these females. In almost all females heterozygous for the AMH *null* mutation the number of follicles fell in between the numbers found in wild-type and AMH *null* females. In 4-month-old AMH *null* females serum inhibin levels were higher and FSH levels were lower compared to those in wild-type females. In contrast, inhibin levels were lower in 13-month-old AMH *null* females, and FSH levels were unchanged compared to those in wild-type females. Furthermore, the weight of the ovaries was twice as high in the 4-month-old AMH *null* females as in age-matched wild-type females.

We conclude that AMH plays an important role in primordial follicle recruitment, such that more primordial follicles are recruited in AMH *null* mice than in wild-type mice; the mice heterozygous for the AMH *null* mutation take an in-between position. Consequently, the ovaries of AMH *null* females and those of females heterozygous for the AMH *null* mutation will show a relatively early depletion of their stock of primordial follicles. The female AMH *null* mouse may thus provide a useful model to study regulation of primordial follicle recruitment and the relation between follicular dynamics and ovarian aging. (*Endocrinology* 140: 5789–5796, 1999)

ANTI-MÜLLERIAN HORMONE (AMH), also called Müllerian-inhibiting substance, is a member of the transforming growth factor- β superfamily of peptide growth and differentiation factors (1). During male fetal sex differentiation, AMH is synthesized by testicular Sertoli cells and induces degeneration of the Müllerian ducts, which form the anlagen of the uterus, the oviducts, and the upper part of the vagina (2). During female fetal development, no ovarian AMH production occurs (3). However, AMH messenger RNA (mRNA) expression is detected in ovarian granulosa cells from postnatal day 3 onward (3). Immunohistochemical (4, 5) and mRNA *in situ* hybridization (3, 6) studies in rats revealed specific expression of AMH and its type II receptor (AMHR II) in granulosa cells of mainly nonatretic preantral and small antral follicles, whereas the signal was lost in nonatretic large antral follicles and atretic follicles of all size classes (6). During the estrous cycle, no marked changes were detected in the patterns of AMH and AMHR II mRNA expression, except at estrus, when a heterogeneous decrease in

the expression of both mRNAs was found in nonatretic preantral follicles compared with the more homogeneous expression pattern on other days of the cycle (6). In cultured rat granulosa cells, exogenous AMH inhibits biosynthesis of aromatase and decreases LH receptor number (7). Furthermore, AMH opposes the proliferation of cultured granulosa-luteal cells (8, 9).

The marked changes in AMH and AMHR II mRNA expression and the reported effects of AMH in *in vitro* culture systems indicate that AMH may play a role during follicle development in the postnatal ovary. However, little is known about the exact function of AMH in the ovary. In the present study the function of AMH in the postnatal ovary has been studied with the help of an AMH *null* mouse model generated by Behringer *et al.* (10). As in female animals, AMH and its sole receptor (AMHR II) (11, 12) are predominantly found in the postnatal ovary, nonovarian effects of the deletion of the *amh* gene are unlikely to occur, and therefore, the AMH *null* mouse provides an excellent model for examining the function of AMH in the mouse ovary. AMH *null* males develop Müllerian duct derivatives, including oviducts, a uterus, and a vagina, in addition to a complete male reproductive system. AMH *null* females have macroscopically normal uteri, oviducts, and ovaries. Furthermore, these females are fertile and have litters of normal size (10). Yet, we hypothesized that AMH might be involved in subtle and

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long term aspects of control of follicle development and/or follicle selection. To investigate whether AMH exerts effects on the composition of the follicle population, we determined the entire follicle population in wild-type mice [AMH(+ / +)], mice heterozygous for the AMH *null* mutation [AMH(+ / -)] and AMH *null* mice [AMH(- / -)] at 25 days, 4 months, and 13 months of age. Other parameters of reproductive function, such as serum FSH and inhibin levels and ovarian and uterine weights, were also determined.

We found that AMH is an important regulator of primordial follicle recruitment and that ovaries of AMH *null* mice show depletion of primordial follicles at an earlier age.

Materials and Methods

Animals

A single male and a single female AMH(+ / -) mouse were obtained from The Jackson Laboratory (Bar Harbor, ME). This pair was used to generate F₁ mice. Next, several F₁ AMH(- / -) males and females were cross-bred with female and male C57B6 mice to obtain AMH(+ / -) mice on a C57B6 genetic background. Subsequently, these mice were used to make AMH(+ / +), AMH(+ / -), and AMH(- / -) mice, which were used for follicle population analysis. The mice were kept under standard animal housing conditions in accordance with the NIH Guidelines for the Care and Use of Experimental Animals. Lights were on from 0730–2030 h. AMH(+ / +) mice, AMH(+ / -) mice, and AMH(- / -) mice of 25 days (n = 4), 4 months (n = 4), and 13 months (n = 5) of age were used for this study. The 4- and 13-month-old animals were killed on the day of estrus, which was determined by placing the females individually with an AMH(+ / +) male of proven fertility in the afternoon, followed by a check for a copulatory plug the next morning. Females with a copulatory plug were killed on the same day at 1600 h by decapitation, after which blood was collected immediately. Females 25 days old, which were still prepubertal, were also killed at 1600 h. After bleeding, the ovaries were removed, weighed, and fixed overnight in Bouin's fluid for histological examination of the follicle population. The uterus was also removed and weighed.

The fixed ovaries were embedded in paraffin after routine histological procedures, and 8- μ m sections were mounted on slides and stained with hematoxylin and eosin. Blood samples were stored 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 and inhibin.

Determination of mouse AMH genotype

To analyze the genotype of the mice, genomic DNA was isolated by incubating tail tissue overnight at 55 C in 500 μ l 50 mM Tris-HCl (pH 8.0), 100 mM EDTA (pH 8.0), 900 μ g/ml proteinase K (Roche Molecular Biochemicals, Mannheim, Germany), and 0.5% (wt/vol) SDS. The next day, 500 μ l phenol (Merck & Co., Darmstadt, Germany) dissolved in Tris-HCl (pH 8.0) were added, and the mixture was shaken vigorously for 15 sec. Subsequently, the solution was centrifuged at 13,000 rpm for 10 min. To the supernatant, 200 μ l phenol and 200 μ l chloroform-isoamyl alcohol (24:1) were added. After shaking vigorously, the solution was centrifuged for 10 min at 13,000 rpm. To the supernatant, 2 vol 100% ethanol were added, and the precipitated DNA was washed with 70% ethanol. After the DNA was air-dried for a few minutes, it was dissolved in 100 μ l TE buffer (10 mM Tris-HCl, pH 7.5, and 1 mM EDTA, pH 8.0). Approximately 1 μ g DNA was used in the PCR reactions. Primers KO568 (5'-GGAACACAAGCAGAGCTTCC-3') and KO810 (5'-GAGACAGAGTCCATCACG-ACC-3') were used to determine the presence of the wild-type allele and were suggested by The Jackson Laboratory (http://lena.jax.org/resources/documents/imr/protocols/amh_ko.html). Primer KO568 anneals to nucleotide sequence 568–587 located in exon 1 of the *amh* gene (numbering according to GenBank sequence, accession number X63240), whereas primer KO810 anneals to antisense sequence 810–789 located in intron 1, resulting in a PCR product of 243 bp. In animals containing the AMH *null* allele, part of exon 1, intron 1, and exon 2 are replaced by the pCM1 neo-cassette. Therefore, the PCR product is only produced in DNA of AMH(+ / +)

and AMH(+ / -) animals, which contain the complete wild-type allele. Primers p126 (5'-CTCGTCAAGAAGGCGATA-3') and p127 (5'-GGGATCGGCAT-TGAACA-3') were used to determine the presence or absence of the AMH *null* allele, *i.e.* the pCM1 neo-cassette (13). Primer p126 anneals to the antisense sequence 1057–1041, whereas primer p127 anneals to nucleotide sequence 265–281 in the pCM1 neo-cassette, resulting in a PCR product of 793 bp. Only AMH(+ / -) and AMH(- / -) animals, which contain the pCM1 neo-cassette, will produce this PCR product. For the PCR reaction, 50 ng of primers KO568 and KO810 or 100 ng of primers p126 and p127 were used in a PCR buffer containing 1.2 mM dithiothreitol, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.25 mM deoxy-NTPs (Pharmacia Biotech, Uppsala, Sweden), 0.5 mM spermidine (Sigma, St. Louis, MO), and 0.2 U Supertaq (Sphearo Q, Leiden, The Netherlands) in a total volume of 25 μ l.

The PCR reaction mixtures were preheated at 94 C for 5 min followed by 30 PCR cycles (1-min denaturation at 94 C, 1-min annealing at 58 C for the wild-type allele and 1-min annealing at 62 C for the *null* allele, 1-min extension at 72 C) and a final extension step of 10 min at 72 C. The DNA control PCR was carried out as previously described by Slegtenhorst *et al.* (14). The products were electrophoresed on a 1.5% agarose gel.

Estrous cycle length

Daily vaginal smears were taken of female mice belonging to the three different genotypes for a continuous period of 40 days, beginning at 3–4 months of age (n = 10/group), to assess cycle length and the regularity of the cycle. Dried smears were examined microscopically, and the stage of the estrous cycle was determined according to the criteria of Allen (15). The females were housed individually in cages placed next to a cage containing an adult AMH(+ / +) male.

Ovarian histology and follicle counting

Serial 8- μ m sections of both ovaries were used for follicle counting. All primordial follicles were counted in every second section. Based on the mean diameter of the follicle, which was determined by measuring two perpendicular diameters in the section in which the nucleolus of the oocyte was present, the growing follicles were divided into two classes, *i.e.* small and large follicles. The small follicle class contains preantral and small antral follicles with a diameter smaller than 310 μ m, and the large follicle class contains antral follicles with a diameter larger than 310 μ m. The number of large follicles was very small in females of all three ages. This was expected, as in immature 25-day-old animals growing follicles become atretic before they reach this stage of follicular development (16) and the cycling animals of 4 and 13 months of age were killed on the day of estrus, a time point during the estrous cycle when no large follicles have developed (17, 18).

Nonatretic and atretic follicles were counted separately. The criteria for atresia were the presence of pyknotic nuclei in the granulosa cells and/or degeneration of the nucleus of the oocyte (18, 19). For all three genotypes we found degenerating, *i.e.* fragmented oocytes, which are the remnants of atretic follicles (19). No diameter could be determined for these follicle remnants, because the layer of granulosa cells could not be distinguished very well from the surrounding interstitial tissue. To prevent double counting of these follicle remnants, which we called atretic oocytes, these oocytes were counted in every tenth section. The atretic oocytes were classified as a separate group.

In addition, the total number of fresh corpora lutea was counted in the 4- and 13-month-old animals. Newly formed corpora lutea could be distinguished from the older ones by their smaller size of luteal cells.

Measurements of serum FSH and inhibin

Serum FSH was determined by RIA using rat FSH as a ligand and antibodies against ovine FSH (20). All results are expressed in terms of NIDDK rat FSH RP-2. The intraassay variation was 8.8%, and all samples were measured in one assay.

Serum inhibin-like immunoreactivity was estimated using the method described by Robertson *et al.* (21), with a bovine follicular fluid preparation with an arbitrary potency of 1 U/ μ g protein as standard (22). The antibody was raised against purified 32-kDa bovine follicular

fluid inhibin and cross-reacts with free α -subunits. All samples were run in the same assay, and the intraassay coefficient of variation was 8.2%.

Statistical analysis

Results are presented as the mean \pm SEM. The data were evaluated for statistical differences by one-way ANOVA, followed by Duncan's new multiple range test using SPSS 7.5 (SPSS, Inc., Chicago, IL) computer software. Differences were considered significant at $P \leq 0.05$.

Results

Estrous cycle length

Vaginal smears were taken daily for a period of 40 days to determine the length and regularity of the estrous cycle in AMH(+/+), AMH(+/-), and AMH(-/-) female mice. No differences were found among these three groups of mice in either the length or the regularity of the estrous cycle. In all three groups some animals showed at least one prolonged estrous cycle (varying from 8–22 days) in which several metestrous or diestrous smears were obtained. However, no consistent differences among the three genotypes were observed. In all groups regular estrous cycles with a length of 4–6 days were found (results not shown).

Weights of ovaries and uterus

Uterine and ovarian weights were determined to obtain an indication of whether AMH had some effect on development of the uterus and the ovary. Furthermore, uterine weight is an estrogen-sensitive parameter and thus may also give an indication of ovarian function.

At all ages no statistical difference was found in uterine weight among the three genotypes (Table 1). No difference in the weight of the ovaries among the three groups was found at 25 days and 13 months of age. At the age of 4 months, however, the weights of the two ovaries were about 1.8-fold higher in the AMH(-/-) females than in AMH(+/+) mice. It is of interest to note that the ovarian weight of AMH(+/-) females fell in between the weights of the ovaries of AMH(+/+) and AMH(-/-) mice (Fig. 1).

Ovarian morphology

The ovaries of all genotypes contained primordial, pre-antral, and small antral follicles, both nonatretic and atretic. In 4- and 13-month-old animals both fresh and old corpora lutea were present, indicating an active estrous cycle. At all ages, atretic oocytes were found in the interstitium (Fig. 2C).

TABLE 1. Uterine weight in AMH (+/+), AMH (+/-), and AMH (-/-) female mice of 25 days, 4 months, and 13 months of age

Age	Genotype	Uterus wt (mg)
25 days (n = 4)	AMH (+/+)	16.0 \pm 3.0
	AMH (+/-)	17.0 \pm 3.0
	AMH (-/-)	11.0 \pm 1.0
4 months (n = 4)	AMH (+/+)	130.0 \pm 8.0
	AMH (+/-)	114.9 \pm 9.2
	AMH (-/-)	88.9 \pm 7.0
13 months (n = 5)	AMH (+/+)	210.5 \pm 32.2
	AMH (+/-)	213.8 \pm 18.7
	AMH (-/-)	165.6 \pm 24.5

Values represent the mean \pm SEM.

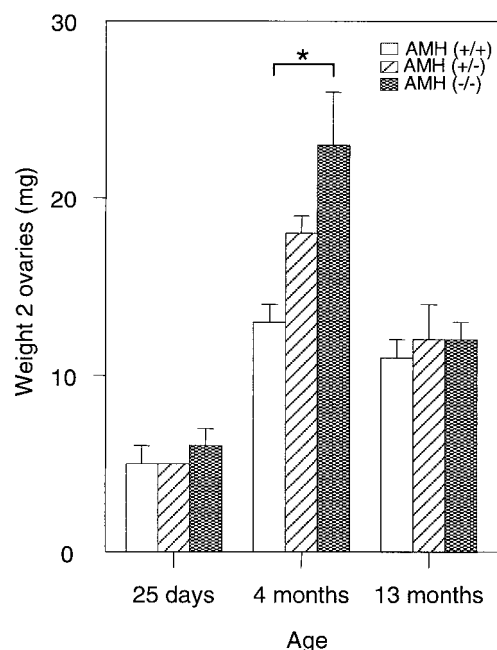


FIG. 1. Ovarian weight in 25-day-, 4-month-, and 13-month-old AMH(-/-), AMH(+/-), and AMH(+/+) female mice. The combined weight of both ovaries is given. At 4 months of age, ovarian weight was significantly higher in AMH(-/-) females than in AMH(+/+) females. Data represent the mean \pm SEM (n = 4–5). The asterisk indicates a statistically significant difference ($P \leq 0.05$).

Figure 2, A and B, illustrates that in a 25-day-old AMH(-/-) mouse ovary many small follicles were found, whereas a 13-month-old AMH(-/-) mouse ovary contained very few small follicles. At this latter age, the main part of the ovary consists of interstitial tissue and corpora lutea. For ovaries of age-matched AMH(+/+) and AMH(+/-) females similar histology was observed, as reported previously by Behringer *et al.* (10).

Follicle counts

To test the hypothesis that AMH might play a role in follicle maturation or selection, the entire follicle population in 25-day-, 4-month-, and 13-month-old AMH(+/+), AMH(+/-), and AMH(-/-) female mice was determined (Fig. 3, A–C). Note the differences in scale of the y-axis among the different graphs in Fig. 3, A–C.

In 25-day-old females no significant difference in the number of primordial follicles among AMH(+/+), AMH(+/-), and AMH(-/-) females was detected (Fig. 3A). However, compared with the ovaries of AMH(+/+) females of 4 and 13 months of age, the ovaries of age-matched AMH(-/-) females contained a significantly smaller number of primordial follicles (Fig. 3, B and C). In fact, at 13 months of age the number of primordial follicles was reduced in AMH(-/-) females to 38 ± 15 , whereas in the AMH(+/+) females the average number of primordial follicles was 225 ± 52 (Fig. 3C).

For the category of nonatretic small follicles the AMH(-/-) females at the age of 25 days showed a marked increase of approximately 1.5-fold compared with that in the AMH(+/+) mice (Fig. 3A). In 4-month-old AMH(-/-) females an approximately 3-fold increase in the number of

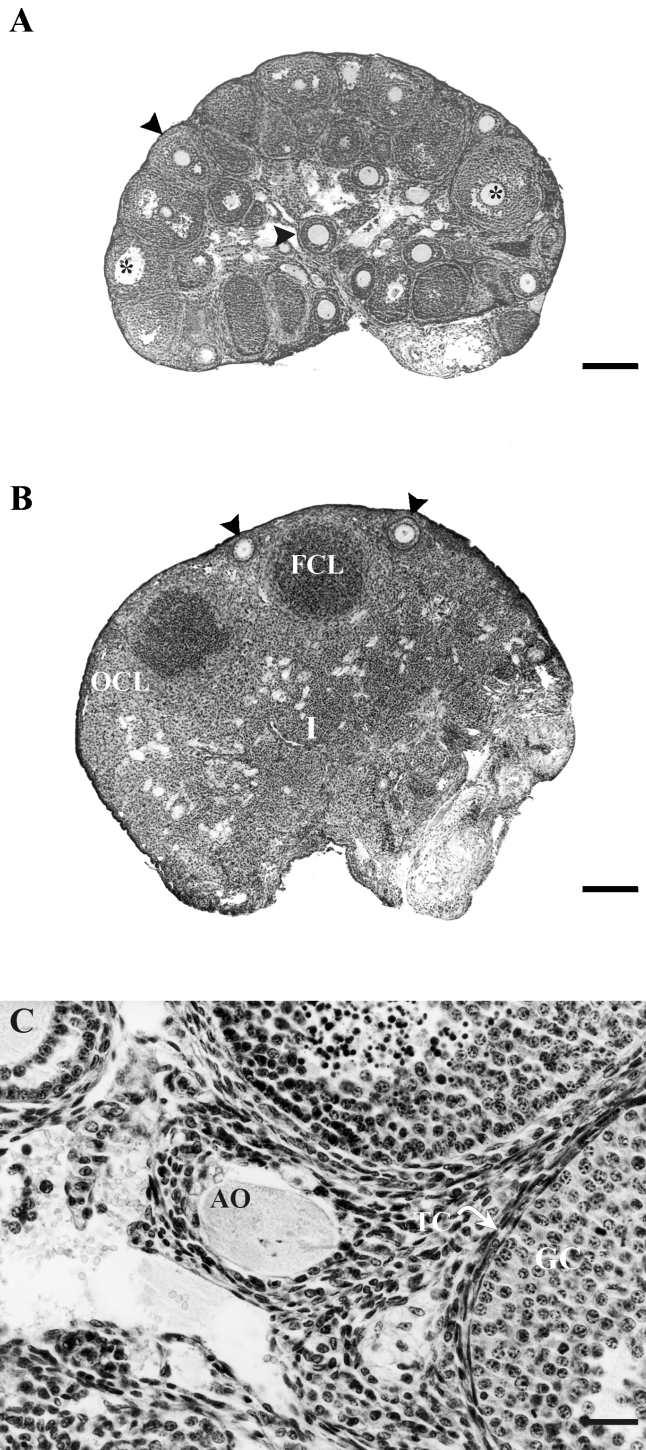


FIG. 2. Photomicrographs of ovarian histology. A, Ovary of a 25-day-old AMH(-/-) female mouse. Many nonatretic (arrowhead) and atretic (asterisk) small follicles are present, whereas no corpora lutea can be detected. Scale bar, 250 μ m. B, Ovary of a 13-month-old AMH(-/-) female mouse. The ovary mainly consists of interstitium (I), and old (OCL) and fresh corpora lutea (FCL). Only a few small follicles are present (arrowhead). Scale bar, 250 μ m. C, Atretic oocyte from a 25-day-old AMH(-/-) mouse ovary. The oocyte (AO) has resumed its meiotic division and has lost its round shape. Around the oocyte no clear granulosa and thecal cell layers can be distinguished. Both the granulosa cell layer (GC) and the thecal cell layer (TC) are present in the follicle adjacent to the atretic oocyte. Scale bar, 50 μ m.

nonatretic small follicles was found compared with that in the AMH(+/+) females (Fig. 3B). This 3-fold increase in follicle number was also found for the atretic small follicles. In contrast, significantly fewer nonatretic and atretic small follicles were counted in the 13-month-old AMH(-/-) females (Fig. 3C). The number of atretic oocytes in 4-month-old AMH(-/-) females was about 4-fold larger than that in AMH(+/+) females (Fig. 3B). In the three groups of mice at the three different ages the average numbers of 0.9 ± 0.2 nonatretic large follicle and 0.8 ± 0.2 atretic large follicle were found (results not shown).

In almost all instances the number of follicles present in AMH(+/-) females fell in between the numbers found in the AMH(+/+) and AMH(-/-) females (Fig. 3, A-C).

Number of fresh corpora lutea

To determine whether there are any differences among the three genotypes in the number of ovulations during the last estrous cycle, the number of fresh corpora lutea were counted in the 4- and 13-month-old females. At both 4 and 13 months of age no significant differences among the three groups of mice were found, and an average of 10 fresh corpora lutea were present (results not shown).

Measurement of serum FSH and inhibin levels

In 4-month-old AMH(-/-) females the serum inhibin level was 2-fold higher than that in AMH(+/+) females of the same age, whereas in 13-month-old AMH(-/-) females the inhibin serum level was 2-fold lower (Table 2). No statistically significant difference was found in inhibin values in 25-day-old females of the three genotypes. For the serum FSH level, a statistically significant difference was found only for AMH(-/-) females of 4 months of age, which showed a decreased level compared with AMH(+/+) animals (Table 2).

Discussion

Although AMH has a clear function during male fetal development (23), its action in the female is still undefined. Although in the female, AMHRII mRNA expression is already present in the ovary and the mesenchymal cells surrounding the Müllerian ducts before birth (24, 25), AMH mRNA expression is only found after birth in the ovary. Postnatally, both mRNA species are highly expressed in granulosa cells of mainly nonatretic preantral and small antral follicles (3, 6). This expression pattern does not change during the estrous cycle, except at estrus, when a more heterogeneous pattern becomes apparent and the expression of AMH mRNA is lower in some nonatretic preantral follicles. This expression pattern may indicate a role for AMH in the regulation of follicle selection or maturation (6). Such a role is supported by the findings that AMH has inhibitory effects on granulosa cell proliferation (8, 9), aromatase activity, and LH receptor expression (7).

In the present study a possible role for AMH in the ovary was examined by comparing the entire ovarian follicle population of AMH(+/+) mice with the follicle populations of AMH(-/-) and AMH(+/-) mice. Ovaries of 25-day-, 4-month, and 13-month-old females were examined. These

FIG. 3. Follicle population in 25-day-, 4-month-, and 13-month-old AMH(-/-), AMH(+/-), and AMH(+/+) female mice. **A**, Follicle populations in 25-day-old AMH(-/-), AMH(+/-), and AMH(+/+) female mice. Significantly more nonatretic small follicles were detected in AMH(+/-) and AMH(-/-) females than in AMH(+/+) females. No significant difference in the number of primordial follicles, atretic small follicles, or atretic oocytes was observed among the three groups of mice. **B**, Follicle population in 4-month-old AMH(-/-), AMH(+/-), and AMH(+/+) female mice. Significantly fewer primordial follicles and significantly more nonatretic small follicles were found in AMH(-/-) and AMH(+/+) females than in AMH(+/-) females. Significantly more atretic small follicles were found in AMH(-/-) females than in the two other groups of females, whereas significantly more atretic oocytes were found in AMH(-/-) and AMH(+/-) females than in AMH(+/+) females. **C**, Follicle population in 13-month-old AMH(-/-), AMH(+/-), and AMH(+/+) female mice. Significantly fewer primordial and nonatretic and atretic small follicles were found in AMH(-/-) females than in AMH(+/+) females. Data represent the mean \pm SEM (n = 4-5). An asterisk indicates a statistically significant difference ($P \leq 0.05$).

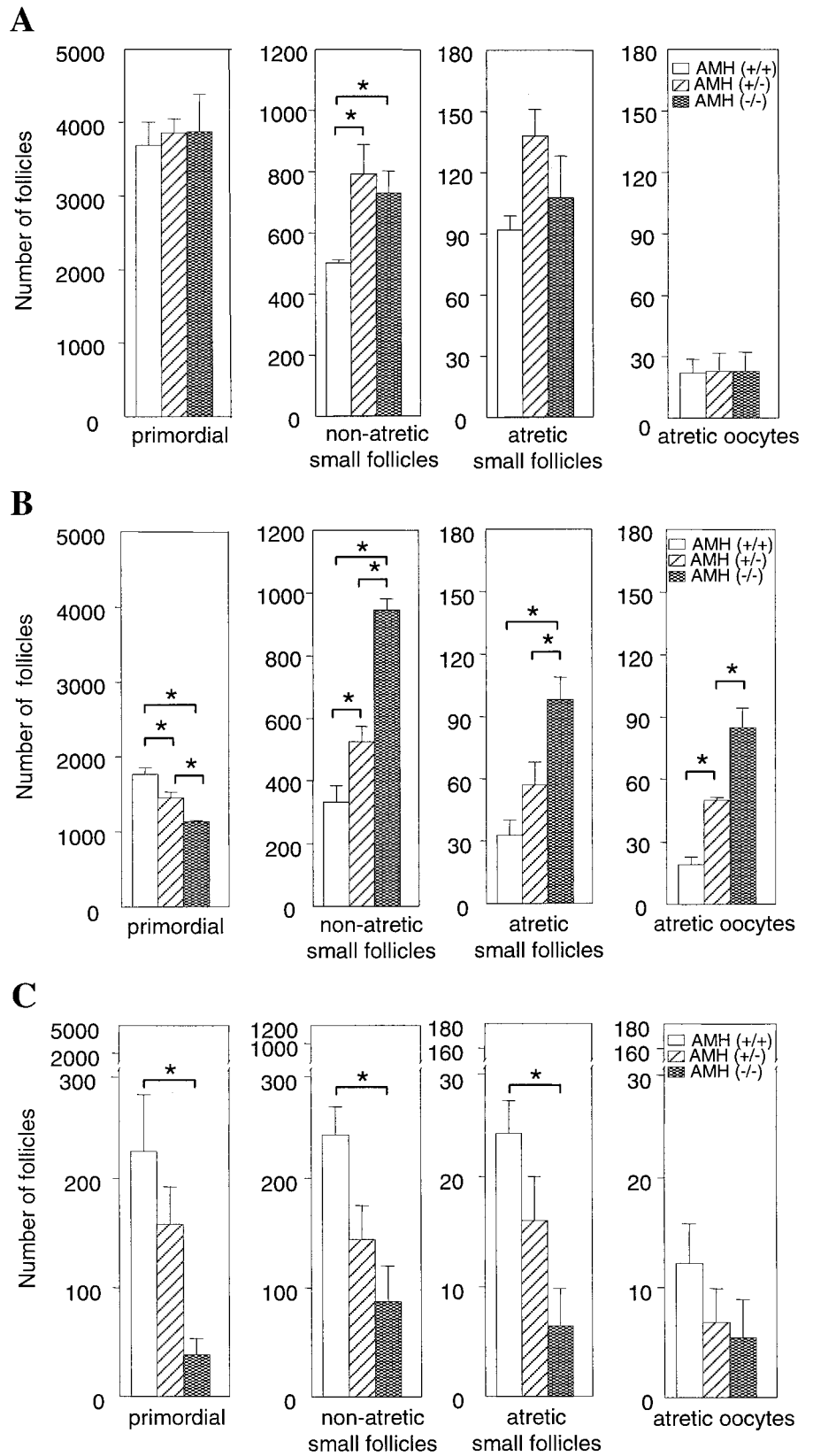


TABLE 2. Serum inhibin immunoreactivity and FSH levels in AMH (+/+), AMH (+/-), and AMH (-/-) female mice of 25 days, 4 months, and 13 months of age

Age	Genotype	Inhibin α -subunit (U/ml)	FSH (ng/ml)
25 days (n = 4)	AMH (+/+)	33.9 \pm 5.8	24.1 \pm 5.3
	AMH (+/-)	28.9 \pm 4.8	17.7 \pm 1.5
	AMH (-/-)	25.0 \pm 2.2	15.0 \pm 1.8
4 months (n = 4)	AMH (+/+)	20.6 \pm 1.6	39.7 \pm 2.6
	AMH (+/-)	35.2 \pm 1.5 ^a	31.5 \pm 2.8
	AMH (-/-)	43.5 \pm 4.0 ^a	27.9 \pm 2.9 ^a
13 months (n = 5)	AMH (+/+)	21.5 \pm 1.6	28.8 \pm 2.6
	AMH (+/-)	12.6 \pm 3.8 ^a	31.3 \pm 2.9
	AMH (-/-)	11.5 \pm 0.9 ^a	37.9 \pm 3.8

Values represent the mean \pm SEM.

^a Significantly different from AMH (+/+) in the same age group ($P < 0.05$), evaluated by Duncan's new multiple range test.

ages were chosen to represent three different stages in the reproductive life of the female mouse. At 25 days of age, female mice are prepubertal and do not have estrous cycles; at 4 months of age, the females are at the height of their reproductive life; and at 13 months of age, the females have almost reached reproductive cessation (26).

Upon characterization of the complete follicle population in AMH(+/+), AMH(+/-), and AMH(-/-) animals, the results indicate that the number of primordial follicles decreases with age in the three groups of mice. However, in AMH(-/-) females, the pool of primordial follicles decreases faster than in AMH(+/+) mice. Although in 25-day-old females no significant differences were found in the number of primordial follicles between AMH(+/+) and AMH(-/-) females, at 4 and 13 months of age, the number of primordial follicles was significantly smaller in AMH(-/-) females. The absence of a difference in the primordial follicle number in prepubertal mice indicates that AMH may not have a role in the pronounced wave of attrition of follicles that occurs before regular estrous cycles start (27). One way by which the smaller number of primordial follicles in AMH(-/-) females of 4 and 13 months of age may be explained is a loss of primordial follicles from the stock through degeneration by attrition. The extent of this phenomenon, however, is difficult to measure, because adequate morphological markers of degeneration in primordial follicles are lacking (28). Furthermore, degenerated primordial follicles may be eliminated very rapidly from the ovary, preventing an accurate determination of their number (29). An alternative explanation for the smaller number of primordial follicles found in 4- and 13-month-old AMH(-/-) females compared with AMH(+/+) females could be an increased number of estrous cycles within a certain time period in AMH(-/-) females. However, no difference in the length or the regularity of the estrous cycle among the three genotypes at 3–4 months of age was detected. Yet another explanation would be that in AMH(-/-) animals, more primordial follicles are recruited to enter the pool of growing follicles. This explanation is favored on the basis of the present observations of increased numbers of nonatretic and atretic small follicles in AMH(-/-) females of 25 days and 4 months of age compared with the age-matched AMH(+/+) control mice.

The larger number of small follicles in 4-month-old AMH(-/-) females might lead to a larger number of ovulations per cycle and subsequently to larger litters. However, we have not found any difference in the size of the litters derived from AMH(-/-), AMH(+/-), or AMH(+/+) females (results not shown), which is consistent with the previously published report (10). However, to exclude the possibility of increased embryonic death *in utero* in 4-month-old AMH(-/-) females, we determined the number of ovulations by counting the number of fresh corpora lutea that emerge from the preovulatory follicles after recent ovulation. Again, no difference was detected in the number of fresh corpora lutea between the ovaries of 4-month-old AMH(-/-) females and the age-matched females from the two other groups, which excludes a higher ovulation rate in the absence of AMH. These results suggest that in the absence of AMH action, increased atresia of small follicles may result in loss of an increased percentage of growing follicles, so that finally the number of these follicles that reach the preovulatory stage is approximately equal in AMH(-/-) and AMH(+/+) mice. Indeed, in 4-month-old AMH(-/-) ovaries we found significantly more atretic small follicles than in AMH(+/+) ovaries. However, the number of nonatretic small follicles showed a similar increase. Thus, in the AMH(-/-) females more atretic small follicles were found, probably because in these animals more nonatretic small follicles were being produced. In addition, the number of atretic oocytes, which are thought to arise from atretic follicles, is larger in AMH(-/-) females of 4 months of age, indicating that in AMH(-/-) females the larger number of small nonatretic follicles is compensated by more atretic follicles. Thus, increased atresia results in similar numbers of preovulatory follicles and ovulations in AMH(-/-) and AMH(+/+) female mice. Higher rates of atresia may also exist at other days of the cycle, which we have not studied; therefore, further studies are necessary to elucidate this point.

From 4 to 13 months of age the number of nonatretic small follicles in AMH(-/-) females shows a marked decline. This is in accordance with the very small number of primordial follicles that are still present in the ovaries of 13-month-old AMH(-/-) females. This smaller number of nonatretic small follicles is not associated with a smaller number of fresh corpora lutea still being formed in these females. This may be explained by more efficient growth of follicles to the preovulatory stage, as indicated by the significantly lower percentage of atretic small follicles in the 13-month-old AMH(-/-) females.

It is of interest to note that in almost all animals the number of follicles per class in the AMH(+/-) females fell in between the numbers found for AMH(+/+) and AMH(-/-) females. For other gene knockout animal models, such as activin *null* mice or FSH *null* mice (30, 31), such a gene dosage effect has not been reported. Moreover, Müllerian duct regression in male AMH(+/-) mice occurs to the same extent as in AMH(+/+) males (10). The AMH gene dose dependency observed in the present study may indicate that ovarian AMH production or secretion is not under stringent feedback control, but, rather, depends on the intrinsic activity of the gene itself.

The results presented herein indicate that AMH exerts an inhibitory effect on recruitment of primordial follicles into

the pool of growing follicles. A direct effect of AMH on the primordial follicles themselves cannot be excluded, although no information is available that clearly demonstrates AMH type II receptor expression in these follicles. Alternatively, the primary action of AMH may be autocrine in nature, as granulosa cells of small follicles express both AMH and its type II receptor. The small follicles may be stimulated by AMH to produce a factor(s) that acts on primordial follicles.

Little is known about the factors that control the release of primordial follicles from their quiescent state. Primordial follicles can be considered the ovarian follicle stock, and this reserve of dormant follicles is not renewable (26). Throughout neonatal life and continuing through the reproductive life span, follicles leave the stock of primordial follicles in a continuous stream and begin to grow, thus depleting the original pool of primordial follicles with age (26, 29). There are indications that the recruitment of primordial follicles into the growing pool is influenced by the total size of the nongrowing pool of primordial follicles itself (32). Thus, a decrease in the number of remaining primordial follicles is accompanied by an increase in their depletion rate (29). Despite the fact that the gonadotropins FSH and LH predominantly act on recruited antral follicles (33), several studies show that gonadotropins influence the size of the primordial follicle pool. Hypophysectomy, for example, slows the loss of primordial follicles, implicating a role of pituitary factors, such as the gonadotropins, in the control of growth initiation of primordial follicles (34). FSH may accelerate the initiation of the growth of primordial follicles. Unilateral ovariectomy in aged rats causes increased FSH serum levels and concomitant increased loss of primordial follicles (35), whereas in aging rats, in which primordial follicles are lost at an accelerated rate (36), both LH and FSH levels are increased compared with the levels in young females (37). In transgenic mice that maintain a chronically elevated serum LH level, the primordial follicle pool is depleted faster (38). A direct effect of FSH and LH on the primordial follicles themselves, however, appears to be unlikely, as several studies were unsuccessful in detecting full-length LH and FSH receptor mRNA expression in primordial follicles (39–42). In contrast, gonadotropins are not essential for the initiation of primordial follicle growth. In FSH *null* females primordial follicles are recruited and grow into preantral follicles (31), and primordial follicle recruitment also occurs in cultured newborn mouse ovaries without the addition of gonadotropins to the culture medium (43).

Despite their advanced age and the small number of primordial follicles present in their ovaries, we found that the 13-month-old females of the three different genotypes had not yet reached the period of acyclicity. Although the estrous cycle in these animals was not checked with the help of vaginal smears, we conclude this from the fact that in the ovaries of 13-month-old females an average of 10 fresh corpora lutea were found, which is the same average number as found in 4-month-old females.

The ovaries from 4-month-old AMH(–/–) mice were heavier than the ovaries of control animals. Although most often the total mass of the corpora lutea largely determines the weight of an ovary, the numbers of corpora lutea were similar in the animals of the three genotypes, suggesting that the increase in ovarian weight in the AMH(–/–) females is

caused by the 3-fold larger number of small follicles. In 25-day- and 13-month-old animals no significant differences were observed in ovarian weight among the three genotypes, which is in line with the much smaller differences in the number of small follicles found in these animals. No difference was found among the three groups of mice in uterine weight, which indicates that the estrogen levels in the AMH(+/+), AMH(+/-), and AMH(-/-) are similar.

The large number of small follicles in the 4-month-old AMH(–/–) mice may also explain the changes in serum levels of FSH (decrease) and inhibin immunoreactivity (increase) in these animals. In mouse preantral follicles, both inhibin A and inhibin B are produced (44, 45), whereas small antral follicles mainly produce inhibin B, and large antral follicles mainly produce inhibin A (46, 47). Despite the fact that only the inhibin immunoreactivity level was measured in the serum samples, we suggest that the increased number of small follicles in the AMH(–/–) females is also responsible for an increase in bioactive dimeric inhibin A or B, which, in turn, suppresses both FSH synthesis and FSH release from the pituitary gland (48). The reduced FSH level in AMH(–/–) females of 4 months of age might prevent the larger number of small antral follicles in these animals to develop to the preovulatory follicle stage. Indeed, if the FSH level was not lower in AMH(–/–) females, an increase in litter size in these mice might have been expected. The larger number of growing follicles accompanied by a decrease in FSH is an intriguing observation, as FSH stimulates preantral follicle development (49–51), although the vast majority of the effects of FSH are on the recruited, antral pool of follicles (33). A possible explanation might be that small follicles may be more sensitive to FSH in the absence of AMH. Alternatively, AMH may be instrumental in inhibition of the production and/or activity of a factor(s) important for preantral follicle development. Candidates for such factors are growth and differentiation factor-9 (GDF-9) and bone morphogenetic protein-15 (BMP-15), which both are members of the transforming growth factor- β superfamily. GDF-9 is an oocyte-specific growth factor, and in mice lacking GDF-9 there is an early block in folliculogenesis at the one-layer primary follicle stage (52). Recently, it has been shown that recombinant GDF-9 is able to enhance the growth and differentiation of cultured small preantral follicles (53). BMP-15 is most closely related to and shares a coincident expression pattern with GDF-9, which could imply that BMP-15 is also necessary for preantral follicle development (54). Thus, AMH might have an effect on expression of GDF-9 and/or BMP-15, thereby influencing preantral follicle development.

In conclusion, the study of ovaries of AMH *null* mice indicates that ovarian AMH directly or indirectly prevents or inhibits primordial follicles to enter the pool of growing follicles. As impaired recruitment of primordial follicles may be the underlying cause of human diseases such as premature ovarian failure (55), it will be of great interest to study the role of AMH in the physiology of the human ovary.

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