Anti-Müllerian Hormone Inhibits Initiation of Primordial Follicle Growth in the Mouse Ovary

ALEXANDRA L. L. DURLINGER, MARIA J. G. GRUIJTERS, PIET KRAMER, BAS KARELS, HOLLY A. INGRAHAM, MARK W. NACHTIGAL, JAN TH. J. UILENBROEK, J. ANTON GROOTEGOED, AND AXEL P. N. THEMMEN

Department of Endocrinology and Reproduction (A.L.L.D., M.J.G.G., P.K., J.Th.J.U., J.A.G., A.P.N.T.), Faculty of Medicine and Health Sciences, Erasmus University Rotterdam, 3000 DR Rotterdam, The Netherlands; Department of Physiology (H.A.I.), Center for Reproductive Sciences, University of California-San Francisco, California 94143-0444; and Department of Pharmacology (M.W.N.), Faculty of Medicine, Dalhousie University, Halifax, Nova Scotia, Canada B3H 2Y9

Recruitment of primordial follicles is essential for female fertility; however, the exact mechanisms regulating this process are largely unknown. Earlier studies using anti-Müllerian hormone (AMH)-deficient mice suggested that AMH is involved in the regulation of primordial follicle recruitment. We tested this hypothesis in a neonatal ovary culture system, in which ovaries from 2-d-old C57Bl/6J mice were cultured for 2 or 4 d in the absence or presence of AMH. Ovaries from 2-d-old mice contain multiple primordial follicles, some naked oocytes, and no follicles at later stages of development. We observed that in the cultured ovaries, either nontreated or AMH-treated, follicular development progressed to the same extent as in in vivo ovaries of comparable age, confirming the validity of our culture system. However, in the presence of AMH, cultured ovaries contained 40% fewer growing follicles compared with control ovaries. A similar reduction was found after 4 d of culture. Consistent with these findings, we noted lower inhibin α-subunit expression in AMH-treated ovaries compared with untreated ovaries. In contrast, expression of AMH ligand type II receptor and the expression of oocyte markers growth and differentiation factor 9 and zona pellucida protein 3 were not influenced by AMH.

Based on the results, we suggest that AMH inhibits initiation of primordial follicle growth and therefore functions as an inhibitory growth factor in the ovary during these early stages of folliculogenesis. (Endocrinology 143: 1076–1084, 2002)

For female mammals, the finite number of primordial follicles available during reproductive life is established during fetal development (human) (1) or just after birth (mouse, rat) (2, 3). Factors involved in the initiation of primordial follicle growth, a process also known as initial folliculogenesis, include stem cell factor (SCF, or Kit ligand) (5), growth and differentiation factor 9 (GDF9) (6), basic fibroblast growth factor (bFGF) (7), and nerve growth factor (NGF) (8) promote initiation of primordial follicle growth. Also, anti-Müllerian hormone (AMH) has been implicated in primordial follicle recruitment from analysis of AMH null ovaries (9). AMH is a member of the TGFβ superfamily of growth and differentiation factors (10) and is responsible for Müllerian duct regression during male fetal development (11). This ligand-receptor system is also found in the ovary with expression of both the AMH ligand and its type II receptor (AMHRII) postnatally in granulosa cells of mainly nonatretic preantral and small antral follicles (12, 13). In addition, AMHRII is also expressed in the prenatal ovary (14). Examination of ovarian follicles in AMH-deficient female mice revealed lower numbers of primordial follicles and more growing follicles compared with wild-type littermates. These findings led to the proposal that AMH inhibits primordial follicle recruitment (9).

In this study, we examined the ability of AMH to inhibit primordial follicle recruitment in cultured neonatal mouse ovaries. To this end, we developed a neonatal ovary culture system, in which follicular development progresses to the same extent as in in vivo ovaries of comparable age. In this system, we characterized primordial follicle recruitment by the determination of the number of primordial and growing follicles in neonatal ovaries after 2 and 4 d of culture in the presence or absence of recombinant AMH. The effects of AMH on primordial follicle recruitment was also investigated by assessing AMHRII and inhibin α-subunit transcript expression and additional oocyte-specific markers, such as GDF9 and zona pellucida protein 3 (ZP3).

Collectively, results from these studies suggest that AMH is a potent inhibitor of primordial follicle recruitment.

Materials and Methods

AMH production

Human embryonic kidney 293 (HEK293) cells were stably transfected with a cDNA encoding His-tagged rat AMH inserted in the pRc/cyto-megalovirus expression vector. The AMH cDNA contained an optimized cleavage site that yields maximal amounts of cleaved mature AMH (15, 16). HEK293 cells were cultured in DMEM/F-12 (Life Technologies, Inc., Paisley, Scotland, UK) supplemented with 5% 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 and proteins with a molecular mass above 10 kDa were concentrated ap-
proximately 35-fold using a Centrifrap10 filter system (Millipore Corp., Bedford, MA).

The amount of AMH was measured by ELISA making use of the His-tag, with the Bio-Rad Laboratories, Inc. (Hercules, CA) 3,3′,5′-tetramethylbenzidine peroxidase enzyme immunoassay substrate kit. The primary antibody, pentaHis monoclonal antibody (raised in rabbit; QIAGEN GmbH, Hilden, Germany), was used at 100 ng/ml. The secondary antibody, a goat-antimouse IgG peroxidase conjugate (Sigma, St. Louis, MO), was used at 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 wild-type HEK293 cells was used as control medium.

The presence or absence of AMH in the media used in the experiments was investigated by Western blotting using a primary polyclonal antibody against AMH (raised in goat; C-20, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at a 1:2000 dilution and a secondary peroxidase-conjugated mouse antigoat/sheep antibody (Sigma) at a dilution of 1:10000.

The AMH preparations (800 U/ml) were shown to be bioactive using a Mullerian duct regression assay (15, 16) (data not shown).

**Animals and ovary organ culture**

C57Bl/6j female mice were used in all experiments described in this report. For the ovary organ culture experiments, ovaries of 2-d-old females were used because these ovaries predominantly contain prordial follicles, some naked oocytes and no growing follicles. Day of birth was denoted as d 1. The animals were obtained from the Animal Facility of the Faculty of Medicine and Health Sciences of the Erasmus University Rotterdam and were kept under standard animal housing conditions in accordance with the NIH guidelines for the Care and Use of Experimental Animals. Animals were killed by decapitation.

Ovaries of 2-d-old mice were either immediately fixed in Bouin’s fixative for morphological examination and follicle counting, or snap-frozen in liquid nitrogen for RNA isolation, or placed in culture. An organ culture method was used as described by Cooke et al. (17), which was modified in our laboratory with the help of Dr. Judith Emmen (18). The ovaries, together with some surrounding tissue, were removed from the abdomen under a dissection microscope. The tissue was placed in watch-glasses containing PBS. With the help of two syringe needles, the tissue surrounding the ovaries, including the capsule in which the ovary is enclosed (periovarian sac), was removed. Because large interanimal variation in the number of follicles per ovary was observed (see Results), ovaries of each animal were matched and one was used for control cultures and the other for treated cultures. Ovaries were cultured in a four-well culture plate (Nunc plate, Applied Scientific, South San Francisco, CA). Each well contained 0.5 ml DMEM/F-12 medium with GLUTAMAX-I (Life Technologies, Inc.) supplemented with 1% (vol/vol) charcoal-stripped FCS, insulin (10 μg/ml), and transferrin (10 μg/ml; Sigma). Penicillin (250 IU/ml), streptomycin (0.25 mg/ml), and fungizone (3.1 μg/ml) (BioWhittaker, Walkersville, MD) were added to the culture medium to prevent bacterial contamination. No effect of the charcoal stripped FCS was found in the sensitive fetal Mullerian duct regression assay, whereas by Western blotting no AMH could be detected in the charcoal-stripped FCS. Each ovary was placed in a small droplet of medium (approximately 15 μl) on a piece of 0.4-μm Millicell-cellular matrix filter (Millipore Corp.) of approximately 1 cm², floating on the culture medium. The ovaries were cultured at 37°C in a humidified atmosphere containing 5% CO₂ for 2 or 4 d. Of each pair of ovaries (derived from one animal), one ovary was cultured in medium containing concentrated medium of wild-type HEK293 cells (control), whereas the other ovary was cultured in medium containing concentrated medium of HEK293 cells producing rat AMH (800 U/ml).

After 2 d of culture, the medium was removed and fresh medium was added. At the end of the culture period, the ovaries were either fixed for 2 h in Bouin’s fixative or snap frozen in liquid nitrogen and stored at −80°C. Ovaries of 2-, 4-, and 6-d-old C57Bl/6j mice were used for comparison of the development of follicles in the culture experiments, hereby focusing on the developmental stage of the follicles. No comparison was made regarding the number of primordial and growing follicles between cultured ovaries and freshly isolated ovaries of comparable age.

**Ovarian histology and follicle counting**

To investigate the development of ovarian follicles in the cultured ovaries and to compare it with follicle development in vivo, fresh and cultured ovaries were fixed for 2 h in Bouin’s fixative, embedded in paraffin after routine histological procedures, and 8-μm serial sections were mounted on slides and stained with hematoxylin and eosin.

Primordial follicles are nongrowing follicles and consist of an oocyte partially or completely encapsulated by flattened squamous pregranulosa cells. Early primary follicles have initiated development and contain at least one cuboidal (enlarged) granulosa cells (19). In addition, primordial follicles and early primary follicles were distinguished by size: follicles with a mean diameter less than or equal to 20 μm were classified as primordial follicles, whereas follicles with a mean diameter greater than 20 μm were classified as growing follicles (primary and secondary follicles). This borderline of 20 μm was established in a pilot experiment. The mean diameter of the follicles was determined by measuring two perpendicular diameters (20). All primordial follicles were counted in every second section, whereas in every second section of the growing follicles only those in which the nucleus of the oocyte was clearly visible were counted. We did not attempt to distinguish between atretic and growing follicles because at this stage of follicle development, atresia is not apparent by histology. Both 2- and 4-d culture experiments were performed twice.

**Immunohistochemistry of AMH and inhibin α-subunit**

To determine the onset of AMH and inhibin α-subunit protein expression in vivo and in cultured neonatal mouse ovaries, immunohistochemistry was performed on freshly isolated 1- to 6-d-old mouse ovaries and on 2- to 4-d-old mouse ovaries cultured for 2 or 4 d. Of every group, four ovaries were examined, which were derived from different animals.

The ovaries were fixed for 2 h in Bouin’s fixative, embedded in paraffin, and 8-μm sections were made. Sections were coated with 3-aminopropyl triethoxysilane(Sigma). After deparaffinization, sections were quenched for 12 min in 3% H₂O₂/methanol solution to block endogenous peroxidase activity and transferred to PBS. For AMH immunohistochemistry, the sections were microwaved for 3 × 5 min at 700 W in 0.01 M citric acid monohydrate buffer, pH 6.0 (Merck, Darmstadt, Germany), cooled down to room temperature (RT), and subsequently rinsed in PBS. For AMH immunohistochemistry, sections were incubated with normal rabbit serum in 5% (wt/vol) BSA (Dako Corp., Glastrup, Denmark) for 15 min at RT, and for inhibin α-subunit immunohistochemistry, the sections were preincubated with normal swine serum in 5% BSA (Dako Corp.) for 15 min at 37°C. For AMH immunohistochemistry, the preincubation step was followed by incubation at 4°C overnight with primary polyclonal antibody against AMH isolated from mature pooled human AMH (C-20; Santa Cruz Biotechnology, Inc.), diluted 1:1000 in 5% BSA in PBS. The antibody was raised against a peptide mapping at the carboxy terminus of human AMH, which differs from the corresponding mouse sequence, by a single amino acid. For inhibin α-subunit immunohistochemistry, the preincubation step was followed by incubation at 37°C for 30 min with primary polyclonal antibody AS173-11, diluted 1:40 in 5% BSA in PBS. This primary polyclonal antibody was raised against amino acids 37–55 of the rat inhibin pre-pro-α-subunit. After incubation, the sections were rinsed in PBS and subsequently treated for 30 min at RT either with goat anti-rabbit antibody (dilution: 1:400; Dako Corp.) for AMH immunohistochemistry or with biotinylated swine anti-rabbit antibody (dilution: 1:400; Dako Corp.) for inhibin α-subunit immunohistochemistry. Finally, a treatment with streptavidin-biotin-peroxidase complex (diluted 1:200 in PBS; Dako Corp.) for 30 min at RT followed. Peroxidase activity was developed with 0.07% 3,3′-diaminobenzidine tetrahydrochloride (Fluka, Basel, Switzerland) for 5–7 min. In each experiment, control sections were incubated with 5% BSA/PBS in the absence of the primary antibody (negative control). Hema-toxylin/eosin was used for counterstaining. Ovaries from 30-d-old mice, containing many AMH and inhibin α-subunit-positive follicles, were used as a positive control.

**RNA isolation and RNase protection assay**

Freshly isolated 2-, 4-, and 6-d-old ovaries or 2- to 4-d-old ovaries that were cultured for 2 or 4 d in the absence or presence of added AMH were snap
frozen in liquid nitrogen and stored at −80°C. Two separate RNase protection assays were performed. In each assay, different sets of total RNA were used. For each assay, total RNA was isolated from 20 ovaries per culture condition, 20 ovaries of 2-, 4-, and 6-d-old mice and 5 ovaries of 30-d-old. Total RNA was isolated using Trizol Reagent (Life Technologies, Inc.), which is an improvement of the single-step RNA isolation method developed by Chomczynski and Sacchi (21). The isolated RNA was dissolved in 10 μl deionized H2O and stored by −80°C.

Mouse ZP3 and mouse inhibin α-subunit DNA templates for in vitro transcription were generated by RT-PCR. The RT-PCR reaction was carried out on 2.5 μg total RNA, extracted from 30-d-old mouse ovaries, using random hexamers. A sample of the RT reaction product was used in the PCR reaction. With the help of ZP3 primer 5'-GACTTCAGC-GTTGCTTGG-3', annealing to nucleotide sequence 735–753 of the Zp3 gene (numbering according to GenBank sequence), and 5'-CACGTC-CA-ACTTC-ACAG-3', annealing to antisense sequence 1174–1196, a 440-bp fragment was generated. For the inhibin α-subunit, PCR primers 5'-GCA-ATCCACCACACATACG-3', annealing to sense sequence 2937–2954, and 5'-GAAACTCCGGTTGGTACAG-3', annealing to antisense sequence 884–866, were used to generate a 511-bp fragment. These fragments were inserted into the PCR2.1-TOPO vector. A fragment of 521 bp of the PCR2.1-TOPO vector containing the ZP3 PCR product was removed from PCR2.1-TOPO vector by cutting the plasmid with BamHI and XhoI, whereas a fragment of 538 bp containing inhibin α-subunit PCR product was removed by cutting the PCR2.1-TOPO vector with EcoRI. Both fragments were subsequently subcloned in pBlueScript KS AccI-Sau3AI fragment of the rat GAPDH cDNA.

Mouse GDF9 DNA template (22) was kindly donated by Dr. O. Ritvos (Helsinki, Finland). This template was used to obtain a PCR fragment of 411 bp, containing a part of 293-bp of mouse GDF9 construct and a part of pGEM-T vector containing the T7 polymerase site. The primers used in this PCR reaction were primer 5'-CAAGGTTITCCCCAGTCA-3', annealing to the sense sequence 2937–2954 located within the pGEM-T vector and primer 5'-CITGCCATGGAACACTTGCTC-3', annealing to antisense sequence 490–471 located within the GDF9 construct. This PCR product was used as template for the construction of a GDF9 [32P]-uridine triphosphate-labeled antisense RNA probe. Mouse AMHRII antisense probe was generated as described before (23). The control glyceraldehyde 3-phosphate dehydrogenase (GAPDH) RNA probe was synthesized using a construct containing 163 bp sequence 884–866, were used to generate a 511-bp fragment. These fragments were inserted into the PCR2.1-TOPO vector. A fragment of 521 bp of the PCR2.1-TOPO vector containing the ZP3 PCR product was removed from PCR2.1-TOPO vector by cutting the plasmid with BamHI and XhoI, whereas a fragment of 538 bp containing inhibin α-subunit PCR product was removed by cutting the PCR2.1-TOPO vector with EcoRI. Both fragments were subsequently subcloned in pBlueScript KS AccI-Sau3AI fragment of the rat GAPDH cDNA.

RNase protection assays were performed on total RNA, extracted from fresh and cultured mouse ovaries, as described by Baarends (14). GAPDH was used as a control for RNA loading. The relative amount of protected mRNA band was quantified through exposure of the gels to a phosphor screen (Molecular Dynamics, Inc., B & I Systems, Zoetermeer, The Netherlands) and using PhosphorImager, and ImageQuant software. The mRNA levels were normalized to those for GAPDH mRNA and are expressed as a percentage of the mRNA level of 2-d-old ovaries, which was considered 100%.

Two separate assays were performed, using RNA derived from two separate culture experiments. In every assay, 5 μl of the RNA samples were hybridized with inhibin α-subunit and GAPDH probes, and the remaining 5 μl with probes for AMHRII, GDF9, ZP3 and GAPDH. Total RNA of 30-d-old mouse ovaries was used as a control.

Statistical analysis

Results are presented as the mean ± sem. The data were evaluated for statistical differences by an independent samples t test using SPSS, Inc.9.0 (SPSS, Inc., Chicago, IL) computer software. Differences were considered significant at P < 0.05.

Results

AMH production

To ensure that no AMH is present in the concentrated control medium, and that AMH is present in the concentrated medium of rat AMH-producing HEK293 cells, the concentrated media were examined by Western blot analysis using an AMH antibody. Figure 1 shows the result of this analysis. There is no AMH in lane 1, containing concentrated control medium, whereas two bands representing AMH protein can be seen in lane 2 containing concentrated medium of rat AMH-producing HEK293 cells. The lower band is the monomeric form of mature AMH (12 kDa), whereas the top band is nonreduced AMH dimer (24 kDa). The absence of larger bands indicates complete cleavage of the proform of AMH.

Culture medium from both control and AMH-treated cultures was also examined by Western blot analysis to check for the presence of endogenous AMH and to make sure that the added rat AMH is not degraded during the culture. No AMH was found in the control cultures, whereas nondegraded AMH was present in the AMH-treated cultures (results not shown).

Ovary organ culture and ovarian morphology

The culture system was evaluated by comparing the morphology of 2-d-old ovaries cultured for 2 or 4 d with the morphology of in vitro ovaries of corresponding age, which were isolated from 4- and 6-d-old mice, respectively. This evaluation focused on the developmental stage of the follicles, whereas no comparison was made regarding the number of different stages of follicles.

Ovaries of 2-d-old mice, which were used for the ovary culture experiments, predominantly contain primordial follicles, some naked oocytes, and no growing follicles (Fig. 2A). Histological examination of the ovaries after 2 or 4 d of culture indicated that the culture conditions had no pronounced detrimental effect on the ovarian tissue, i.e. almost no necrosis or apoptotic cells were found (Fig. 2B). After 2 d of culture, the ovaries contained many primordial follicles but also some primary follicles with different sizes (Fig. 2D), whereas after 4 d of culture, the ovaries contained in addition to primordial and many primary follicles also some early secondary follicles (Fig. 2F). Most primordial follicles were located in the cortical part of the ovary, whereas the primary and secondary follicles (further referred to as growing follicles) were mostly found in the central part of the ovary (Fig. 2B). As a further validation of the culture system, we compared ovaries of similar ages, i.e. ovaries cultured for 2 or 4 d were compared with ovaries of 4- or 6-d-old mice, respect-
The most significant difference was a larger diameter of primary follicles in ovaries cultured for 2 d than primary follicles in 4-d-old ovaries (Fig. 2C), whereas follicle development in ovaries cultured for 4 d were not different from ovaries from 6-d-old mice (Fig. 2E). It also seems that more follicles reach the primary stage in ovaries cultured for 2 d than in 4-d-old ovaries. However, no direct statements regarding the numbers can be made because no follicle counting was performed in the freshly isolated ovaries.

The presence of AMH in the culture medium did not have detrimental effect on the tissue of the cultured ovaries (results not shown).

**AMH and inhibin α-subunit immunohistochemistry**

To further evaluate the condition of the cultured ovaries and to determine the onset of AMH and inhibin α-subunit protein expression in vivo, an immunohistochemical study was performed on freshly isolated 1- to 6-d-old mouse ovaries, and in ovaries from 2-d-old mice cultured for 2 or 4 d.

In freshly isolated ovaries of 2-d-old mice, containing naked oocytes, primordial follicles, or interstitial tissue no specific AMH protein expression was detected (Fig. 3A). In vivo AMH protein expression was first found in a few granulosa cells of the smallest primary follicles from 4-d-old mice (Fig. 3C), whereas in larger primary follicles all granulosa cells show AMH protein expression. High AMH protein expression was found in the granulosa cells of primary and early secondary follicles of 6-d-old mice (Fig. 3E).

Cultured ovaries exhibited an identical AMH expression pattern compared with freshly isolated ovaries, i.e. expression was found in granulosa cells of primary and secondary follicles. However, the expression level of AMH was higher in the primary follicles of ovaries cultured for 2 d than that in freshly isolated ovaries of 4-d-old animals (compare Fig. 3, C and G). No difference was found in AMH protein expression level between freshly isolated 6-d-old ovaries and ovaries cultured for 4 d (results not shown). The negative control showed that some background staining was found in the oocytes and in the interstitium (Fig. 3I).

The inhibin α-subunit protein expression pattern was identical to the AMH protein expression both in vivo and in vitro (Fig. 3, B–J), although the inhibin α-subunit protein expression was somewhat less abundant. A very low amount of background staining was found in the oocytes (Fig. 3J).

The presence of AMH in the culture medium did not affect the expression of either AMH or inhibin α-subunit (results not shown).

Histological and immunohistochemical evaluation of cultured and freshly isolated ovaries of comparable age revealed that follicle development in vivo and in vitro are similar, and therefore our neonatal ovary organ culture system...
FIG. 3. Immunohistochemical localization of AMH and inhibin α-subunit in freshly isolated (control) and cultured neonatal C57Bl/6J mouse ovaries. A and B, Section of a 2-d-old control mouse ovary. No AMH (A) and inhibin α-subunit (B) protein expression is found in naked oocytes (no), primordial follicles (pr), and interstitial tissue (I). Scale bar, 20 μm. C and D, Section of a 4-d-old control mouse ovary. AMH protein expression (C) and inhibin α-subunit protein expression (D) are found in several granulosa cells of early primary follicles. Scale bar, 20 μm. E and F, Section of a 6-d-old control mouse ovary. Abundant AMH protein expression (E) and inhibin α-subunit protein expression (F) are found in granulosa cells of primary follicles (P) and early secondary follicles (S). Scale bar, 20 μm. G and H, Section of a 2-d-old mouse ovary cultured for 2 d in the absence of added AMH. High AMH protein expression (G) and inhibin α-subunit protein expression (H) are found in granulosa cells of primary follicles (P). Scale bar, 20 μm. I and J, Section of a 4-d-old control mouse ovary. For AMH some background staining is found in the interstitium (I) and the oocytes, whereas for inhibin α-subunit very low background staining is only found in the oocytes (J). Scale bar, 20 μm.
was applied to examine the effect of AMH on the initiation of primordial follicle recruitment.

**Effect of AMH on initiation of primordial follicle growth**

To determine the effect of AMH on the recruitment of primordial follicles, the numbers of primordial, primary and secondary (growing) follicles were counted in ovaries cultured for 2 or 4 d in the absence or presence of AMH.

In the ovarian culture experiment, the effect of different culture conditions (+/− AMH) were tested by comparing the number of primordial and growing follicles between ovaries derived from the same animal (paired ovaries) because no large differences exist in the number of primordial follicles between two ovaries derived from the same animal (results not shown). To optimize the contact between the ovarian tissue and the culture medium, the periovarian sac was removed before the 2-d-old ovaries were used in the ovarian culture experiments. To test whether the removal of the periovarian sac has any impact on the number of primordial follicles, the number of primordial follicles was counted in paired ovaries of two 2-d-old mice, of which one of the ovaries the periovarian sac was removed. Removal of the periovarian sac resulted in the loss of primordial follicles because approximately 3000 (2982 ± 53) primordial follicles were found in 2-d-old ovaries with the periovarian sac in place, and about 1400 (1384 ± 37) after removal of the periovarian sac.

After 2 or 4 d of culture, no difference was found in the total number of follicles per cultured ovary, when the ovaries were derived from the same female (results not shown). However, variation did occur between cultured ovaries isolated from different animals. This interanimal variation was mainly due to a difference in the number of primordial follicles, ranging from 691–2281 primordial follicles per ovary. Moreover, primordial follicles were also lost during the culture period because significantly more primordial follicles were found in the ovaries after 2 d of culture (1893 ± 104) than after 4 d of culture (1401 ± 79).

The total numbers of primordial and growing follicles after 2 and 4 d of culture in the presence or absence of AMH are shown in Table 1. After both 2 and 4 d of culture, no effect of AMH on the number of primordial follicles was found. However, significantly fewer growing follicles were found in the AMH-treated ovaries compared with the control ovaries. After normalization to percentages, we found that after 2 d of culture the number of growing follicles cultured in the presence of AMH was approximately 59 ± 4% of the control (Fig. 4A), whereas after 4 d of culture this was approximately 66 ± 6% (Fig. 4B).

After 2 d of culture, for both treatment groups, most growing follicles reached a diameter of 30 μm and some a diameter of 40 μm. After 4 d of culture, most growing follicles reached a diameter of 40 μm and some a diameter of 50 μm. The presence of AMH did not affect the maximal diameter reached by the follicles during the culture period.

**RNase protection assay**

To investigate whether the decrease in number of growing follicles was reflected in the levels of expression of mRNA for markers of follicle differentiation, the mRNA expression level for the granulosa cell markers, inhibin α-subunit and AMHRII, and for the oocyte-specific markers, GDF-9 and ZP3, in freshly isolated 2-, 4-, and 6-d-old ovaries and in cultured ovaries was examined using RNase protection assay. Total RNA from 30-d-old mice was used as a control.

Phosphorimage analysis of the RNase protection assay is shown in Fig. 5A and B. Quantitative analysis revealed that AMHRII mRNA expression is present in all ovaries examined and does not change with age or under different culture conditions (Fig. 5C). The mRNA expression level of inhibin α-subunit increased from d 2 to d 6. In the ovaries cultured for 2 d in the presence of AMH, a decrease in inhibin α-subunit expression level of 68% was found, compared with the ovaries cultured in the absence of AMH. After 4 d of culture this decrease was 46% (Fig. 5C).

In the freshly isolated ovaries an increase in the mRNA expression level from d 2 to d 6 was found for both oocyte-specific markers (GDF9 and ZP3). No difference was found in mRNA expression level between ovaries cultured in the presence or absence of added AMH, although a slight decrease was found for ZP3 expression after 2 d of culture in the presence of AMH (Fig. 5C).

**Discussion**

In the mouse, primordial follicles are formed just after birth, and this pool of primordial follicles constitutes the

![Fig. 4](image-url)

**Fig. 4.** Growing follicles as a percentage of the control in 2-d-old mouse ovaries cultured for 2 d (A) or 4 d (B) in the absence or presence of AMH. The number of growing follicles found in ovaries cultured in the presence of AMH is shown as a percentage of the control. N represents the number of ovaries that were investigated. Data represent the mean ± SEM. The asterisks indicate a statistically significant difference (P ≤ 0.05).
complete supply of ovarian follicles during reproductive life. Initiation of primordial follicle growth occurs from the moment that these follicles arise in the ovary, although not all primordial follicles start to grow at the same time and some will remain dormant for months in rodents (2, 3) and even for years in the human (1). The factors that regulate this initiation of primordial follicle growth are still largely unknown. Recently, it was shown in a culture system for neonatal rat ovaries that SCF and bFGF can stimulate the recruitment of primordial follicles (5, 7), whereas in vitro treatment with GDF9 enhances the progression of primordial and primary rat follicles into small preantral follicles (6). Studying the follicle population of NGF-deficient mice revealed that in these mice fewer primary follicles were formed, whereas an almost normal complement of primordial follicles existed, suggesting that NGF is important for the initiation of primordial follicle growth (8). These are the first studies, which show that ovarian growth factors play an important role in initiation of primordial follicle growth. In our study of ovaries from AMH-deficient females, it became evident that also AMH affects initiation of primordial follicle growth (9).

In the present study, the effect of AMH on primordial follicle recruitment was tested using ovaries from 2-d-old mice in a neonatal ovary organ culture system. Ovaries of 2-d-old mice provide a useful system for the study of factors
that influence the initiation of primordial follicle growth because they contain many primordial follicles, only few naked oocytes, and no growing follicles. In this culture system, not all primordial follicles started to grow, but similar to observations in vivo (24) and in another ovary culture system (25), some of the primordial follicles located in the central part of the ovary were the first to initiate growth.

After 2 and 4 d of culture, fewer growing follicles were found in ovaries cultured in the presence of AMH than in the control ovaries, which indicates that AMH indeed inhibits initial recruitment. By immunohistochemistry it was shown that AMH is already produced by early primary follicles, making AMH a very early marker of ovarian follicle growth. This means that from postnatal d 4, about 1 d after the first primary follicles are formed in mouse ovaries, these ovaries produce a small amount of AMH. Also in the cultured ovaries, endogenous AMH was found in the early primary follicles that arise after 1 d of culture. Therefore, the effect of AMH found in the culture model may be an underestimation of the actual effects.

To exclude the possibility that the lower number of growing follicles found in AMH-treated ovaries is caused by an AMH-induced retardation of early primary follicle growth, we also looked at the maximal mean diameter that was reached in control and AMH-treated ovaries. Because under both culture conditions the growing follicles reached the same maximal diameter, we conclude that AMH has no effect on the pace of early primary follicle growth.

The inhibition of initiation of primordial follicle growth is probably due to a direct effect of AMH on the primordial follicle. Using RNase protection assay, we show that AMHRII mRNA expression, essential for an effect of AMH, is present in ovaries of 2-d-old mice. In both mouse and rat, ovarian AMHRII mRNA expression is even found before birth (our observations) (13, 14). In addition, in situ hybridization studies showed ovary-specific AMHRII expression in neonatal ovaries. However, exact localization of the radioactive AMHRII probe to the pregranulosa cells was not possible because this probe is best visualized using a microscope with darkfield, whereby it is very difficult to discern specific structures or cell types in neonatal ovaries (13) (Durlinger, A. L. L., unpublished observations). At later stages of ovarian development, AMHRII expression is limited to the granulosa cells of mainly nonatretic preantral and small antral follicles (13) (Durlinger, A. L. L., unpublished observations), although recently AMHRII transcripts have also been detected in the internal theca cell layer of preantral follicles (16).

As soon as primordial follicle growth is initiated, as indicated by a change in appearance of the pregranulosa cells, not only AMH, but also α-inhibin protein is produced by the granulosa cells of the early primary follicles and the inhibitory effect of AMH on primordial follicle recruitment is reflected by a decrease in α-inhibin, used as a marker of early follicle development. However, a functional role of α-inhibin produced by these primary follicles is unclear at the present. More insight in the role of inhibin in early follicle development could come from detailed analysis of the follicle population of inhibin-deficient females, before they develop ovarian tumors (26).

The number of primordial follicles that start to grow within a certain time period was not dependent on the total number of primordial follicles present in the ovary at the beginning of the culture experiment. It appears that within a certain time period the same number of primordial follicles starts to grow, irrespective of the size of the pool of primordial follicles. The same result was found in another in vitro study in which prenatal and neonatal mouse ovaries were placed in culture (27). Krarup et al. (28), however, state that their in vivo study shows that the fraction of a pool of primordial follicles that initiate growth does depend on the actual size of the pool primordial follicles. The discrepancy between our results and of Bybskov et al. (27) and the results of Krarup et al. (28) could be explained by the fact the ovarian culture experiments are in vitro studies, in which many possible factors influencing the initiation of primordial follicle growth are eliminated, while Krarup et al. (28) performed an in vivo study.

Although the amount of AMH (800 U/ml) used in the herein described culture experiments caused complete Müllerian duct regression in vitro (Durlinger, A. L. L., unpublished observations), the recruitment of primordial follicles was not completely blocked. This observation is in accordance with the findings in AMH-deficient females, in which not all primordial follicles initiated growth. Thus, AMH is not the only regulator of initial recruitment. Indeed, the stimulatory effect of SCF (5), GDF9 (6), bFGF (7), and NGF (8) on the initiation of primordial follicle growth show that activation of primordial follicle growth is under the influence of both stimulatory and inhibitory regulation. It would be interesting to culture neonatal ovaries with SCF, GDF9, bFGF, or NGF in the presence of AMH to study the possible functional interactions on initial recruitment. Indications for an interaction between SCF and GDF9 already exist because SCF mRNA expression is highly elevated in mice lacking GDF9, suggesting that GDF9 is one of the oocyte-secreted factors that negatively regulate SCF expression (29). Besides AMH, SCF, GDF9, bFGF, and NGF, probably many other as yet unidentified factors are involved, and their role in initial recruitment is still not established. Identification of receptors for hormones and growth factors expressed by either the oocyte or the pregranulosa cells of primordial follicles will give more insight in the process of initial recruitment.

Acknowledgments

The authors would like to thank Tamara Prins for describing ovarian follicle development in neonatal C57Bl/6j mice, Miriam Verhoeft-Post for generating the AMH-producing HEK293 cells, Anke McCluskey-Dankbar for assistance with the RNase protection assay, Dr. Olli Ritvos for donating the mouse GDF9 DNA template, and Dr. Jenny Visser for critical reading of the manuscript.

Received July 20, 2001. Accepted November 13, 2001.
Address all correspondence and requests for reprints to: Alexandra L. Durlinger, Department of Endocrinology & Reproduction, Faculty of Medicine and Health Sciences, Erasmus University Rotterdam, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands. E-mail: durlinger@endov.lgg.eur.nl.

References

15. Nachtigal MW, Ingraham HA 1996 Bioactivation of Müllerian-inhibiting sub-
stance during gonadal development by a kex2/subtilisin-like endoprotease. Proc Natl Acad Sci USA 93:7711–7716
29. Elvin JA, Yan C, Wang P, Nishimori K, Matzuk MM 1999 Molecular character-
ization of the follicle defects in the growth and differentiation factor 9-deficient ovary. Mol Endocrinol 13:1018–1034

Durlinger et al. • AMH Inhibits Primordial Follicle Recruitment