0013-7227/02/$15.00/0
*Printed in U.S.A.*

Endocrinology 143(3):1076–1084


# 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 recruitment (4), have long remained unknown. Recently, however, it was shown that 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/cytomegalovirus 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-

Abbreviations: AMH, Anti-Müllerian hormone; AMHII, AMH ligand type II receptor; bFGF, basic fibroblast growth factor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GDF9, growth and differentiation factor 9; NGF, nerve growth factor; RT, room temperature; SCF, stem cell factor; ZP3, zona pellucida protein 3.

proximately 35-fold using a Centriprep10 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′5,5′-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 Müllerian 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 primordial 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 $\mu$g/ml), and transferrin (10 $\mu$g/ml; Sigma). Penicillin (250 IU/ml), streptomycin (0.25 mg/ml), and fungizone (3.1 $\mu$g/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 Müllerian 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 $\mu$l) on a piece of 0.4-$\mu$m Millicel-cellular matrix filter (Millipore Corp.) of approximately 1 $cm^2$, floating on the culture medium. The ovaries were cultured at 37 C in a humidified atmosphere containing 5% $CO_2$ 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-$\mu$m 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 $\mu$m were classified as primordial follicles, whereas follicles with a mean diameter greater than 20 $\mu$m were classified as growing follicles (primary and secondary follicles). This borderline of 20 $\mu$m 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 nonatretic and atretic 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 $\alpha$-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-d-old mouse ovaries cultured for 2 or 4 d. Of every group, four ovaries were examined, which were derived from two different animals.

The ovaries were fixed for 2 h in Bouin's fixative, embedded in paraffin, and 8-$\mu$m sections were made. Sections were mounted on slides coated with 3-aminopropyl triethoxysilane (Sigma). After deparaffinization, sections were quenched for 12 min in 3% $H_2O_2$/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, the sections were preincubated with normal rabbit serum in 5% (wt/vol) BSA (DAKO Corp., Glastrup, Denmark) for 15 min at RT, and for inhibin $\alpha$-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 MIS (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 $\alpha$-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-$\alpha$-subunit. After incubation, the sections were rinsed in PBS and subsequently treated for 30 min at RT either with biotinylated rabbit antigoat antibody (dilution: 1:400; DAKO Corp.) for AMH immunohistochemistry or with biotinylated swine antirabbit antibody (dilution 1:400; DAKO Corp.) for inhibin $\alpha$-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). Hematoxylin/eosin was used for counterstaining. Ovaries from 30-d-old mice, containing many AMH and inhibin $\alpha$-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-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 $H_2O$ 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 primers 5′-GACTTCCACG-GTTGCCTTG-3′, annealing to nucleotide sequence 735–753 of the *Zp3* gene (numbering according to GenBank sequence), and 5′-GCAGTC-CAG-CCTTCC-ACAG-3′, annealing to antisense sequence 1174–1156, a 440-bp fragment was generated. For the inhibin α-subunit, PCR primers 5′-GCCATCCCAACACATACG-3′, annealing to sense sequence 374–391, and 5′-GAAACTGGGAGGGTGTACG-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 *Bam*HI and *Xho*I, whereas a fragment of 538 bp containing inhibin α-subunit PCR product was removed by cutting the PCR2.1-TOPO vector with *Eco*RI. Both fragments were subsequently subcloned in pBluescript KS and used to generate a [$^{32}$P]-uridine triphosphate-labeled antisense RNA probe.

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′-CAGGGTTTTCCCAGTCAC-3′, annealing to the sense sequence 2937–2954 located within the pGEM-T vector and primer 5′-CTGCCATGGAACACTTGCTC-3′, annealing to the antisense sequence 490–471 located within the GDF9 construct. This PCR product was used as template for the construction of a GDF9 [$^{32}$P]-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 *Acc*I-*Sau*3AI 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 & L Systems, Zoetermeer, The Netherlands) and using PhosphorImager, and ImageQuant (Molecular Dynamics, Inc.) as computer analysis 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 $P \leq 0.05$.

## 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 vivo* 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 of 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, respec-

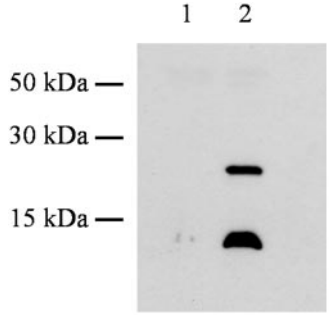


FIG. 1. Western blot analysis of concentrated wild-type HEK293 and rat AMH-producing HEK293 cells. No AMH protein expression is found in concentrated medium of wild-type HEK293 cells (lane 1), whereas AMH protein is found in concentrated medium of rat AMH-producing HEK293 cells (lane 2).

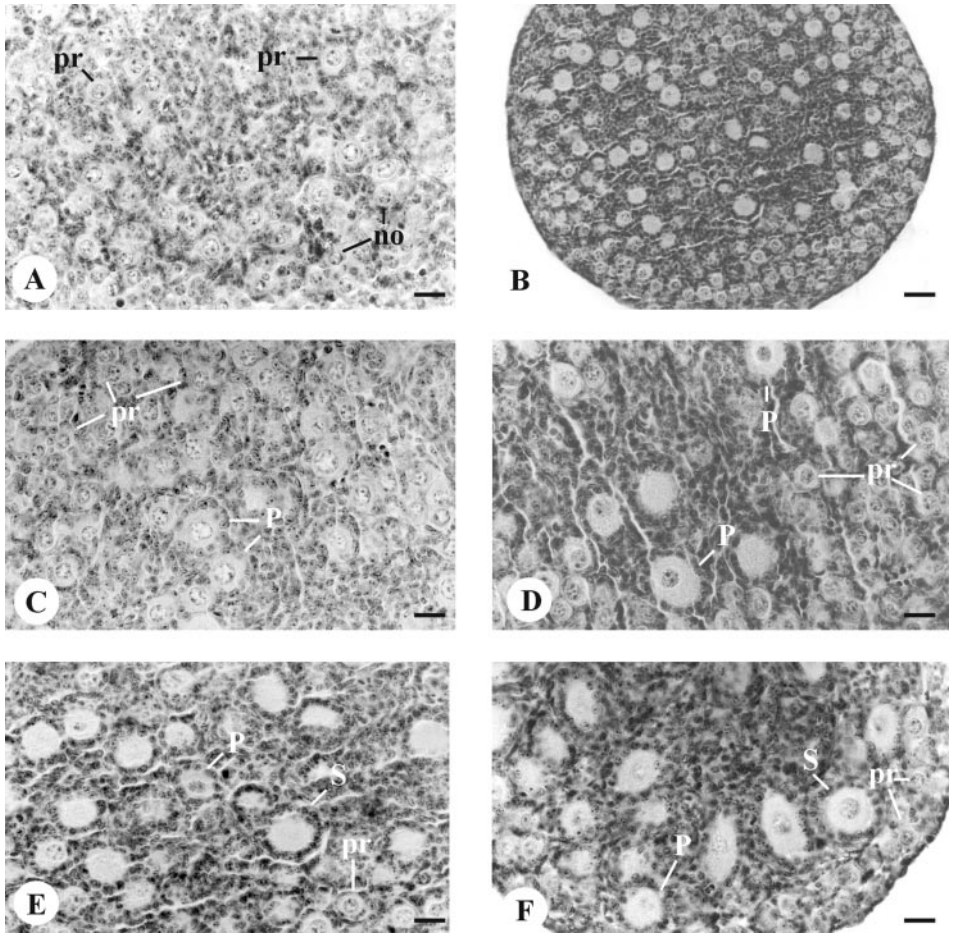


FIG. 2. Photomicrographs of cultured and noncultured neonatal mouse ovaries. A, Section of an ovary of a 2-d-old mouse. Many primordial follicles (pr) and some "naked" oocytes (no) are present. *Scale bar*, 20 $\mu$m. B, Section of a 2-d-old mouse ovary cultured for 2 d. The present culture conditions had no detrimental effect on tissue quality because almost no necrotic and apoptotic cells were found. Most primordial follicles are located in the cortex of the ovary, whereas the primary and secondary follicles are predominantly found in the central part of the ovary. *Scale bar*, 50 $\mu$m. C, Section of an ovary of a 4-d-old mouse. Many primordial (pr) and some primary (P) follicles are found. *Scale bar*, 20 $\mu$m. D, Section of a 2-d-old mouse ovary cultured for 2 d. After 2 d of culture the ovaries contained many primordial follicles (pr) and primary follicles (P) of different sizes. *Scale bar*, 20 $\mu$m. E, Section of an ovary of a 6-d-old mouse. Besides primordial (pr) and primary (P) follicles, also some early secondary (S) follicles are present. *Scale bar*, 20 $\mu$m. F, Section of an ovary of a 2-d-old mouse cultured for 4 d. In addition to primordial (pr) and primary (P) follicles, also some early secondary follicles are found (S) (C). *Scale bar*, 20 $\mu$m.

tively. 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

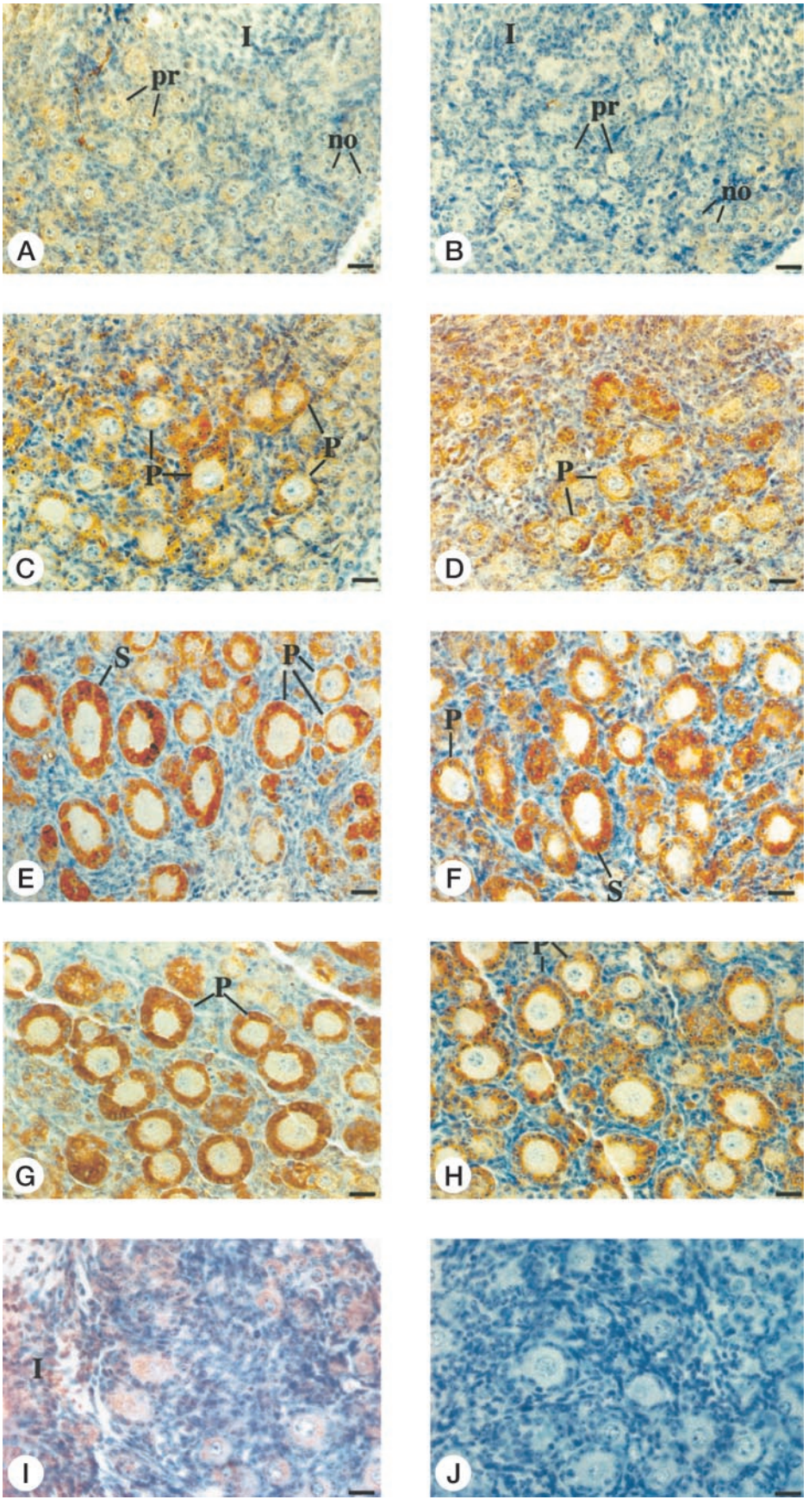


FIG. 3. Immunohistochemical localization of AMH and inhibin $\alpha$-subunit in freshly isolated (control) and cultured neonatal C57Bl/6J mouse ovaries. A and B, Section of a 2-d-old control mouse ovary. No AMH (A) and inhibin $\alpha$-subunit (B) protein expression is found in naked oocytes (no), primordial follicles (pr), and interstitial tissue (I). *Scale bar*, 20 $\mu$m. C and D, Section of a 4-d-old control mouse ovary. AMH protein expression (C) and inhibin $\alpha$-subunit protein expression (D) are found in several granulosa cells of early primary follicles. *Scale bar,* 20 $\mu$m. E and F, Section of a 6-d-old control mouse ovary. Abundant AMH protein expression (E) and inhibin $\alpha$-subunit protein expression (F) are found in granulosa cells of primary follicles (P) and early secondary follicles (S). *Scale bar*, 20 $\mu$m. 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 $\alpha$-subunit protein expression (H) are found in granulosa cells of primary follicles (P). *Scale bar*, 20 $\mu$m. 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 $\alpha$-subunit very low background staining is only found in the oocytes (J). *Scale bar*, 20 $\mu$m.

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 of 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).

**TABLE 1.** The number of primordial and growing follicles after 2 and 4 d of culture in the absence or presence of added AMH

| Days of culture | −/+ AMH | No. of primordial follicles | No. of growing follicles |
|---|---|---|---|
| 2 (N = 9) | − AMH | 1968 ± 82 | 81 ± 4 |
| 2 (N = 9) | + AMH | 1808 ± 141 | 50 ± 2[a] |
| 4 (N = 11) | − AMH | 1369 ± 148 | 153 ± 8 |
| 4 (N = 11) | + AMH | 1434 ± 155 | 97 ± 8[a] |

The numbers of follicles found in two independent experiments are pooled and shown as mean ± SEM ($P < 0.05$). [a] Indicates a significant difference from the control. N, Number of ovaries examined.

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.

PhosphorImager analysis of the RNase protection assay is shown in Fig. 5, A 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

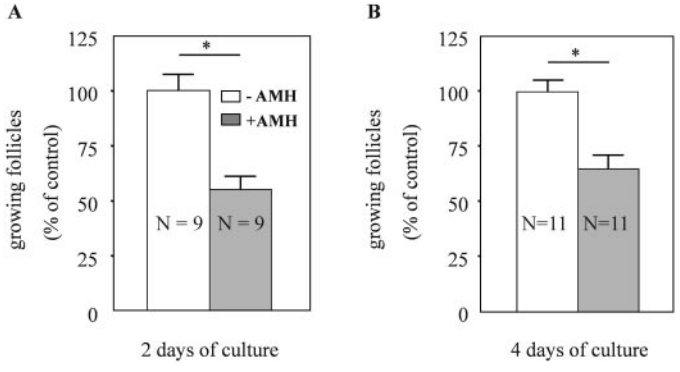


FIG. 4. Growing follicles as a percentage of the control in 2-d-old mouse ovaries cultured for 2 d (A) or 4 d (B) in the absence or presence of AMH. The number of growing follicles found in ovaries cultured in the presence of AMH is shown as a percentage of the control. N represents the number of ovaries that were investigated. Data represent the mean ± SEM. The *asterisks* indicate a statistically significant difference ($P \leq 0.05$).

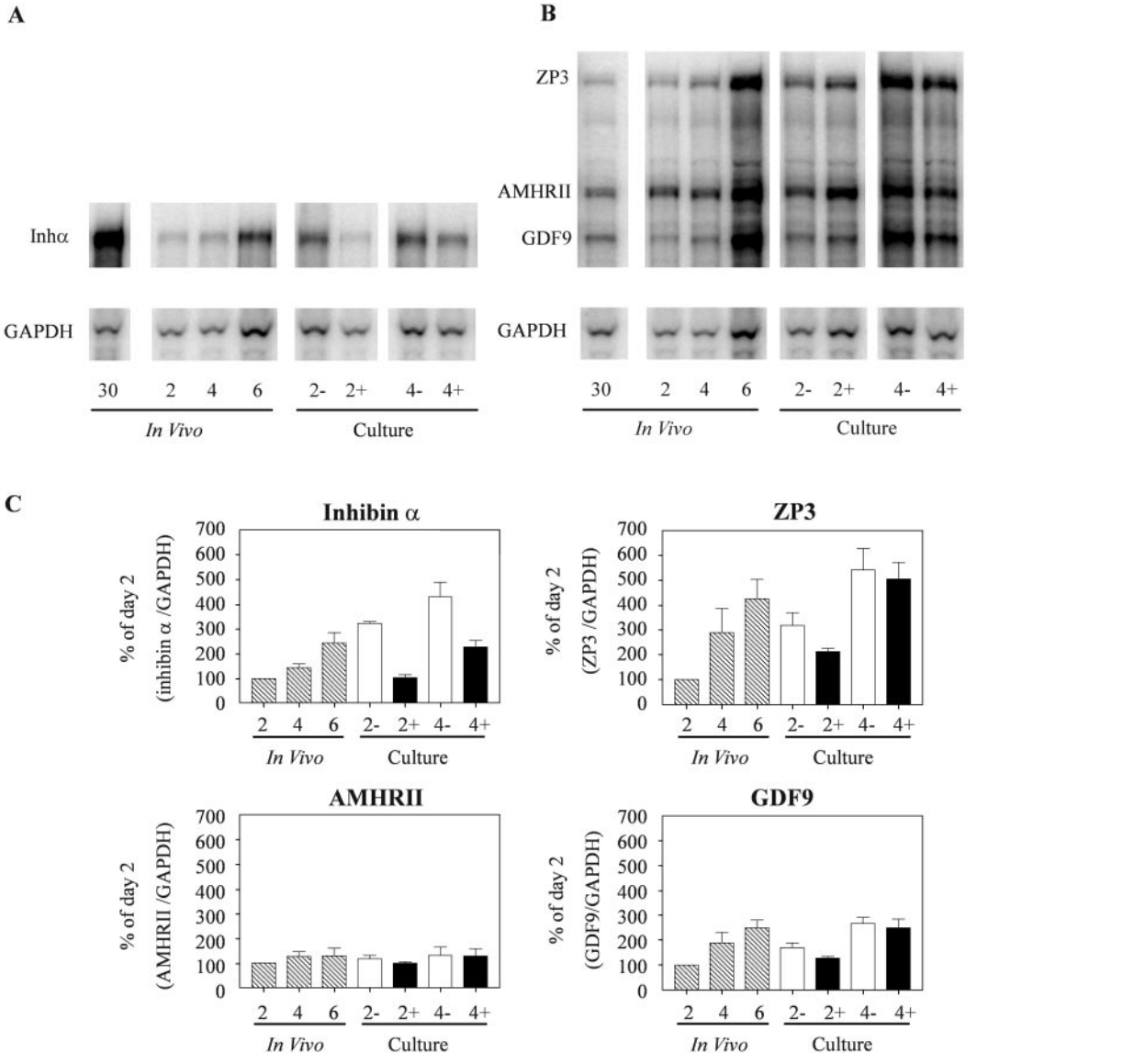


FIG. 5. Effect of AMH on expression of GDF9, ZP3, AMHRII, and inhibin α-subunit mRNAs. A and B, PhosphorImager analysis of a representative RNase protection assay for inhibin α-subunit and GAPDH mRNA expression (A) and for ZP3, AMHRII, GDF9 and GAPDH mRNA expression (B) in neonatal ovaries of 2-, 4-, and 6-d-old control mice, and in 2-d-old ovaries cultured for 2 or 4 d in the presence (+) or absence (−) of AMH. RNA from 30-d-old mice is used as a control. C, Quantification of inhibin α-subunit, ZP3, AMHRII, and GDF9 mRNA levels in 2-, 4-, and 6-d-old control mouse ovaries, and in 2-d-old mouse ovaries cultured for 2 or 4 d in the absence or presence of added AMH. All these mRNA levels were normalized to that of GAPDH mRNA. Values are represented as a percentage of 2-d-old ovaries (considered 100%) and are the mean ± SEM of two independent experiments. For every culture condition, total RNA of 20 ovaries was pooled, whereas total RNA of 20 ovaries of 2-, 4-, and 6-d-old animals and of 5 ovaries of 30-d-old animals were pooled.

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 $\alpha$-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 $\alpha$-inhibin, used as a marker of early follicle development. However, a functional role of $\alpha$-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 Byskov *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 Verhoef-Post for generating the AMH-producing HEK293 cells, Anke McLuskey-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. 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.fgg.eur.nl.

## References

1. **Peters H** 1969 The development of the mouse ovary from birth to maturity. Acta Endocrinol (Copenh) 62:98–116

2. **Peters H** 1978 In: Jones RE, ed. The vertebrate ovary. New York: Plenum Press; 121–144
3. **Ueno S, Kuroda T, Maclaughlin DT, Ragin RC, Manganaro TF, Donahoe PK** 1989 Müllerian inhibiting substance in the adult rat ovary during various stages of the estrous cycle. Endocrinology 125:1060–1066
4. **McGee EA, Hsueh AJW** 2000 Initial and cyclic recruitment of ovarian follicles. Endocr Rev 21:200–214
5. **Parrott JA, Skinner MK** 1999 Kit-ligand/stem cell factor induces primordial follicle development and initiates folliculogenesis. Endocrinology 140:4262–4271
6. **Vitt UA, McGee EA, Hayashi M, Hsueh AJW** 2000 *In vivo* treatment with GDF9 stimulates primordial and primary follicle progression and theca cell marker CYP17 in ovaries of immature rats. Endocrinology 141:3814–3820
7. **Nilsson E, Parrott JA, Skinner MK** 2001 Basic fibroblast growth factor induces primordial follicle development and initiates folliculogenesis. Mol Cell Endocrinol 25:123–130
8. **Dissen GA, Romero C, Hirshfield AN, Ojeda SR** 2001 Nerve growth factor is required for early follicular development in the mammalian ovary. Endocriology 142:2078–2086
9. **Durlinger ALL, Kramer P, Karels B, de Jong FH, Uilenbroek JTJ, Grootegoed JA, Themmen APN** 1999 Control of primordial follicle recruitment by anti-Müllerian hormone in the mouse ovary. Endocrinology 140:5789–5796
10. **Cate RL, Mattaliano RJ, Hession C, Tizard R, Farber NM, Cheung A, Ninfa EG, Frey AZ, Gash DJ, Chow EP, Fisher RA, Bertonis JM, Torres G, Wallner BP, Ramachandran KL, Ragin RC, Manganaro TF, MacLaughlin DT, Donahoe PK** 1986 Isolation of the bovine and human genes for Müllerian inhibiting substance and expression of the human gene in animal cells. Cell 45:685–698
11. **Jost A** 1947 Recherches sur la differenciation sexuelle de l'embryon de lapin. Arch Anat Microsc Morphol Exp 36:271–315
12. **Hirobe S, He WW, Lee MM, Donahoe PK** 1992 Müllerian inhibiting substance messenger ribonucleic acid expression in granulosa and Sertoli cells coincides with their mitotic activity. Endocrinology 131:854–862
13. **Baarends WM, Uilenbroek JTJ, Kramer P, Hoogerbrugge JW, van Leeuwen EC, Themmen APN, Grootegoed JA** 1995 Anti-Müllerian hormone and anti-Müllerian hormone type II receptor messenger ribonucleic acid expression in rat ovaries during postnatal development, the estrous cycle, and gonadotropin-induced follicle growth. Endocrinology 136:4951–4962
14. **Baarends WM, van Helmond MJ, Post M, van der Schoot PJ, Hoogerbrugge JW, de Winter JP, Uilenbroek JTJ, Karels B, Wilming LG, Meijers JH, Themmen APN, Grootegoed JA** 1994 A novel member of the transmembrane serine/threonine kinase receptor family is specifically expressed in the gonads and in mesenchymal cells adjacent to the Müllerian duct. Development 120: 189–197
15. **Nachtigal MW, Ingraham HA** 1996 Bioactivation of Müllerian-inhibiting substance during gonadal development by a kex2/subtilisin-like endoprotease. Proc Natl Acad Sci USA 93:7711–7716
16. **Ingraham HA, Hirokawa Y, Roberts LM, Mellon SH, McGee E, Nachtigal MW, Visser JA** 2000 Autocrine and paracrine Müllerian-inhibiting substance hormone signaling in reproduction. Recent Prog Horm Res 55:53–68
17. **Cooke PS, Young PF, Cunha GR** 1987 A new model system for studying androgen-induced growth and morphogenesis *in vitro*: the bulbourethral gland. Endocrinology 121:2161–2170
18. **Emmen JMA, McLuskey A, Adham IM, Engel W, Grootegoed JA, Brinkmann AO** 2000 Hormonal control of gubernaculum development during testis descent; gubernaculum outgrowth *in vitro* requires both Insl 3 and androgen. Endocrinology 141:4720–4727
19. **Pedersen T, Peters H** 1968 Proposal for classification of oocytes and follicles in the ovary of the mouse. J Reprod Fertil 17:555–557
20. **Van Cappellen WA, Meijs-Roelofs HM, Kramer P, Van den Dungen HM** 1989 Ovarian follicle dynamics in immature rats treated with a luteinizing hormone-releasing hormone antagonist (Org. 30276). Biol Reprod 40:1247–1256
21. **Chomczynski P, Sacchi N** 1987 Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162: 156–159
22. **Laitinen M, Vuojolainen K, Jaatinen R, Ketola I, Aaltonen J, Lehtonen E, Heikinheimo M, Ritvos O** 1998 A novel growth differentiation factor-9 (GDF-9) related factor is co-expressed with GDF-9 in mouse oocytes during folliculogenesis. Mech Dev 78:135–140
23. **Visser JA, McLuskey A, Verhoef-Post M, Kramer P, Grootegoed JA, Themmen APN** 1998 Effect of prenatal exposure to diethylstilbestrol on Müllerian duct development in fetal male mice. Endocrinology 139:4244–4251
24. **Byskov AG, Lintern-Moore S** 1973 Follicle formation in the immature mouse ovary: the role of the rete ovarii. J Anat 116:207–217
25. **Eppig JJ, O'Brien MJ** 1996 Development *in vitro* of mouse oocytes from primordial follicles. Biol Reprod 54:197–207
26. **Matzuk MM, Kumar TR, Shou W, Coerver KA, Lau AL, Behringer RR, Finegold MJ** 1996 Transgenic models to study the roles of inhibins and activins in reproduction, oncogenesis, and development. Recent Prog Horm Res 51: 123–154; discussion 155–127
27. **Byskov AG, Guoliang X, Andersen CY** 1997 The cortex-medulla oocyte growth pattern is organized during fetal life: an *in-vitro* study of the mouse ovary. Mol Hum Reprod 3:795–800
28. **Krarup T, Pedersen T, Faber M** 1969 Regulation of oocyte growth in the mouse ovary. Nature 224:187–188
29. **Elvin JA, Yan C, Wang P, Nishimori K, Matzuk MM** 1999 Molecular characterization of the follicle defects in the growth and differentiation factor 9-deficient ovary. Mol Endocrinol 13:1018–1034