2005

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MOLECULAR REPRODUCTION AND DEVELOPMENT 71:480–488 (2005)

Evidence for a Role for Anti-Müllerian Hormone in the Suppression of Follicle Activation in Mouse Ovaries and Bovine Ovarian Cortex Grafted Beneath the Chick Chorioallantoic Membrane

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ABSTRACT The first critical transition in follicular development, the activation of primordial follicles to leave the pool of resting follicles and begin growth, is poorly understood, but it appears that the balance between inhibitory and stimulatory factors is important in regulating the exodus of follicles from the resting pool. There is evidence that anti-Müllerian hormone (AMH; also known as MIS) inhibits follicle activation in mice, but whether it plays a similar role in non rodent species is not known. When pieces of bovine ovarian cortex, rich in primordial follicles, are cultured in serum-free medium, most follicles initiate growth, but when cortical pieces are grafted beneath the chorioallantoic membrane (CAM) of chick embryos, follicle activation does not occur. Since embryonic chick gonads of both sexes produce and secrete high levels of AMH, the hypothesis that the AMH in the chick circulation inhibits follicle activation was tested. In Experiment 1, whole newborn mouse ovaries were grafted beneath the CAM (placed "in ovo") or cultured in vitro for 8 days. In vitro (or after 8 days in vivo) follicles activated and proceeded to the primary or secondary stage, but activation was suppressed in ovo. This inhibition was reversed if ovaries were removed from beneath the CAM and cultured in vitro. In contrast, when ovaries from mice null mutant for the AMH type II receptor were CAM-grafted in Experiment 2, follicle activation occurred in a similar fashion to activation in vitro. This finding strongly implicates AMH as the inhibitor of follicle activation in ovo. Since chick embryonic gonads are the source of circulating AMH, chicks were gonadectomized in Experiment 3, prior to grafting of pieces of bovine ovarian cortex beneath their CAMs. Bovine primordial follicles activated in the gonadectomized chicks, similar to the results for mice lacking the AMH type II receptor. Taken together these experiments provide strong evidence that AMH is the inhibitor of mouse follicle activation present in the circulation of embryonic chicks and provide indirect, and hence more tentative, evidence for AMH as an inhibitor of bovine follicle activation. *Mol. Reprod. Dev. 71: 480–488, 2005.*

Key Words: ovary; ovarian follicle; follicular development; primordial follicle; anti-Müllerian hormone; cattle; mice

INTRODUCTION

In mammalian ovaries, primary oocytes, the prospective female gametes, are stored within non growing primordial follicles. In some species primordial follicles form during fetal life (e.g., primates, cattle, and sheep), whereas in other species follicle formation does not occur until after birth (e.g., mice, rats, and pigs). The mechanisms involved in the activation process, the transformation from the resting primordial to the growing primary follicle, and in the timing that allows some follicles to start growing while others remain inactive are still largely unknown, especially for larger mammalian species (reviewed in Fortune, 2003).

Our laboratory has developed two experimental models that can be used to study primordial follicle activation in large mammals. The first is a serum-free organ culture system for pieces of ovarian cortex, the ovarian compartment where primordial follicles reside. In this in vitro system, primordial follicles in bovine and baboon ovarian cortex activate and, contrary to the situation in vivo where only a few follicles activate at any one time, almost all the primordial follicles develop to the primary stage within 1–2 days (Wandji et al., 1996, 1997; Fortune et al., 2000). This suggests

Grant sponsor: USDA; Grant numbers: 00-35203-9151, 03-35203-13532.

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Received 28 December 2004; Accepted 10 January 2005
Published online 13 May 2005 in Wiley InterScience (www.interscience.wiley.com).
DOI 10.1002/mrd.20338

that gonadotropins or other extra-ovarian factors are not required for primordial follicle activation and highlights the potential importance of inhibitory factors to the regulation of follicle activation in vivo. The second experimental model consists of grafting ovarian cortical pieces beneath the chorioallantoic membrane (CAM) of 6-day-old chick embryos. In this situation, bovine cortical pieces are rapidly vascularized and primordial follicles remain healthy, but the wholesale spontaneous activation observed in vitro does not occur "in ovo" (Cushman et al., 2002), suggesting that chick embryos produce an inhibitor of follicle activation.

It is likely that follicle activation in vivo is controlled by a balance of stimulatory and inhibitory factors. The results obtained with both the in vitro and in ovo experimental systems described above highlight the importance of inhibitory factors and release from their effects in primordial follicle activation in cattle, a species of practical interest and an excellent model for human ovarian function. In mice, anti-Müllerian hormone (AMH, also known as Müllerian inhibiting substance or MIS), a member of the transforming growth factor β superfamily, has been implicated in the inhibition of follicle activation. *Amh* null mice are fertile, but their store of primordial follicles is depleted earlier in life than in control animals (Durlinger et al., 1999) and addition of AMH to cultures of mouse ovaries inhibited follicular activation (Durlinger et al., 2002). We have hypothesized that the complete inhibition of follicle activation observed in bovine cortical pieces in ovo is due to inhibitory effects of AMH in the chick circulation, since both gonadal and circulating concentrations of AMH are high for much of chick embryonic life (Hutson et al., 1981; Teng, 1987).

In the current study, we have explored the nature of the inhibitory factor that suppresses follicle activation in ovo, using a combination of experiments with neonatal mouse ovaries and bovine ovarian cortical tissue in different experimental situations (in ovo, in vitro, and in vivo). Although large mammals such as domestic animals and humans are the practical targets of interest in studying the regulation of follicle growth initiation, mice are very useful experimental models. The small size of the ovaries, which allows the culture of whole ovaries, the fact that follicle formation and the initiation of follicle growth occur within a short period after birth, and the availability of mice in which specific gene functions have been deleted provide experimental advantages not available for larger mammals. Specifically, these experiments were designed to (1) determine the effects of culture in ovo on whole mouse ovaries, compared with culture in vitro and with the in vivo situation, (2) determine, using mice lacking the gene for the AMH type II receptor (*Amhr2*), whether AMH is the (or a) inhibitor of follicle activation in ovo, and (3) determine whether the gonads, which are the source of AMH in chick embryos, are the source of the factor that inhibits activation of bovine primordial follicles in ovo.

MATERIALS AND METHODS

Animals and Isolation of Ovarian Tissue

For Experiment 1, female and male C57BL/6J mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and bred. Neonatal female pups were killed by decapitation either within 24 hr of birth (for control, in vitro, and in ovo groups) or at 8 days of age (in vivo group). The ovaries were dissected from the pups and either fixed immediately (control and in vivo groups), placed in organ culture (in vitro group), or grafted beneath the CAM of 6-day-old chick embryos (in ovo group), as described further in the sections below. For Experiment 2, mice heterozygous for a null mutation in the *Amhr2* gene (Jamin et al., 2002) were obtained from Dr. R. Behringer (M.D. Anderson Cancer Center, Houston, TX) and bred to obtain homozygous mutant and wild type progeny. The ovaries were dissected from female pups within 24 hr of birth and cultured in vitro or in ovo by methods described below. For Experiment 3, pieces of bovine ovarian cortex were obtained during the last third of gestation, as described previously (Wandji et al., 1996) and then cultured in vitro or in ovo. All use of live animals was in accordance with procedures approved by the Cornell University Animal Care and Use Committee.

Experimental Designs

Experiment 1. Experiment 1 was designed to test two hypotheses: (1) that follicle activation would be inhibited in whole mouse ovaries grafted beneath the chick CAM, similar to previous results for isolated pieces of bovine ovarian cortex, and (2) that more follicles would leave the resting pool in mouse ovaries cultured in vitro compared to litter mates in vivo. To test these hypotheses, ovaries from newborn mice were either fixed immediately (control) or grafted beneath the CAM of chick embryos (in ovo) or placed in organ culture (in vitro) in Experiment 1A. After 8 days in vitro, in ovo, or in vivo (i.e., pups left with their mothers for 8 days) ovaries were retrieved and fixed for histological morphometry as described below.

Based on the results of Experiment 1A, Experiment 1B was conducted to determine if primordial follicles in CAM grafts retain the ability to activate if the grafts are removed and cultured in vitro. Therefore, ovaries placed beneath the CAM were retrieved 2 days after grafting and then either placed in vitro for 8 days (crossover) or fixed immediately for histology (day 2 control).

Experiment 2. This experiment was designed to test the hypothesis that the inhibition of follicle activation observed in Experiment 1 was due to the high circulating levels of AMH in chick embryos. Ovaries were isolated 12–36 hr after birth from wild type female pups and litter mates null mutant for the *Amhr2* gene. One ovary of each pair was grafted beneath the CAM of a chick embryo and the other was placed in organ culture. After 4 days in ovo or in vitro, ovaries were retrieved and fixed for histological studies. Tail tissue of the pups was used for genotyping, as described below.

Experiment 3. Since the results of Experiments 1 and 2 suggested that the inhibitor of follicle activation in the circulatory system of embryonic chicks is AMH, we hypothesized that AMH is also the inhibitor of follicle activation in CAM-grafted pieces of bovine ovarian cortex. Because mutants deficient in AMH or its type II receptor are not available for cattle, we took an indirect approach to testing this hypothesis. Since AMH is produced by embryonic chick ovaries and testes, destruction of the chick gonads prior to CAM grafting of bovine cortical pieces should allow follicle activation to occur in bovine ovarian cortex in ovo, if the hypothesis is correct. In this experiment bovine cortical pieces, isolated as described previously (Wandji et al., 1996) were treated in one of five ways: (1) fixed immediately (day 0 control), (2) placed in culture (in vitro control), (3) grafted beneath the CAM of intact chick embryos (in ovo control), (4) grafted beneath the CAM of chick embryos whose gonads had been destroyed by electrocautery, or (5) grafted beneath the CAM of sham-cauterized chick embryos (control for cautery). Cortical pieces in groups 2–5 were collected after 2 days and fixed for histological analysis.

Culture In Vitro

Ovaries of newborn mice or bovine cortical pieces were cultured in 24-well Costar plates (Corning, Inc., Corning, NY) on uncoated culture plate inserts (Millicell-CM, 0.4 μm pore size, Millipore Corporation, Bedford, MA) with 300 μl of Waymouth medium MB 752/1 (Invitrogen, Carlsbad, CA) supplemented with 50 μg streptomycin sulfate and 75 μg penicillin/ml (Invitrogen), ITS+ (6.25 μg insulin, 6.25 μg transferrin, 6.25 ng selenious acid, 1.25 mg BSA, and 5.35 μg linoleic acid per ml; BD Biosciences, Bedford, MA) and 25 mg/L pyruvic acid (Sigma Chemical Co., St. Louis, MO). Tissue was cultured for the periods of time specified for each experiment in a humidified incubator with 95% air:5% CO_2 at 38–38.5°C. Every other day, 200 μl of medium was withdrawn and replaced with fresh medium.

Culture In Ovo and Gonadectomy

Fertilized chicken eggs were obtained from the Cornell University Poultry Farm and incubated at 38°C and 60% humidity. Prior to the experiments, a window was made in the shells of 3-day-old fertilized chick eggs. The shell was gently opened and a piece of shell removed; the eggs were then sealed with a piece of Scotch-3M tape and returned to the incubator. CAM grafting was performed using 6-day-old eggs, as described previously for bovine ovarian cortical pieces (Cushman et al., 2002). Briefly, neonatal mouse ovaries or pieces of bovine ovarian cortex were placed beneath the developing CAM, between the CAM and the yolk sac (one ovary or cortical piece per embryo). The grafts were retrieved after 2, 4, or 8 days in ovo. Retrieved grafts were either fixed immediately for histological studies or, in Experiment 1B, placed in vitro for a further 8 days and then retrieved for histological analysis.

In Experiment 3, some chick embryos were gonadectomized or sham cauterized on day 5 of incubation, prior to CAM-grafting on day 6. The ground lead of a surgical electrocautery unit was introduced into the thin albumen of the egg through a pinhole in the shell. The embryo was elevated through the window of the egg with a smooth glass rod, with care taken to avoid damage to extraembryonic membranes and blood vessels. The embryo was cauterized along both sides of its spine to ablate the gonadal ridge as well as some of the associated metanephros, using a weak coagulating current. Very little bleeding resulted from this procedure. The embryo was then lowered back into the egg, the window in the shell was re-sealed with fresh tape, the pinhole was sealed with paraffin, and the egg was returned to the incubator. Sham-operated controls received cautery treatment to a hind limb. The success of gonadal destruction was confirmed at the end of the experiment, when CAM grafts were retrieved.

Histology and Morphometric Analysis of Mouse Ovaries and Bovine Cortical Pieces

Ovaries were fixed for 1 hr in 2.5% glutaraldehyde, 2.5% formaldehyde in 0.075M cacodylate buffer, pH 7.3 (Tousimis Research Corporation, Rockville, MD). The ovaries were embedded in LR White plastic (EMS, Fort Washington, NJ) and 2 μm sections were cut with a glass knife. To avoid counting the same follicles twice, every other set of 10 consecutive sections was mounted on gelatin-coated slides and stained with toluidine blue. Only the largest section within each set of 10 was selected for counting and only follicles in which the oocyte nucleus was present were counted. Follicles were classified and measured using an inverted microscope with Hoffman modulation contrast optics. The image was projected onto a video monitor and the diameters of all healthy follicles and their enclosed oocytes were measured using a computer-driven image analysis program (NIH Image; NIH, Bethesda, MD).

Follicles were classified as (1) primordial, when there was a single layer of flattened granulosa cells around the oocyte, (2) primary, when a single layer of cuboidal granulosa cells enclosed the oocyte, or (3) secondary, when the oocyte was surrounded by two or more complete layers of granulosa cells. In addition, follicles were classified as either healthy or atretic. Follicles with intact basal lamina, an oocyte with no more than three cytoplasmic vacuoles, an intact germinal vesicle and a healthy nucleolus were classified as healthy. Follicles in which the oocyte had >3 cytoplasmic vacuoles and the beginning of chromatin condensation were considered to be early atretic. Follicles with fragmentation of the oocyte cytoplasm and nucleolus and heavy condensation of the oocyte chromatin were classified as moderately atretic. Advanced atresia was marked by complete fragmentation or absence of the oocyte.

Genotyping

Female progeny of parents heterozygous for a null mutation of the *Amhr2* gene were genotyped to identify

homozygous wild type and mutant pups. Genomic DNA was isolated by incubating tail tissue of newborn mice at 55°C for 3 hr in 500 μl 50 nM Tris-HCl pH 8.0, 100 mM EDTA pH 8.0, 100 mM NaCl, 1% SDS, 1.2 mg/ml proteinase K (Ambion; Austin, TX), neutralized with phenol/chloroform/isoamyl alcohol (25:24:1) for 1 hr and centrifuged (14,000 rpm) for 5 min. The upper phase was transferred to a fresh microfuge tube and the DNA was precipitated with an equal volume of 100% ethanol. The DNA was pelleted by centrifugation (14,000 rpm) for 5 min. The supernatant was removed and the pellet washed with cold (−20°C) 70% ethanol, followed by centrifugation (14,000 rpm) for 5 min. The supernatant was discarded and the DNA pellet air-dried and then dissolved in 100 μl TE (10 mM Tris-HCl, pH 7.5, and 1 mM EDTA, pH 8.0).

PCR was performed on 1 μl of DNA using the following sets of primers: MISr2-Fw8 (5′-AGGTGGGTCAGACC-CAGAGC-3′) and MISr2-Rv9 (5-′GCATGACCTCCT-TCCTGGATT-3′) to determine the presence of the wild-type allele (PCR product of 223 bp); Cre-up (5′-TCCAATTTACTGACCGTACACCAA-3′) and Cre-dn (5′-CCTGATCCTGGCAATTTCGGCTA-3′) to determine the mutant band (PCR product of 500 bp). All chemicals for PCR were from Invitrogen (Life Technologies, Rockville, MD). The PCR reaction mixture was preheated for 5 min at 95°C followed by 35 amplification cycles (denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, extension at 72°C for 45 sec). The products were visualized by ethidium bromide staining after electrophoresis on a 2.5% agarose gel.

Statistical Analysis

Mean numbers of total primordial, primary and secondary follicles per section, mean numbers of healthy primordial, primary and secondary follicles per section, and mean diameters of healthy follicles and oocytes were calculated for each mouse ovary and for the cortical pieces from each fetal bovine ovary. If Hartley's test indicated heterogeneity of variance among means, values were transformed to logarithms before further analysis, but only non transformed data are presented in the figures. Differences among treatment groups were tested by one-way ANOVA, followed by Duncan's multiple range test when the ANOVA indicated a significant difference.

RESULTS

Experiment 1: Follicular Populations in Mouse Ovaries In Vivo, In Vitro, and In Ovo

On the day of birth, mouse ovaries contained only primordial follicles, as expected (Fig. 1, day 0). After 8 days in vivo, some primordial follicles had activated and developed to the primary or secondary stage. In vitro, some primordial follicles also had activated and progressed to the primary or secondary stage, but the number of primary follicles and the total number of growing follicles were higher in ovaries maintained in vitro, compared to contemporaneous ovaries in vivo ($P < 0.05$). Ovaries grafted beneath the chick CAM (in ovo) became well vascularized and the small number of growing follicles present after 8 days did not differ from ovaries on day 0 ($P > 0.05$), indicating that, in contrast to the in vitro and in vivo groups, activation did not occur in ovo. In all groups, >90% of follicles were healthy (data not shown). In contrast to the differences observed in numbers of follicles (Fig. 1), neither follicle nor oocyte diameters differed among treatment groups, within each follicular stage (Fig. 2).

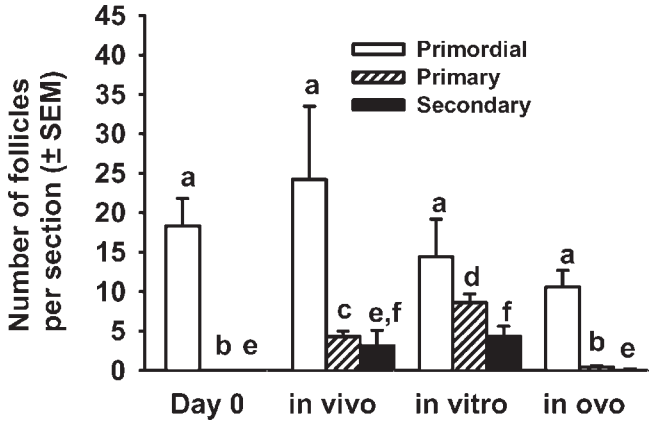

Fig. 1. Numbers of primordial, primary and secondary follicles (mean per section ± SEM) in mouse ovaries on the day of birth (day 0), or after 8 days in vivo, in vitro, or in ovo. Within each follicular stage, bars with no common superscripts are different ($P < 0.05$; N = 4 ovaries per group).

To determine, if the suppression of follicle activation in mouse ovaries in ovo is a reversible effect, ovaries were retrieved after 2 days in ovo and were then either fixed for histological assessment or transferred to the in vitro culture system for an additional 8 days. Ovaries switched from in ovo to in vitro culture (crossover) had more primary and total growing follicles at the end of culture in vitro compared to numbers after 2 days in ovo ($P < 0.05$, Fig. 3), showing that mouse primordial follicles in CAM grafts retain the ability to activate. Diameters of follicles and their oocytes did not differ between the day 2 in ovo and crossover groups, but diameters of secondary follicles and their oocytes in the in vitro group were greater than in the in ovo and crossover groups and the diameters of the primary follicles and their oocytes in the in vitro group were greater than the in ovo group (Fig. 4).

Experiment 2: Role of AMH Type II Receptor in Suppression of Follicle Activation In Ovo

To test the hypothesis that the inhibition of activation observed in ovo is due to the high level of circulating AMH in chick embryos, one ovary of newborn *Amhr2* (+/+) and (−/−) mice was placed in ovo and the other ovary was cultured in vitro. Ovaries were retrieved and fixed after 4 days in ovo or in vitro. In the in vitro group, most follicles were at the primordial stage, but, as expected, a number of follicles had activated (Fig. 5B). There was no effect of genotype on numbers of primordial or growing (primary plus a few secondary) follicles in vitro. All

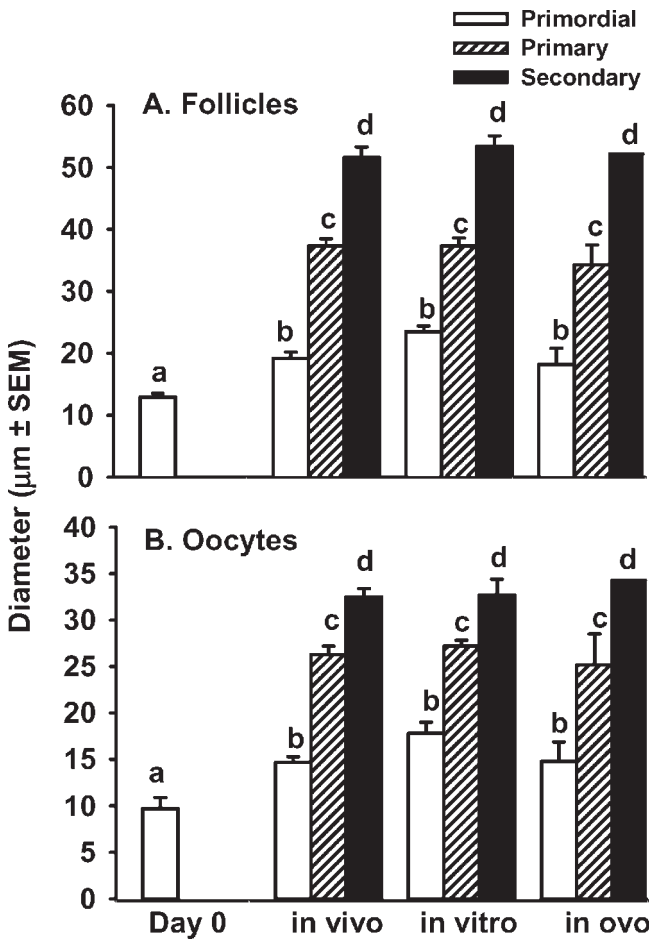

Fig. 2. Mean diameters (μm ± SEM) of healthy primordial, primary and secondary follicles and their oocytes in mouse ovaries on the day of birth (day 0), or after 8 days in vivo, in vitro, or in ovo. Within each follicular stage, bars with no common superscripts are different ($P < 0.05$; N = 4 ovaries per group).

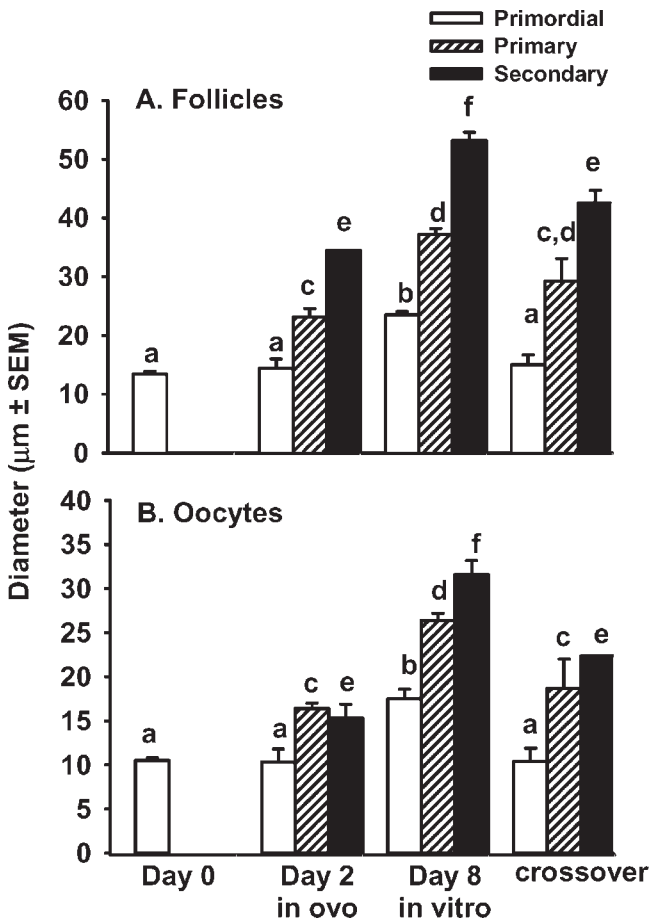

Fig. 4. Mean diameters (μm ± SEM) of healthy primordial, primary and secondary follicles and their oocytes in mouse ovaries on the day of birth (day 0) or after 2 days in ovo, 8 days in vitro, or 2 days in ovo followed by 8 days in vitro (crossover). Within each follicular stage, bars with no common superscripts are different ($P < 0.05$; N = 5 ovaries per group).

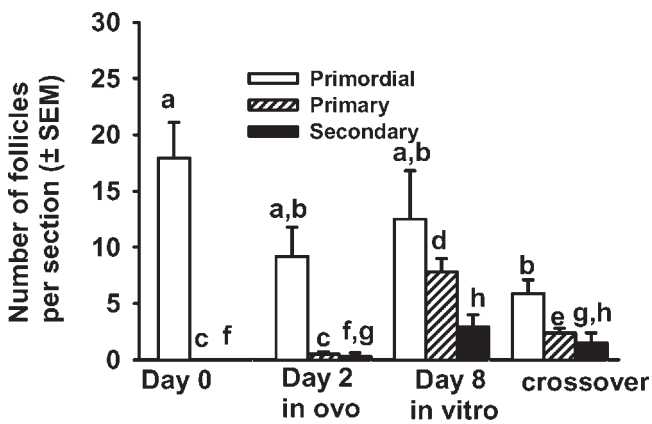

Fig. 3. Numbers of primordial, primary and secondary follicles (mean per section ± SEM) in mouse ovaries on the day of birth (day 0) or after 2 days in ovo, 8 days in vitro, or 2 days in ovo followed by 8 days in vitro (crossover group). Within each follicular stage, bars with no common superscripts are different ($P < 0.05$; N = 5 ovaries per group).

ovaries grafted beneath the chick CAM became vascularized and were healthy when they were retrieved after 4 days. Contrary to the situation in vitro, where follicles of both wild type and *Amhr2* null mutant ovaries had activated, activation occurred in ovo only in ovaries from *Amhr2* (−/−) mice (Fig. 5A). *Amhr2* (−/−) ovaries had significantly more growing follicles compared to the *Amhr2* (+/+) group, showing that the absence of a functional *Amhr2* gene is associated with activation of primordial follicles when ovaries are grafted beneath the CAM of chick embryos.

Experiment 3: Effects of Gonadectomy on Follicle Activation in Bovine Cortical Pieces In Ovo

The results of Experiment 2 above provide evidence that AMH in the embryonic chick circulation inhibits activation of primordial follicles in whole mouse ovaries grafted in ovo. Experiment 3 was designed to test the hypothesis that AMH is also the factor that inhibits activation in pieces of bovine ovarian cortex in ovo. Since AMH is produced by the developing testes and ovaries of embryonic chicks, this hypothesis was tested by gona-

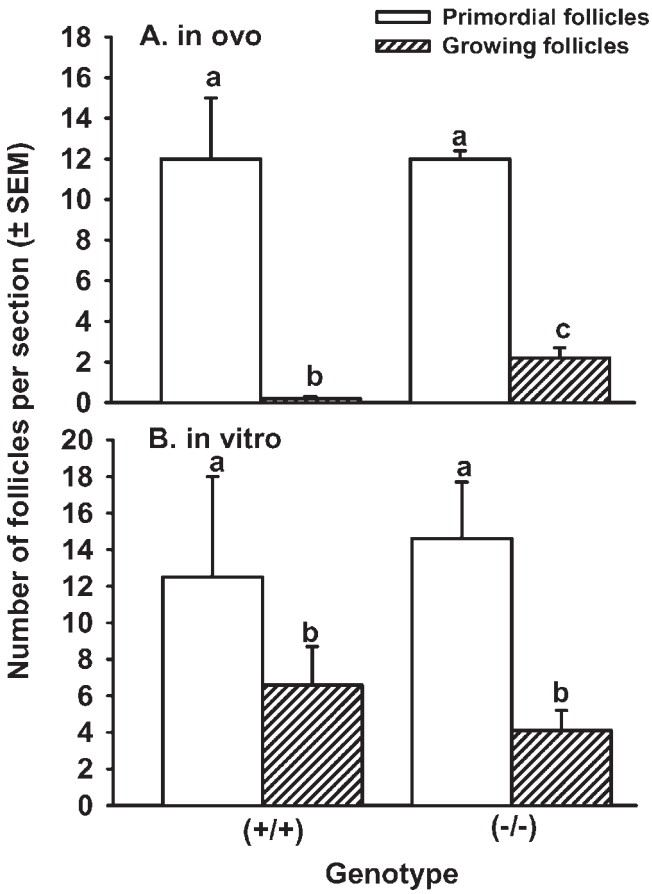

Fig. 5. Effect of presence (+/+) or absence (−/−) of the gene for the anti-Müllerian hormone (AMH) type II receptor on the numbers of primordial and primary follicles (mean per section ± SEM), after 4 days in ovo (**A**) or 4 days in vitro (**B**). Within each follicular stage, bars with no common superscripts are different ($P < 0.05$; N = 2 ovaries per group).

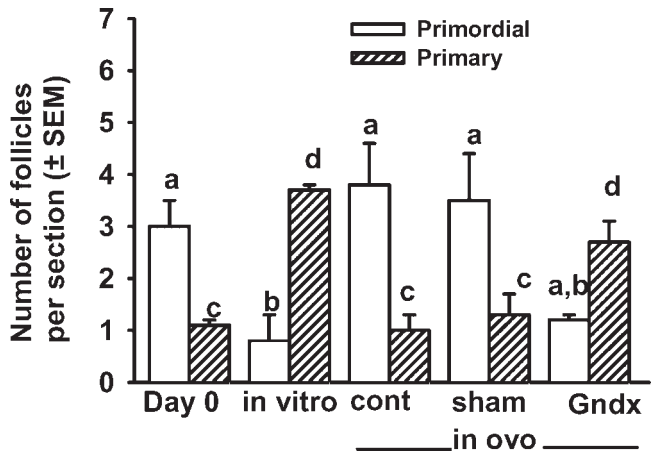

Fig. 6. Effect of gonadectomizing embryonic chicks on follicle activation in chorioallantoic membrane (CAM)-grafts of bovine ovarian cortex. Numbers of primordial and primary follicles (mean per section ± SEM) on day 0, after 2 days in vitro, or 2 days after CAM-grafting (in ovo) into intact (control), sham-operated, or gonadectomized (Gdnx) 6-day-old chicks. Within each follicular stage, bars with no common superscripts are different ($P < 0.05$; N = 2–3 separate experiments per group).

dectomizing chicks prior to CAM grafting bovine ovarian cortical pieces, with intact and sham operated chicks (cautery of non gonadal area) serving as controls. As expected, freshly isolated bovine cortical pieces had mostly primordial follicles and smaller numbers of primary follicles (Fig. 6, day 0). After 2 days in vitro, the number of primordial follicles had decreased by about 75%, whereas primary follicles had increased concomitantly, indicating that activation occurred in culture as expected. The numbers of primordial and primary follicles in cortical pieces CAM-grafted to intact or sham operated controls were not different from day 0 controls, indicating that activation did not occur in these situations (i.e., when chick gonads were intact). In contrast, when cortical pieces were transplanted to gonadectomized chicks, the number of primordial follicles after 2 days in ovo was not different from cultures in vitro (Fig. 6). In addition, in cortical pieces retrieved from gonadectomized chicks, the number of primary follicles was about 2.5 times greater than in day 0 controls ($P < 0.05$) and not different from in vitro controls ($P > 0.05$), suggesting that, in the absence of gonads, activation had occurred (Fig. 6). The numbers of healthy primordial and primary follicles followed a pattern similar to the total numbers of follicles and there was no effect of treatment on follicle or oocyte diameters (data not shown).

DISCUSSION

The current results provide the first insight into the potential role of AMH in follicle activation in a large mammalian species and also provide new evidence to support the postulated role of AMH in activation of mouse follicles. Since the ovaries of large mammals cannot be cultured intact and null mutants are not available, we used a variety of approaches in ovo with whole mouse ovaries and bovine cortical pieces. The results showed that the inhibition of follicle activation previously documented for bovine ovarian cortical pieces grafted beneath the CAM of chick embryos (Cushman et al., 2002) is also evident when whole newborn mouse ovaries are placed in ovo. In addition, the inhibition was overcome by CAM-grafting ovaries of mice lacking functional AMH receptors (*Amhr2* null mutant mice) into intact chicks and by grafting bovine cortical pieces beneath the CAM of gonadectomized chicks. The results of these two different experimental approaches implicate AMH secreted by chick embryonic gonads as the inhibitor of follicle activation in ovo. Taken together, these results suggest that AMH is an inhibitor of follicle activation in large mammals, such as cattle, as well as in rodents.

Although several lines of evidence suggested that the activation of primordial follicles is regulated by negative, as well as positive, factors, little was known about how inhibition is effected in vivo until Durlinger et al. (1999) analyzed follicular development in *Amh* null mutant mice. AMH (also known as MIS) has long been

recognized as the hormone produced by fetal testes that regresses the Müllerian ducts during differentiation of the male reproductive tract (see reviews by Josso et al., 2001; Teixeira et al., 2001). Although *Amh* null mutant mice exhibited the expected deficits in male development, homozygous mutant females initially appeared normal (Behringer et al., 1994). However, careful analyses of *Amh* null mutant mice by Durlinger et al. (1999) revealed that, although the ovaries of prepubertal and young adult mice have more growing preantral and small antral follicles than wild type controls, the stock of primordial follicles becomes depleted earlier in life. Further support for AMH as an inhibitor of follicle activation was derived from the finding that addition of AMH to cultures of newborn mouse ovaries partially inhibited the initiation of follicle growth (Durlinger et al., 2002).

Results of previous experiments in our laboratory suggest that AMH might also inhibit follicle activation in cattle. When bovine or baboon ovarian cortical pieces, rich in primordial follicles, are cultured in vitro, most primordial follicles activate, but progression to the secondary stage is rare (Wandji et al., 1996, 1997). We hypothesized that grafting cortical pieces beneath the chick CAM, where they could become vascularized, would promote growth beyond the primary stage. Contrary to this expectation, the follicle activation that occurs in vitro was absent in ovo, although the cortical pieces were rapidly vascularized and follicles remained healthy for 10 days in ovo (Cushman et al., 2002). Since chick embryonic gonads of both sexes produce high levels of AMH (Hutson et al., 1981; Teng, 1987), the inhibition of follicle activation in ovo suggested that AMH may negatively regulate follicle activation in large mammalian species like cattle. However, addition of recombinant human AMH to cultures of bovine cortical pieces did not block the wholesale spontaneous activation that occurs in vitro, although it did attenuate the growth of activated, primary follicles (Cushman and Fortune, 2003). This finding led us to take a more indirect approach in the current experiments to testing the hypothesis that AMH is a negative regulator of follicle activation in cattle, using the CAM-graft model with whole, wild type mouse ovaries and mouse ovaries lacking the AMH receptor *Amhr2* and with bovine cortical pieces transplanted into gonadectomized chick embryos.

When newborn mouse ovaries were grafted beneath the CAM, the activation of follicles, which was observed after 8 days in vivo or in vitro, was absent. This result is consistent with our previous finding that primordial follicles in pieces of bovine ovarian cortex grafted beneath the CAM did not active (Cushman et al., 2002), in contrast to the wholesale activation that occurs when cortical pieces are cultured in vitro (Wandji et al., 1996). Thus, the inhibitory effect exerted in ovo was observed in both the absence (cattle; Cushman et al., 2002) and presence (mice; current study) of the medullary compartment of the ovary, suggesting that absence of the medullary compartment is not a factor in the inhibition of activation in CAM-grafted bovine cortical pieces. In ovo, mouse ovaries became vascularized and >90% of follicles were maintained in a healthy state, similar to results in vivo and in vitro. That the CAM graft situation does not exert deleterious effects on mouse primordial follicles is also evidenced by the fact that activation occurred when mouse ovaries were retrieved from the CAM after 2 days in ovo and cultured in vitro in a crossover experiment. This is similar to previous results when bovine ovarian pieces were "crossed over" from in ovo to in vitro culture (Cushman et al., 2002).

Interestingly, more follicles in intact mouse ovaries activated in vitro than in vivo. Although the wholesale activation observed when bovine or baboon cortical pieces are cultured (Wandji et al., 1996, 1997) was not observed in mouse ovaries in vitro, a fairly large percentage of follicles was growing after 8 days in vitro or in vivo, about 50% and 25%, respectively. This suggests that a larger proportion of resting follicles activates in the first "wave" of follicular development. If this rate of activation were sustained, the follicle pool would quickly become depleted. The source of ovarian AMH appears to be growing follicles; AMH or its mRNA has been detected in granulosa cells of activated follicles in rats (Hirobe et al., 1994; Baarends et al., 1995), sheep (Bezard et al., 1988), and cattle (Takahashi et al., 1986). Hence, the results of our experiments with newborn mouse ovaries support the idea that the inhibition of follicle activation by AMH is not initiated until a pool of growing follicles is established. The activation of a greater percentage of follicles in mouse ovaries in vitro versus in vivo suggests that ovaries in vitro are exposed to lower concentrations of AMH, perhaps due to its diffusion into the culture medium.

The results with CAM grafts of bovine cortical pieces and whole mouse ovaries, and the reversibility of the inhibitory effect of the in ovo situation, suggest that the chick circulation contains an inhibitor of activation. Because circulating levels of AMH are high in both male and female chick embryos, we hypothesized that AMH is the inhibitory factor. That hypothesis was tested by grafting the ovaries of mice homozygous for a null mutation of the AMH receptor II (*Amhr2-Cre*; Jamin et al., 2002). Like other TGFβ family members, AMH signaling requires that type I and type II receptors form heteromeric complexes. Mutant male mice lacking the AMH type II receptor are a phenocopy of AMH ligand-deficient males, showing that the AMH type II receptor is essential for AMH action (Mishina et al., 1996). Likewise, *Amhr2* mutant females are fertile, as are the female null mutants for AMH protein (Mishina et al., 1996). After 4 days in ovo, activation had occurred in the grafted ovaries of the *Amhr2* mutant mice, similar to activation in vitro in the other ovary of the *Amhr2* mutant mice, whereas no activation occurred in ovo in ovaries of their wild type litter mates. These findings strongly suggest that AMH is the factor in the chick circulation that inhibits the activation of primordial follicles in whole mouse ovaries.

AMH is produced by developing chick gonads and both ovaries and testes have high levels of AMH for much of embryonic life (Hutson et al., 1981; Teng, 1987). Thus, to determine if AMH is also the factor that inhibits follicle activation in bovine cortical pieces grafted in ovo, the effects of gonadectomizing chicks on activation of follicles in grafted pieces of bovine ovarian cortex were determined. In contrast to the almost complete absence of activation observed in intact or sham-cauterized chicks, activation occurred in bovine cortical pieces grafted beneath the CAM of gonadectomized chick embryos, similar to the proportion of follicles that activated in control cortical pieces cultured in vitro. Although, it is possible that chick gonads secrete another factor that inhibits follicle activation in cattle, but not mice, this seems less likely than that AMH is the inhibitor in ovo of both mouse and bovine follicle activation. However, thus far we have been unable to inhibit activation in vitro in bovine ovarian cortical pieces cultured with graded doses of human recombinant AMH (or in preliminary experiments with rat recombinant AMH), although AMH did attenuate the growth of primary follicles, as discussed below (Cushman and Fortune, 2003). However, the current finding that gonadectomizing chick embryos overrides the inhibitory effect of CAM-grafting on follicle activation, taken together with the results obtained with ovaries of *Amhr2* null mutant mice, suggests that AMH negatively regulates follicle activation in cattle. In vitro activation occurs within the first 24–36 hr of culture (Fortune et al., 2000), so failure to observe inhibition by AMH in vitro may be due to failure of this large molecule (about 150 kDa) to diffuse into the bovine ovarian tissue, which is much denser than in rodents, in time to inhibit activation and/or to low potency of the human (and rat) recombinant AMH with bovine tissue. The ability of AMH to attenuate the growth of bovine follicles after activation occurs in vitro (Cushman and Fortune, 2003) argues for the former explanation. In ovo, vascularization occurs rapidly and this may provide a faster delivery of AMH to the ovarian cortical piece. More studies are needed to resolve the apparent contradiction between the results in ovo and in vitro.

In addition to its role as an inhibitor of follicle activation in mice, and perhaps cattle, AMH may also modulate the growth of activated follicles. AMH attenuated the growth in vitro of medium-sized and large preantral follicles in ovaries cultured with FSH (Durlinger et al., 2001) and of bovine follicles activated in vitro (Cushman and Fortune, 2003). Although these results suggest that AMH, in addition to inhibiting follicle activation, also acts to slow the growth of growing follicles, McGee et al. (2001) reported that AMH enhanced FSH-stimulated growth in vitro of medium-sized rat preantral follicles. These apparent species differences remain to be resolved.

The activation of primordial follicles is the first critical transition in follicular development. Although the temporal regulation of this transition in individual follicles is still a complete mystery, there is now evidence that specific factors can stimulate or inhibit this transition in rodent ovaries. Skinner's laboratory, using an experimental model in which neonatal rat ovaries (containing newly formed primordial follicles) are cultured for 4 days with potential regulators of follicle activation, has provided evidence that kit ligand (Parrott and Skinner, 2000), basic fibroblast growth factor (Nilsson et al., 2001), leukemia inhibitory factor (Nilsson et al., 2002), insulin (Kezele et al., 2002), and bone morphogenetic protein-4 (Nilsson and Skinner, 2003) can increase the percentage of growing follicles. Conversely, the wholesale activation that occurs when cortical pieces from fetal or adult bovine ovaries and fetal baboon ovaries are cultured, and the higher proportion of follicles that activated in vitro versus in vivo in neonatal mouse ovaries in the current study, highlight the importance of negative regulators of follicle activation. In vivo, whether a particular follicle activates or remains in the resting pool at a given time could be due to the balance of positive and negative regulators in its immediate environment. To date, the only negative regulator that has been identified is AMH, but whether it plays this role in non rodent species was unknown. The results presented herein provide suggestive, albeit indirect, evidence that AMH also inhibits the initiation of follicle growth in cattle. More direct evidence for this tentative conclusion awaits the development of new experimental approaches and/or the availability of more potent or specific (i.e., bovine) preparations of AMH.

ACKNOWLEDGMENTS

The authors thank Taylor Packing Co. (Wyalusing, PA) for the donation of fetal bovine ovaries, Dr. Richard Behringer (M.D. Anderson Cancer Center, Houston, TX) for providing *Amhr2* mutant mice, and Dr. M. Matzuk for suggesting the use of the mutant mice. Dr. S. Jamin (in Dr. Behringer's laboratory), Dr. T. Gunn (Cornell University), and Dr. M. Jo (University of Kentucky) provided advice on genotyping. Ms. D. Alo and her coworkers provided expert animal care and bred the mutant mice.

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