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Regulation of Ovarian Primordial Follicle Assembly and Development by Estrogen and Progesterone: Endocrine Model of Follicle Assembly

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The assembly of the developmentally arrested primordial follicle and the subsequent transition of the primordial follicle to the primary follicle are critical processes in normal ovarian physiology that remain to be elucidated. Ovarian follicles do not proliferate and the primordial follicles present in the neonate represent the total number of gametes available to a female throughout her reproductive life. The primordial follicles are oocytes surrounded by less differentiated squamous granulosa cells and are derived from oocyte nests, and primary follicles are oocytes surrounded by a single layer of cuboidal granulosa cells that have initiated follicle development. Abnormalities in primordial follicle assembly, arrest, and development (*i.e.* primordial to primary follicle transition) can cause pathological conditions such as premature ovarian failure. In this study newborn rat ovaries were cultured for 7 d. The rate of primordial follicle assembly *in vivo* was identical with the rate *in vitro*. Interestingly, the rate of primordial follicle transition to the primary follicle was found to be 3 times greater in culture. This abnormal rate of primary follicle development in culture suggests the primordial follicle does not arrest in development as observed *in vivo*. To investigate this phenomena newborn rat ovaries were cultured in the presence of progesterone, estradiol or calf serum. Estradiol, progesterone, or calf serum significantly reduced the level of initial primordial to primary follicle transition. Approximately 60% of follicles make the primordial to primary follicle transition in control ovaries and about 30% in treated ovaries. Steroids and calf serum had no effect on the primordial to primary follicle transition in ovaries collected and cultured from postnatal 4-d-old rats, suggesting the effects observed are restricted to the initial wave of primordial to primary follicle transition. Interestingly, progesterone was also found to significantly reduce the rate of primordial follicle assembly. All viable oocytes assembled into primordial follicles in control ovaries and approximately 40% remained unassembled in progesterone-treated ovaries. Progesterone was also found to reduce primordial follicle assembly *in vivo* with 10% of the total follicles remaining unassembled in progesterone injected neonatal animals. Analysis of cellular apoptosis demonstrated that progesterone inhibited the coordinated oocyte apoptosis required for primordial follicle assembly. The hypothesis developed is that high levels of maternal and fetal steroids prevent premature primordial follicle assembly and primordial to primary follicle transition in the embryo. After birth steroid levels fall dramatically and the primordial follicles are free to assemble and initiate development. These observations suggest a novel role for steroids and the maternal-fetal endocrine unit in the control of ovarian primordial follicle assembly and early follicular development. (*Endocrinology* 144: 3329–3337, 2003)

FOLLICULAR ASSEMBLY IS the developmental process by which individual oocytes assemble into primordial follicles. In the neonatal rodent, the process of follicular assembly is precisely coordinated. The embryonic rodent ovary contains no follicles. All the oocytes are arranged in large clusters called nests. Immediately after birth selected oocytes undergo a wave of apoptosis, and surviving isolated oocytes become surrounded by squamous pregranulosa cells forming the primordial follicle (1). This wave of atresia (*i.e.* apoptosis) is speculated to be the mechanism by which primordial follicles are formed (2). The entire process is completed within 4 d of postnatal rodent development (3). Follicular assembly is a similar but more heterogeneous process in large monoovulatory mammals such as the human and cow. In the human and macaque *Macaca mulatta,* follicular assembly begins about halfway through pregnancy (*i.e.* about month 5 in humans) (4). These animals are born with a small population of secondary follicles and nests of unassembled oocytes (5).

Primordial follicles remain arrested and static (*i.e.* for up to 4–5 decades in the human) until they undergo the primordial to primary follicle transition. When this occurs, the squamous granulosa develop a cuboidal morphology. The granulosa cells begin to proliferate, and the follicle sequesters theca from the surrounding stroma (1). The primordial to primary follicle transition is a nonreversible process. The follicle continues growing until its inevitable destruction by atresia or ovulation. Because primordial follicles do not proliferate or grow, the follicle population represents a female's total reproductive potential. Therefore, the rate of follicular assembly and the primordial to primary follicle transition is of critical importance to female reproduction. Abnormal control of primordial follicle assembly or development can lead to abnormal conditions such as premature ovarian failure or premature onset of menopause.

These early stages of follicle development are completely gonadotropin independent in all mammalian species studied. Rodent primordial follicles are fully competent to undergo follicular assembly and the primordial to primary follicle transition in serum-free culture (6, 7). Bovine follicles are also competent to undergo primordial to primary follicle

Abbreviations: MIS, Müllerian inhibitory substance; PR, progesterone receptor; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling.

transition in serum-free culture (8). FSH receptor-null mutant and LH receptor-null mutant mice appear to have normal initiation of follicle development until the preantral stage (9, 10).

Preliminary studies demonstrated the possible existence of a global factor that can retard early folliculogenesis. *In vivo* primordial follicle assembly is finished in about 4 d, and the initial primordial to primary follicle transition takes place at a moderate rate of about 20% of the total follicles. *In vitro* follicular assembly is also finished in about 4 d; however, the primordial-to-primary follicle transition takes place at a greatly accelerated degree of about 70% of the follicles. Observations suggest the primordial follicles that assemble *in vitro* do not undergo developmental arrest and spontaneously initiate transition to the primary stage. This is a much higher rate of spontaneous primordial-to-primary follicle transition than occurs in cultured postnatal d 4 ovaries (1). Therefore, there appears to be a factor specifically in the neonatal rat that retards primordial follicle assembly and development.

During gestation, levels of estrogen and progesterone in the embryonic and neonatal rat are extraordinarily high. After birth the levels of steroids drop precipitously in a manner similar to the kinetics of primordial follicle assembly. For example, estrogen concentrations in the neonatal rat drop from 5×10^{-7} M 4 h after birth to 2×10^{-8} M 48 h after birth (11). Progesterone concentrations drop from 2×10^{-6} M the day before birth to 6×10^{-7} M the day after birth (12). In larger mammals and monoovulators, the high fetal steroid concentrations drop at approximately the same time as the majority of follicular assembly begins. For example, in the macaque *M. mulatta* fetal progesterone serum concentration drops from 4×10^{-7} M at midterm to an undetectable level at term (13). These changes in steroid concentrations are candidates for factors that may coordinate early follicle development in the neonate. The hypothesis tested in the current study is that high concentrations of maternal and fetal estradiol and progesterone retard early follicle development and that a rapid decline of steroid levels (*i.e.* after birth in the rat) allows primordial follicle assembly and development to be initiated.

Materials and Methods

Histology and organ cultures

Postnatal 0-d-old rat ovaries were cultured for 7 d. Whole ovaries were cultured as previously described (14) on floating filters (0.4 μm Millicell-CM, Millipore Corp., Bedford, MA) in 0.5 ml DMEM-Ham's F-12 medium (1:1, vol/vol) containing 1 μg/ml insulin (bovine, Sigma, St. Louis, MO), 0.1% BSA (Sigma), 0.1% albumax (Life Technologies, Inc., Gaithersburg, MD), 2.75 μg/ml transferrin, and 0.05 mg/ml L-ascorbic acid (Sigma) in a 4-well culture plate (Nunc plate, Applied Scientific, South San Francisco, CA). Ovaries were randomly assigned to treatment groups with one to three ovaries per floating filter. Treatments during organ culture were: calf serum (10% vol/vol) (Hyclone Laboratories, Inc., Logan, UT), estradiol (10^{-6} M) (bovine, Sigma), progesterone (10^{-6} M) (bovine, Sigma), mifepristone (10^{-6} M) (Sigma) (*i.e.* RU-486 progesterone receptor steroid antagonist), and ICI 182-780 (10^{-6} M) (Tocris, Ellisville, MO) (*e.g.* estrogen receptor steroid antagonist). Medium was supplemented with penicillin, streptomycin, and gentamicin to prevent bacterial contamination.

Time-course studies involved the culture of seven sets of ovaries for up to 7 d and removing and fixing one set every day. Ovaries were removed from neonatal rats, one pair every day, after birth for 7 d. After culture ovaries were fixed in Bouin's fixative (Sigma) for 2 h. Ovaries were then embedded in paraffin, sectioned at 4 μm, and stained with hemotoxin and eosin. The number of follicles at each developmental stage was counted in two serial sections from the largest cross-section through the center of the ovary and averaged. Previously, the data obtained from this analysis of two middiameter cross-sections have been shown to provide similar data as analysis of compiled data from all serial sections (14). Normally two ovaries were in each treatment group as a replicate. Experiments were repeated three times (therefore, n = 6 for each treatment group). Normally 150–200 follicles were present in an ovary cross-section.

Neonatal steroid injection

Neonatal rats were injected daily after birth to produce a serum steroid concentration of 2×10^{-6} M. Stock solutions of 6×10^{-4} M of the above steroids were made in sesame oil (Sigma). A 20-μl volume of this stock solution was injected sc into approximately 6 g neonatal rat pups daily for 4 d starting the day of birth. After treatment, rats were euthanized and ovaries were dissected, fixed, stained, and analyzed as described above.

Apoptosis assay

Degree of oocyte atresia (*i.e.* apoptosis) was measured with the *in situ* cell death detection kit (Roche Applied Science, Indianapolis, IN). Briefly, neonatal ovaries were cultured for 2 d, at which time the greatest rate of oocyte apoptosis and follicular assembly is observed. Ovaries were cultured in the absence or presence of steroids. Then culture ovaries were fixed, embedded in paraffin, and sectioned. Paraffin embedded slides were then treated per instructions in the detection kit. Use of terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL) *in situ* caused polymerization of fluorescein-labeled nucleotides to the free 3′ ends of DNA that had been cleaved during apoptosis. Therefore, fluorescein-stained cellular nuclei had multiple breaks in their DNA and were undergoing apoptosis at the time of fixation.

RT-PCR

Multiple whole ovaries were dissected from postnatal day 0 (P0) and 4 (P4) ovaries. Adult uterus was removed for a positive control and skeletal muscle for negative control. RNA was extracted using the TRIZOL protocol per manufacturer instructions (Life Technologies, Inc.). Reverse transcription used specific 3′ primers for the progesterone receptor and cyclophilin (1B15). PCR used the following primers made for the ligand-binding domain of the progesterone receptors (PRs) 3′ (5′-GCAACTGGGCAGCAATAACT-3′) and PR 5′ (5′-ACTCCTGGATGAGCCTGATG-3′), which amplified a 500-bp product. Positive control PCR for the cyclophilin (1B15) was done using primers 1B15 3′ (5′-ATTTGCCATGGACAAGATGCCAGGACCTGTATG-3′) and 1B15 5′ (5′-ACACGCCATAATGGCACTGGTGGCAAGTCCATC-3′), which amplified a 105-bp product. The PCR products were electrophoretically analyzed and sequenced to confirm identity.

Statistics

Treatment groups were compared using ANOVA with Dunnett's test to compare treated groups to a selected control (15). Groups were considered significantly different if P was 0.05 or less. All statistics were calculated with the help of JMP version 3.1 software (SAS Institute, Inc., Carey, NC).

Results

Primordial follicle assembly and development kinetics

The initial experiment was to characterize the kinetics of neonatal rat follicle development *in vivo* and *in vitro*. At birth, the vast majority of oocytes were not associated with follicles (Figs. 1 and 2A). *In vivo* the number of primordial follicles in the ovary rose to about 75% within 6 d (Fig. 2A). There was

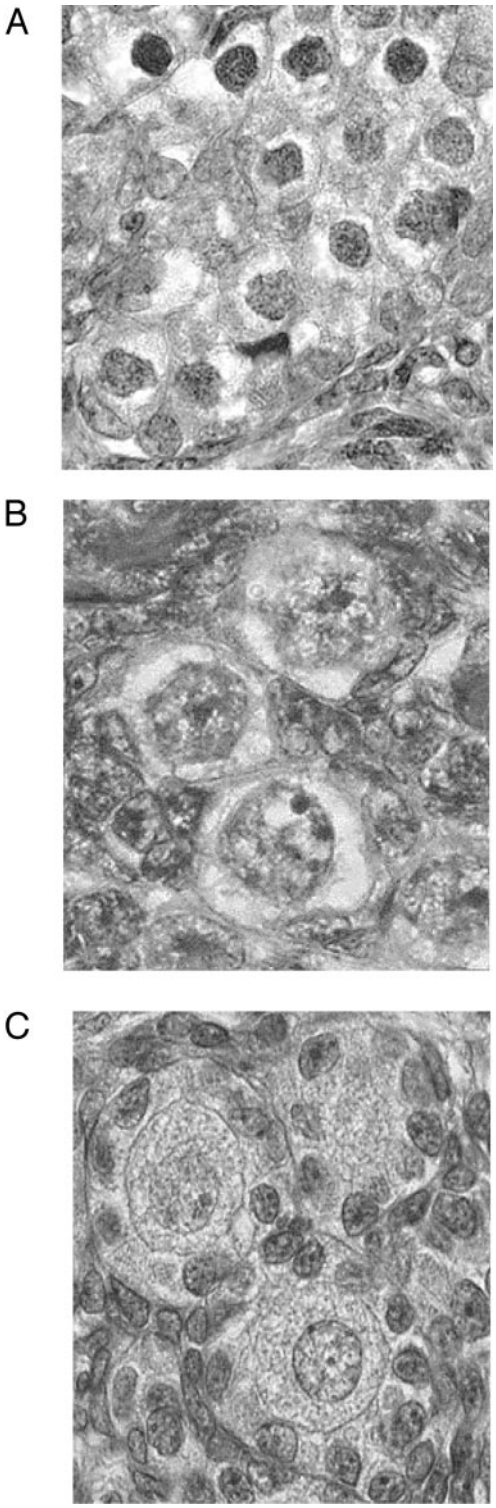

FIG. 1. Follicle morphology: oil immersion micrograph (×400) of follicles at different stages of early development. A, Nest of unassembled follicles in neonatal ovary. B, Primordial follicles in postnatal d 4 ovary with squamous pregranulosa cells. C, Primary follicles in postnatal d 4 ovary with cuboidal granulosa cells.

a gradual increase in primordial to primary follicle transition with the percentage of primary follicles being 25% of all the follicles within 3 d of postnatal development (Fig. 2A). *In vitro* the kinetics of follicular assembly was similar with most of the unassembled follicles disappearing within 4 d after birth. However, the primordial to primary follicle transition (Fig. 1) was greatly accelerated *in vitro* (Fig. 2). After 6 d of culture, 75% of the follicles had moved to the primary stage with only 25% remaining primordial follicles (Fig. 2B). Observations suggest the primordial follicles that assemble *in vitro* did not undergo the normal arrest in development and spontaneously initiate transition to primary follicles. The mean total follicle numbers found *in vitro* and *in vivo* were comparable and approximately 170 per section at 6 d of postnatal development.

Regulation of primordial follicle development

The next experiment was to evaluate estradiol and progesterone's ability to influence early follicle development *in vitro*. Ovaries were dissected from neonatal new born rat pups and cultured for 7 d in the presence of calf serum (10%), estradiol (10^{-6} M), progesterone (10^{-6} M), or both steroids. Ovaries treated with calf serum displayed no change in the proportion of unassembled follicles (Fig. 3A). Estrogen-treated ovaries displayed a moderate but insignificant increase in the proportion of unassembled follicles. Both the progesterone and the progesterone/estradiol-treated ovaries showed significant increases in the number of unassembled follicles, approximately 20% of all oocytes (Fig. 3A). Even though they did not significantly effect follicular assembly, both calf serum and estrogen increased the percentage of primordial follicles to around 60%, compared with approximately 30–40% in control untreated ovaries (Fig. 3B). All the treatments showed a decrease in the percentage of primary follicles (Fig. 3C). The mean number of total follicles did not change with the treatments and was 169 per ovary section. In summary, progesterone inhibited primordial follicle assembly. Estradiol and progesterone had a significant effect on the primordial to primary follicle transition. The calf serum only effected the primordial to primary follicle transition. Assay of 10% calf serum with a specific RIA for progesterone demonstrated no detectable steroid in the calf serum (data not shown), and the manufacturer reported similar results with an estrogen RIA of nondetectable estrogen (Hyclone Laboratories, Inc.). Therefore, the actions of calf serum are steroid independent.

Steroid actions on primordial follicles

The effective concentrations of estradiol and progesterone were determined to assess whether optimal steroid concentrations were used. Newborn ovaries were cultured for 7 d in the presence of varying concentrations of estradiol from 10^{-8} to 10^{-6} M. Estradiol had a maximum effect at 10^{-7} M (Fig. 4A). Newborn ovaries were also cultured for 7 d in the presence of varying concentrations of progesterone from 10^{-8} to 10^{-6} M. The half-maximal effective concentration of progesterone was found to be approximately 10^{-7} M (Fig. 4B).

Newborn ovaries were cultured with progesterone and the PR steroid antagonist mifepristone (*i.e.* RU-486) to determine whether the progesterone nuclear receptor is responsible for progesterone's effects on the organ culture. Mifepristone re-

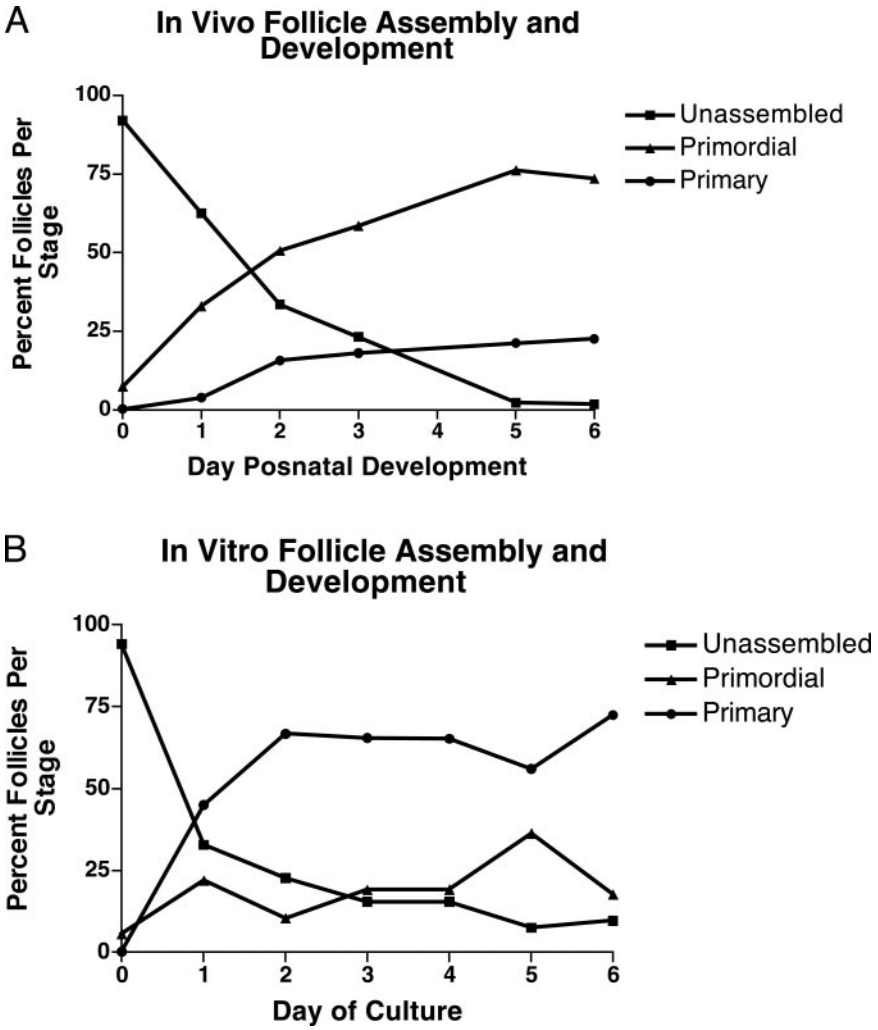

FIG. 2. Primordial follicle assembly and development dynamics: kinetics of early-stage folliculogenesis during a 6-d postnatal development period (A) *in vivo* and (B) during organ culture. Data displayed as percentage of unassembled, primordial, and primary follicles in each stage per day postnatal development or organ culture. The data are representative of four different experiments.

duced the number of unassembled follicles in the progesterone-treated ovaries (Fig. 5B). There was still a higher number of unassembled follicles in the mifepristone/progesterone-treated ovaries than control untreated ovaries. The mifepristone alone was also able to increase the number of unassembled follicles to about 10% of all follicles (Fig. 5B). Although the progesterone nuclear receptor inhibitor was found to partially inhibit progesterone actions, other actions of progesterone appear to also be involved. To confirm the presence of the progesterone nuclear receptor in newborn rat ovaries a PCR procedure was used with specific primers. The progesterone receptor mRNA was detected in newborn rat ovaries (Fig. 6). The identity of this PCR product was confirmed with DNA sequencing (data not shown).

Newborn ovaries were cultured with estradiol and the estrogen receptor antagonist ICI 182-780 to determine whether the estrogen nuclear receptor is responsible for estradiol's effects on the organ culture. The receptor antagonist alone had no effect on the organ culture but was able to completely inhibit estradiol's action on the organ culture (Fig. 5A). The nuclear estrogen receptor appears to be responsible for the effect of estradiol on the primordial to primary follicle transition.

In vivo regulation of primordial follicle assembly

To confirm the organ culture data, corresponding *in vivo* experiments were performed. Newborn rats were injected with estradiol and progesterone daily for 4 d starting immediately after birth. An attempt was made to replicate the culture experiments steroid concentrations *in vivo*. To confirm the actions of steroids, the morphology of the reproductive tract (*e.g.* uterus) was examined and found to be enlarged in steroid-treated animals (Fig. 7). Only progesterone was able to maintain approximately 10% of the oocytes as unassembled follicles (Fig. 8). Estradiol treatments produced results similar to control vehicle-treated animals (Fig. 8). Therefore, the *in vivo* observations supported the organ culture data on the role of progesterone to inhibit primordial follicle assembly. The total follicle numbers per ovary section did not change with treatment and were approximately 120.

Primordial follicle assembly and apoptosis

The apoptosis assay was performed on fixed sections of neonatal ovaries that had been cultured for 2 d. Two days was the midpoint of follicular assembly and optimum oocyte apoptosis. The purpose of the experiment was to determine

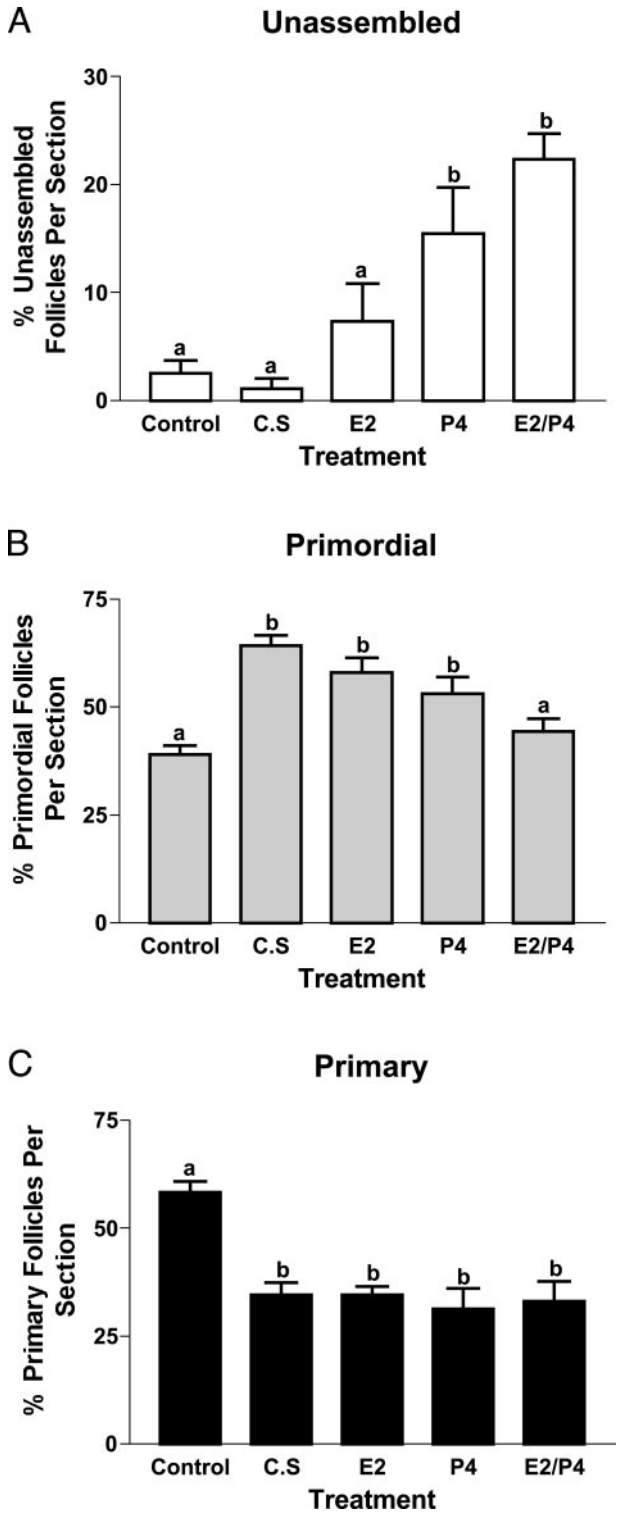

FIG. 3. Regulation of primordial follicle assembly and development: neonatal ovaries cultured for 1 wk in the absence (control) or presence of progesterone (P4) (1 μM), estradiol (E2) (1 μM), combination of estradiol and progesterone (E2/P4), and 10% calf serum to measure its effect on early folliculogenesis. Data are presented as percentage of unassembled (A), primordial (B), and primary (C) follicles at each stage per treatment group. The mean ± SEM is presented from four different experiments with a minimum of three ovaries per experiment. *Different letter subscripts* represent a significant difference from control by Dunnett's test ($P < 0.001$).

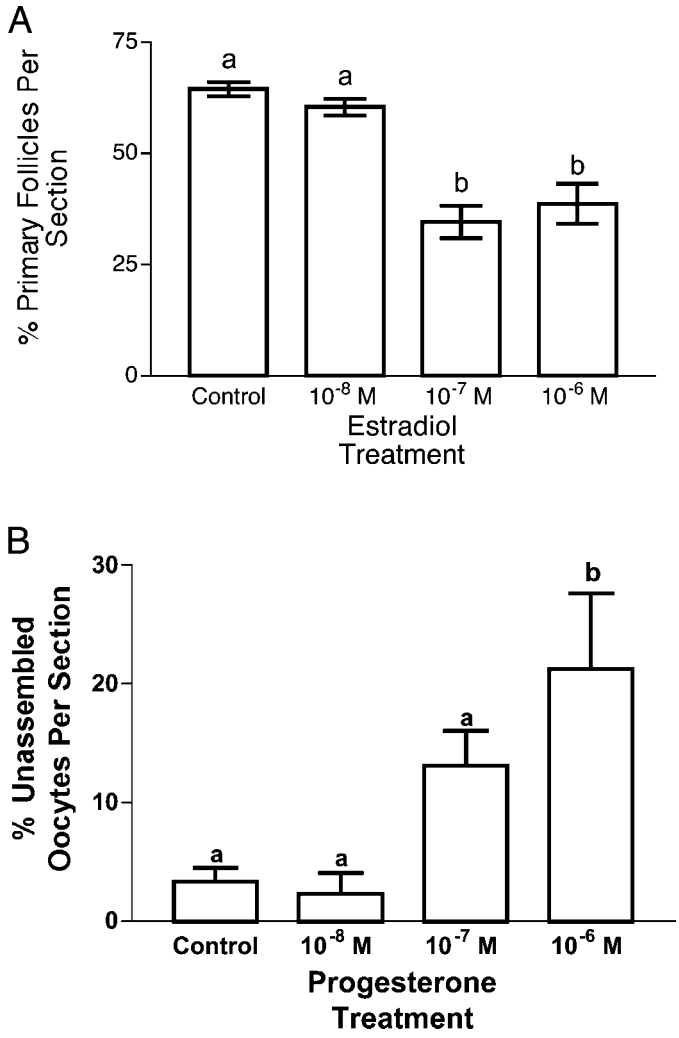

FIG. 4. Steroid dose treatments. A, Effect of different concentrations of estradiol on the primordial to primary follicle transition using ovary organ cultures. Data presented as percentage of primary follicles in the ovary section after treatment with different estradiol concentrations. Estradiol's half-maximal effective concentration was between 10^{-7} and 10^{-8} M. B, Effect of different concentrations of progesterone on primordial follicle assembly using ovary organ culture. Data are presented as percentage of unassembled follicles in the ovary sections after treatment with different progesterone concentrations. Progesterone's half-maximal effective concentration was between 10^{-6} and 10^{-7} M. The mean ± SEM is presented from three different experiments with a minimum of three ovaries per experiment. Control indicates organs incubated with no treatment. *Different letter subscripts* represent a significant difference from control by Dunnett's test ($P < 0.001$).

whether the steroid treatments that retarded primordial follicle assembly also inhibited oocyte apoptosis. Oocyte apoptosis has been postulated to be the mechanism that causes primordial follicle assembly (2). An untreated control ovary displayed what appear to be multiple oocytes in nests undergoing apoptosis (Fig. 9). A progesterone-treated ovary displayed very few oocytes undergoing apoptosis (Figs. 9 and 10). The total mean follicle numbers per section for ovaries incubated in the absence (control) or presence of progesterone were 102 and 113, respectively. The unassembled oocytes were the only population effected by pro-

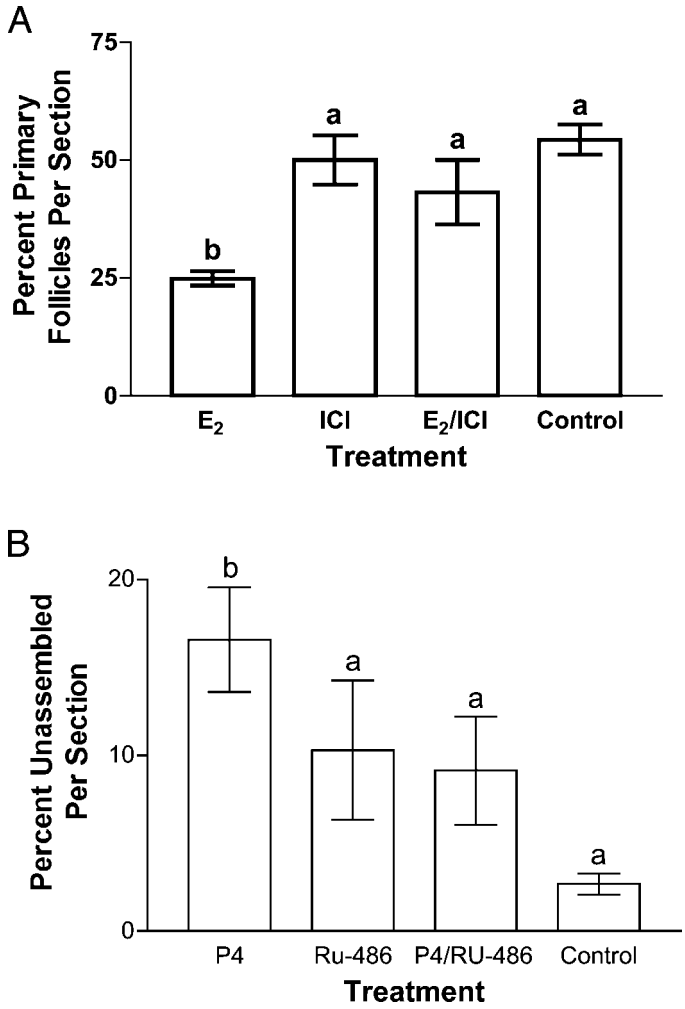

FIG. 5. Steroid actions on primordial follicle. A, To determine whether estradiol actions are due to the nuclear estrogen receptor, neonatal ovaries were cultured with estradiol (E2) (1 μM) and the estrogen receptor antagonist ICI 182-780 (ICI). Data are displayed as the percentage of primary follicles in the ovary sections per different treatment groups. B, To determine whether progesterone action was due to the nuclear progesterone receptor, neonatal ovaries were cultured with progesterone (P4) (1 μM) and progesterone receptor antagonist RU-486 (RU-486) (1 μM). Data are displayed as the percentage of unassembled follicles in the ovary sections per different treatment groups. Mean ± SEM are presented for four different experiments with a minimum of three per section. Control indicated organs incubated in the absence of any treatment. *Different letter subscripts* represent a significant difference from control using Dunnett's test ($P < 0.005$).

gesterone treatment. Therefore, the progesterone appears to inhibit primordial follicle assembly by reducing the rate of oocyte apoptosis (Figs. 9 and 10).

Steroid actions on postnatal ovaries

The final experiment was performed to determine whether the estradiol and progesterone actions observed with the newborn ovary could affect primordial follicles later in development. At the postnatal d 4 period, the ovaries contain negligible unassembled follicles with the primordial and primary stages being predominant. Four-day-old postnatal rat ovaries were cultured with estradiol and progesterone for 2 wk. Neither estradiol nor progesterone was able to effect the primordial to primary follicle transition (Fig. 11). Observations suggest the actions of steroids are confined to the stage of primordial follicle assembly and only the initial wave of primordial to primary follicle transition.

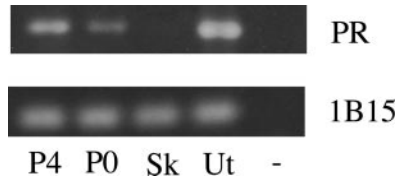

FIG. 6. Progesterone nuclear receptor expression: RT-PCR to find a 500-bp PR transcript in the neonatal rat ovary. RT-PCR for cyclophilin 1B15 used as a control constitutively expressed gene. Adult rat uterus (Ut) was used as positive control and skeletal muscle (Sk) was used as a negative control and (−) indicates a negative control without RT. Test groups were postnatal d 0 rat ovary (P0), and postnatal d 4 rat ovary (P4).

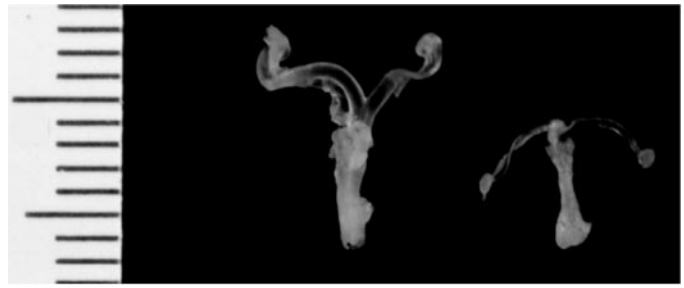

FIG. 7. *In vivo* estradiol treatment: effect of postnatal d 0 *in vivo* daily estradiol treatments on female reproductive tract morphology. *Left*, Reproductive tract of estradiol-treated animal. *Right*, Reproductive tract of untreated control animal. Scale is in millimeters.

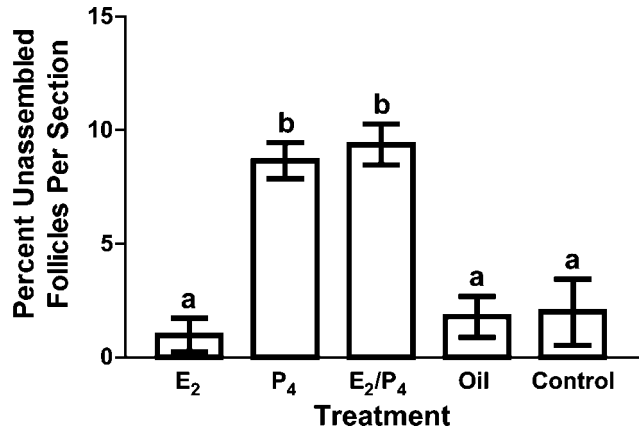

FIG. 8. Steroid actions on primordial follicle assembly *in vivo:* effect of neonatal steroid treatment on primordial follicle assembly *in vivo*. Neonatal rats were injected with estradiol (E2), progesterone (P4), combined estradiol and progesterone (E2/P4), or oil vehicle (oil) for 4 d after birth. Control indicates no injection untreated animals. Data are displayed as percentage of unassembled follicles in ovary sections for each treatment group. Data are presented as the mean ± SEM from six experiments with a minimum of two ovaries per experiment. *Different letter subscripts* represent a significant difference from control by Dunnett's test ($P < 0.001$).

Discussion

The current study demonstrates that progesterone was able to significantly inhibit primordial follicle assembly both

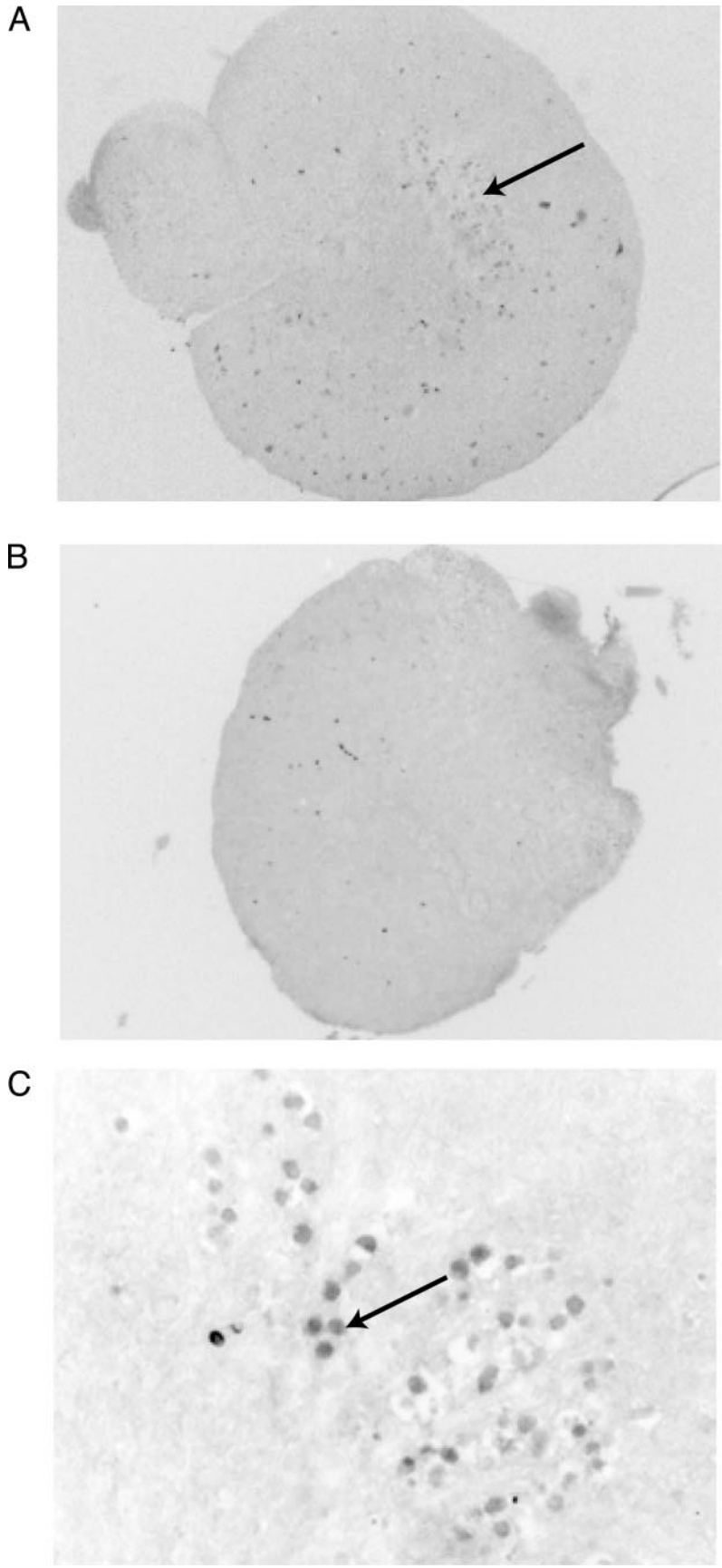

FIG. 9. Progesterone actions on oocyte apoptosis: effect of progesterone on oocyte apoptosis on d 2 of organ culture of neonatal ovaries followed by TUNEL assay of ovary sections. A and C, Control untreated ovary culture sections. *Arrow* points to a nest of apoptotic oocytes. B, Progesterone-treated ovary culture section. Data are representative of a minimum of six ovaries analyzed per treatment.

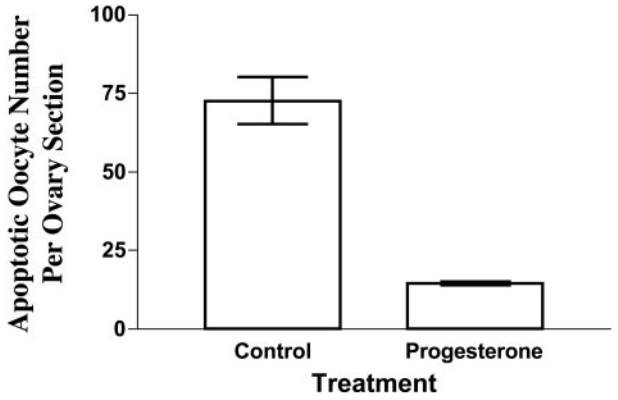

FIG. 10. Oocyte apoptosis: quantitative expression of progesterone's effect on oocyte apoptosis by counting TUNEL-positive cells on sections from control untreated and 1 μM progesterone-treated ovary organ cultures. Data are presented as the mean ± SEM from two experiments with two ovaries each.

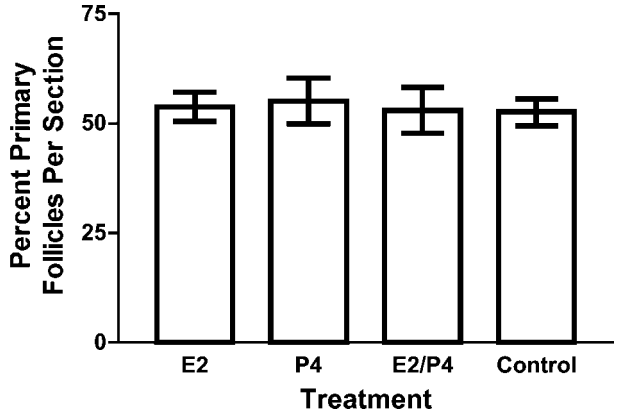

FIG. 11. Steroid actions on postnatal d 4 ovary cultures: effect of 1 μM estradiol (E2) and progesterone (P4) on primordial to primary follicle transition after 2 wk of organ culture of postnatal 4-d-old ovaries. Control indicates untreated organ cultures and E2/P4 combined treatment of estradiol and progesterone. Data are displayed as percentage of primary follicles in ovary sections per treatment group. Data are presented as the mean ± SEM of three experiments with a minimum of three ovaries each.

in vivo and *in vitro*. Progesterone and estradiol both inhibited the primordial to primary follicle transition. The premature high level of primary follicle development observed *in vitro* in comparison to early follicle development *in vivo* was due in part to the absence of estrogen and progesterone. The primordial follicles that assemble *in vitro* do not arrest in development, but instead spontaneously transition to primary follicles. Observations support the hypothesis that a decline in estrogen and progesterone levels in the neonatal ovary allows primordial follicles to assemble and primordial follicle development to be arrested.

The potential presence of the progesterone receptor at this time in ovarian development is ambiguous. It is known that neonatal ovaries contain some substance that can bind progesterone (16). The progesterone receptor antagonist RU-486 was unable to completely inhibit progesterone actions, and the antagonist was found to have some agonist activity. It is possible that progesterone actions are due in part to both the nuclear receptor and a nonnuclear receptor, as has been suggested in other systems (17, 18). The PR-null mutant mice have seemingly normal follicle development until ovulation (19). Although the earliest stages of follicle development have not been studied, the null mutant mouse data suggest that the nuclear PR might not be the only active receptor at this time of ovarian development. The current observations suggest a nonclassical nuclear PR may in part mediate the actions of progesterone on primordial follicle assembly and development. Further analysis is required to fully elucidate the mechanisms of progesterone actions on primordial follicle assembly and development.

Oocyte apoptosis is temporally linked to the process of follicular assembly and is postulated to be the mechanism that drives primordial follicle assembly (2). Observations demonstrate that progesterone treatment of cultured ovaries significantly inhibits the rate of oocyte apoptosis. This suggests a model for how progesterone might retard primordial follicle assembly. Progesterone might be acting as a survival factor that maintains oocyte viability and prevents lone oocytes to develop from nests and become surrounded by pregranulosa to form primordial follicles.

Estrogen receptors, particularly estrogen receptor-β, are known to exist in the pregranulosa of primordial follicles (20). In the neonatal rat, estrogen receptor-α has been detected as early as d 1 (21). Studies with mice with null mutations of the estrogen receptors have not rigorously examined early follicle development. Mutations in the estrogen receptor-α display an ovarian cystic morphology. This is generally attributed to an elevated gonadotropin level in these animals (22), but abnormal early follicle development might also be a factor. Mutations of estrogen receptor-β show no change in folliculogenesis until ovulation. Double mutations of estrogen receptor-α and -β display a partial sex reversed gonad (23). In either case, the phenotype of early follicle development in these animals has not been well documented. The nuclear estrogen receptor antagonist was found to completely attenuate estradiol actions in our organ culture system. Although the media used contained phenol red, the level of estrogenic activity associated with phenol red is orders of magnitude below that was found to be optimal (0.1 μM) in the current culture system (24). Therefore, estrogen effects on the initial wave of primordial to primary follicle transition appear to function through the nuclear estrogen receptor.

The current study suggests estradiol and progesterone actions on the oocyte or pregranulosa cells associated with the primordial follicle inhibit the initial wave of primordial to primary follicle transition. At this early stage of development, negligible secondary follicles are present (25). In the adult ovary, the presence of secondary or antral follicles provides a source for Müllerian inhibitory substance (MIS). MIS can suppress the primordial to primary follicle transition (26, 27). Estrogen actions in more advanced stages of development could be indirectly mediated through substances such as MIS. However, the early stages used in the current study suggest more direct actions of estradiol on the primordial follicle.

Current observations demonstrate a novel function for progesterone and estradiol in the regulation of primordial follicle assembly and development in the neonate. Proges-

terone and, to a lesser degree, estradiol seem competent to retard primordial follicle assembly in the neonatal ovary *in vitro*. Progesterone also is able to retard follicular assembly in the neonate *in vivo*. The model developed is that high levels of steroids in the ovary act to arrest primordial follicle assembly and development, and the decline in steroid levels late in fetal development or after birth allows the initiation of primordial follicle assembly and development. In the nonhuman primate, the decline in fetal steroid levels (13) occurred at the same time primordial follicle assembly and development is initiated. Therefore, the observations made in the current study with a rodent model are speculated to apply to a wide variety of mammalian species including humans. This endocrine model of the regulation of primordial follicle assembly and development has likely evolved to prevent the premature and inappropriate development of primordial follicles. Abnormal primordial follicle assembly can affect the reproductive capacity of the female, pubertal development, menopausal onset, and pathological conditions such as premature ovarian failure. Further studies are required to assess the role of steroids in other species and determine whether specific disease states such as premature ovarian failure may be caused in part from abnormal steroid regulation of this early developmental process.

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