Estradiol, Progesterone, and Genistein Inhibit Oocyte Nest Breakdown and Primordial Follicle Assembly in the Neonatal Mouse Ovary in Vitro and in Vivo

Ying Chen, Wendy N. Jefferson, Retha R. Newbold, Elizabeth Padilla-Banks, and Melissa E. Pepling

In developing mouse ovaries, oocytes develop as clusters of cells called nests or germ cell cysts. Shortly after birth, oocyte nests dissociate and granulosa cells surround individual oocytes forming primordial follicles. At the same time, two thirds of the oocytes die by apoptosis, but the link between oocyte nest breakdown and oocyte death is unclear. Although mechanisms controlling breakdown of nests into individual oocytes and selection of oocytes for survival are currently unknown, steroid hormones may play a role. Treatment of neonatal mice with natural or synthetic estrogens results in abnormal multiple oocyte follicles in adult ovaries. Neonatal genistein treatment inhibits nest breakdown suggesting multiple oocyte follicles are nests that did not break down. Here we investigated the role of estrogen signaling in nest breakdown and oocyte survival. We characterized an ovary organ culture system that recapitulates nest breakdown, reduction in oocyte number, primordial follicle assembly, and follicle growth in vitro. We found that estradiol, progesterone, and genistein inhibit nest breakdown and primordial follicle assembly but have no effect on oocyte number both in organ culture and in vivo. Fetal ovaries, removed from their normal environment of high levels of pregnancy hormones, underwent premature nest breakdown and oocyte loss that was rescued by addition of estradiol or progesterone. Our results implicate hormone signaling in ovarian differentiation with decreased estrogen and progesterone at birth as the primary signal to initiate oocyte nest breakdown and follicle assembly. These findings also provide insight into the mechanism of multiple oocyte follicle formation.

DIFFERENTIATION OF PRIMORDIAL germ cells into oocytes is essential for ovarian differentiation and subsequent female fertility. In the embryonic mouse ovary, germ cells undergo several rounds of mitosis and are classified as oogonia (1). Cytokinesis is incomplete during these cell divisions so that the oogonia form clusters of connected cells called nests or germ cell cysts similar to the germline cysts of invertebrate females such as Drosophila (2). After oogonia stop dividing, they enter meiosis and become oocytes (3). The oocytes remain in clusters, which here will be referred to as oocyte nests, for the rest of fetal development. After birth, oocytes separate and become enclosed in primordial follicles consisting of one oocyte and several somatic granulosa cells. Oocytes in primordial follicles are thought to represent the entire pool of gametes available to a female throughout her reproductive life, although it has been recently suggested that there are postnatal germ line stem cells in the adult ovary (4, 5). Thus, establishment of the primordial follicle pool is essential for mammalian reproduction. The process by which oocyte nests break apart to form primordial follicles is not understood. In mammalian species including the mouse, more than half of oocytes die early in development before follicles are formed (3, 6–9). During this process, some cells in each nest die by programmed cell death, leaving only a third of the total number surviving. In our model for mammalian oocyte nest breakdown (8), one cell of a nest dies causing the nest to break into smaller nests. This is repeated until a few individual oocytes remain. Thus programmed cell death of some oocytes would be required for nests to break down.

Developmental exposure to estrogenic compounds exerts effects on reproductive organs including increased occurrence of multiple oocyte follicles (MOFs) in the adult ovary (10). In normal adult female mouse ovaries, follicles consist of a single oocyte surrounded by layers of granulosa cells; follicles with more than one oocyte are rarely found (11). In contrast, ovaries from adult female mice treated as neonates with natural estrogens or compounds with estrogen-like activity have more MOFs (12–16). MOFs are postulated to be oocyte clusters that did not separate, resulting in more than one oocyte becoming enclosed in a single follicle (12, 13, 17).

Genistein, a phytoestrogen, causes an increase in MOFs (16). Genistein has properties other than estrogenic activity, such as tyrosine kinase inhibition; however, the occurrence of MOFs was not due to this property because another tyrosine kinase inhibitor, lavendustin, did not have this effect (16). In addition, the primary mechanism by which estrogen
elicits its action is through nuclear hormone receptors, estrogen receptor (ER)-α and ERβ (18, 19). To determine whether the estrogenic activity of genistein was involved in the formation of MOFs, mice lacking ERα or ERβ were treated neonatally with genistein. Mice lacking ERβ did not develop MOFs, whereas mice lacking ERα did, further implicating genistein’s estrogenic activity and showing that the effect on the ovary is mediated through ERβ (16). Although effects of estrogenic compounds have been known for many years, the mechanism by which MOFs form is unknown (12, 13). We hypothesize that MOFs are oocyte nests that did not completely break down during neonatal ovarian development. Our model is that normally, exposure of fetal oocytes to maternal estrogen keeps oocytes in nests and at birth estrogen levels drop resulting in nest breakdown. However, when neonatal oocytes are exposed to estrogens, nest breakdown is inhibited.

Mechanisms involved in MOF formation were studied by examining effects of neonatal genistein treatment on oocyte development (20). At postnatal day (PND) 4, genistein-treated mice had significantly more unassembled oocytes, compared with control mice. Thus, genistein disrupted primordial follicle assembly. In addition, genistein treatment inhibited nest breakdown. These results support the idea that MOFs observed in the genistein treated adult ovaries result from incomplete breakdown of oocyte nests during neonatal oocyte development. Genistein treatment also affected oocyte survival because significantly more oocytes were found at PND 4 and 6 in treated mice.

Neonatal treatment of rodents with testosterone and progesterone also results in increased MOFs (13, 21). However, progesterone and testosterone can be converted to estrogen, so it is not known whether these effects are direct or indirect due to conversion to estrogen. Another recent study showed that neonatal progesterone treatment reduced oocyte cell death and primordial follicle assembly in rats supporting the previous mouse studies (22).

We postulate that estrogen signaling plays a role in nest breakdown based on the effects of the plant estrogen, genistein, on developing ovaries. MOFs have been observed in adult females that were treated as neonates with estradiol on PND 1–5 (13). Here we show that the levels of estrogen and other hormones influence nest breakdown but not oocyte death, providing insight into the mechanism by which these hormones induce MOFs.

In vitro ovary organ culture

Ovaries were collected either at 16.5 dpc or PND 1 and placed into culture. Ovaries were cultured in drops of media on 0.4 μm floating filters (Millicell-CM; Millipore Corp., Bedford, MA) in 0.1 mL DMEM/Hams F-12 media supplemented with penicillin-streptomycin, 1% ITS-X (Life Technologies, Inc., Grand Island, NY), 0.1% BSA, 0.1% albumax, and 0.05 mg/ml l-ascorbic acid in 4-well culture plates. Ovaries from an additional group of mice were collected at PND 1 and PND 8 and not cultured for in vivo comparisons.

Chemicals used in the in vitro studies were as follows: estradiol (Sigma Chemical Co., St. Louis, MO); progesterone (Sigma); progesterone (PerkinElmer, Waltham, MA); and genistein (Sigma). All chemicals were dissolved in dimethylsulfoxide (DMSO) at a concentration of 0.1 mM and then added to culture media to achieve the desired final concentration. DMSO was added to media at the same percent as compound treatment (≤0.1%) to serve as a vehicle control.

Ovaries collected at 16.5 dpc were placed in culture and exposed daily to estradiol at 10^{-6} M or DMSO alone as a vehicle control (n = 5–8 ovaries per treatment group). One group of ovaries collected at PND 1 was cultured for 1–7 d in media without hormones and collected after 2, 3, 4, 5, 6, and 7 d of culture (n = 3–5 ovaries per time point). Another group of ovaries collected at PND 1 was cultured for 7 d in the presence of genistein, estradiol, progesterone, or promegestone in media at concentrations ranging from 10^{-10} to 10^{-4} M (n = 3–8 ovaries per treatment group). A third group of ovaries collected at PND 1 was cultured for 7 d in the presence of 10^{-8} M estradiol alone, 10^{-6} M progesterone alone, or both 10^{-6} M estradiol + 10^{-6} M progesterone (n = 6–8 ovaries per treatment group). All ovaries were processed for whole-mount immunohistochemistry.

In vivo estradiol treatment

Pregnant mice delivered pups at 19.5 d gestation (PND 1); pups were separated according to sex, pooled together, and then randomly standardized to eight female pups per litter. Male pups were used in another experiment. To examine the effects of hormone treatment at PND 4, female pups were treated on d 1–4 with estradiol by sc injection at a dose of 5 mg/kg/d in corn oil or left untreated as controls (eight mice per treatment group). Ovaries were collected and processed for whole-mount immunohistochemistry on PND 4. To examine effects of hormones on occurrence of MOFs, another group of female pups was treated on d 1–5 with estradiol at 5 mg/kg/d by sc injection in corn oil or left untreated as controls (eight mice per treatment group). Ovaries were collected and processed for histological and morphological evaluation on PND 19.

Histology and morphological evaluation

Ovaries collected at PND 19 after neonatal exposure to estradiol or left untreated as controls were fixed in 10% neutral buffered formalin for 6 h at 4°C, transferred to 70% ethyl alcohol, and embedded in paraffin. For each animal, three sections (6 μm) were prepared from both ovaries from different depths and stained with hematoxylin and eosin according to standard laboratory procedures (35). Ovaries from eight mice per treatment group were analyzed for the presence of MOFs using light microscopy. The presence of one MOF in a single ovarian section categorized a mouse as positive for MOFs.

Whole-mount immunohistochemistry and fluorescence microscopy

Ovaries collected from in vivo and in vitro experiments were fixed in 5% EM-grade paraformaldehyde (Ted Pella, Inc., Redding, CA) in PBS for 1 h followed by several washes in 5% BSA and 0.1% Triton X-100 in PBS. Whole ovaries were immunostained as previously described (8, 23). The signal transducer and activator of transcription (STAT)-3 (C20) antibody (Santa Cruz Biotechnology, La Jolla, CA) was used at a dilution of 1:500 (23). Propidium iodide or Toto-3 (Molecular Probes, now part of Invitrogen, Carlshbad, CA) was used to label nuclei. Samples were imaged on a Zeiss Pascal Confocal microscope (Carl Zeiss Microlmaging, Inc., Thornwood, NY).

Materials and Methods

Animals

Adult CD-1 female mice were obtained from Charles River Laboratories (Wilmington, MA) and bred to male mice of the same strain. Vaginal plug detection was considered d 0.5 of pregnancy. A group of pregnant mice was killed at 16.5 d postcoitum (dpc) for the in vitro ovary organ culture experiments. The remainder of the pregnant mice delivered pups at 19.5 dpc designated PND 1. Pregnant mice were housed under controlled lighting (12 h light, 12 h dark) and temperature (21–22°C) conditions. All animal procedures complied with an approved National Institute of Environmental Health Sciences/National Institutes of Health animal care protocol and the Syracuse University Institutional Animal Care and Use Committee.
Analysis of oocyte nest breakdown, primordial follicle assembly, and follicle development

Whole ovaries were labeled with an antibody against STAT3, a specific marker for germ cells (23). Ovaries were examined for percent single oocytes relative to the total number of oocytes to assess oocyte nest breakdown (8, 20). The number of individual oocytes relative to the number of oocytes in nests was determined by examining eight optical sections per ovary over each region. These regions were obtained by examining two areas of the ovary and taking four representative, confocal sections at least 20 μm apart in each area. For each region, a single confocal section was examined. In addition, for each region, a stack of 10 sections, 1 μm apart centered around the single section was obtained. This stack of sections was used to determine whether oocytes in the center section were associated with oocyte nests above or below the plane of focus. For primordial follicle assembly and development, the number of each type of follicle per region was determined. For primordial follicle assembly, oocytes were considered unassembled if granulosa cells did not completely surround them or if STAT3 antibody labeling showed the oocytes were associated. Follicles were classified as follows: primordial (oocyte surrounded by several granulosa cells with flattened nuclei), primary (oocyte surrounded by one layer of granulosa cells with cuboidal nuclei), or secondary (oocyte surrounded by more than one layer of granulosa cells).

Determination of germ cell number

The number of oocytes per section was determined by counting the number of oocytes in the eight representative, confocal sections that were collected for analysis of cyst breakdown and determining the average number of oocytes per section.

Statistical analysis

One-way ANOVA was conducted to look at treatment effects on oocyte number, percent single oocytes, follicle assembly, and follicle development. PROC GLM of SAS 9.1 (SAS Institute Inc., Cary, NC) was used to calculate the least-squares means and test specific hypotheses for effects. *P < 0.05 was considered significant.

Results

Characterization of neonatal oocyte development in an in vitro organ culture system

We characterized nest breakdown, oocyte survival, and primordial follicle development in ovary organ culture using ovaries from newborn CD-1 female mice. Before culture (PND 1 or d 0 of culture) and on d 1–7 of culture, ovaries were fixed and labeled for the oocyte marker STAT3 (23). The number of oocytes per section, percent of single oocytes, and relative numbers of different stage follicles were determined. The number of oocytes per section dropped significantly after 1 d of culture (Fig. 1A) similar to nest breakdown in vivo (8). In addition, by d 2, most nests had broken apart (greater than 60%) as in vivo (Fig. 1A). Next, we determined the number of unassembled oocytes and different stages of developing follicles. Before culture, the majority of oocytes were not enclosed in follicles (over 80%, Fig. 1B) but after 7 d in organ culture, most oocytes were in follicles (almost 90%), and some had started growing as evidenced by the appearance of primary and secondary follicles. We also examined follicle development in vivo at PND 8, which would be comparable with 7 d of culture and found that as in culture the majority of oocytes were enclosed in follicles and some had started progressing to the primary and secondary stage (Fig. 1C). However, there were significantly fewer secondary follicles and more primary follicles in vitro, compared with in vivo. This difference may reflect the lack of some factor in the organ culture media that is necessary for optimal follicle development. Finally, during the culture period the ovaries grew in size. Thus, early events of nest breakdown, primordial follicle assembly, and follicle growth proceed similar to in vivo in our organ culture system, but the transition of the first wave of developing follicles from primary to secondary follicles is delayed.

Effects of genistein on oocyte development in ovary organ culture

We have previously shown that treatment of neonatal mice with genistein results in inhibition of nest breakdown and an
increase in oocyte survival (20). Therefore, in the current study, we determined whether genistein would have the same effect on ovaries in organ culture as it did in vivo. Ovaries were collected on PND 1 and placed in culture for 7 d. Ovaries were treated daily with concentrations from $10^{-9}$ to $10^{-4} \text{M}$ genistein, and nest breakdown, oocyte survival, and follicle development were analyzed after whole-mount immunohistochemistry. Control ovaries had approximately 90% single oocytes, whereas genistein-treated ovaries had fewer single oocytes (60–70%) at concentrations of $10^{-8}$ to $10^{-4} \text{M}$ and higher (Fig. 2A); thus, as in vivo, nest breakdown was inhibited. Examples of a cultured control ovary and an ovary treated with $10^{-6} \text{M}$ genistein are shown in Fig. 2, D and E, illustrating the large nests that are present in the treated ovaries. Ovaries treated with the highest concentration of genistein, $10^{-4} \text{M}$, did not grow during the culture period and the structure of the ovary appeared disorganized, suggesting that the tissue did not survive the culture period. There was no significant difference between the number of oocytes per section in control and treated ovaries (Fig. 2B). We also examined follicle assembly and found an increase in the number of un assembled oocytes from 13% to more than 30% (Fig. 2C). Thus, like its effect in vivo, genistein inhibited nest breakdown and follicle assembly in organ culture. However, oocyte survival was not altered in vitro.

**Estradiol alters ovarian differentiation in vivo and in vitro**

To confirm that the alterations in ovarian differentiation are due to estrogen activity, the natural mammalian hormone, estradiol was studied in vivo as well as in vitro. Neonatal mice were treated on d 1–4 with 5 mg/kg d estradiol or vehicle alone, and nest breakdown, oocyte survival, and follicle development were measured after whole-mount immunohistochemistry. We found that the percent of single oocytes was reduced from 74% in control animals to 46% in treated animals at PND4 (Fig. 3A), whereas oocyte number was not affected (Fig. 3B). Primordial follicle assembly was also affected by estradiol treatment of neonates with treated animals having 54% unassembled oocytes, compared with control animals, with only 26% unassembled follicles (Fig. 3C). Thus, estradiol treatment perturbs neonatal ovarian development in vivo.

To determine whether this effect persisted into puberty, mice were again treated with 5 mg/kg d estradiol on d 1–5. Ovaries from mice treated with estradiol exhibited an increased incidence of MOFs at PND 19, compared with control mice (five of eight mice exhibited MOFs in the treated group vs. none of eight in the control), similar to our previous finding of increased MOFs after neonatal genistein treatment (20).

To establish a direct effect of estradiol on ovarian differentiation, ovaries were again collected from CD-1 mice on PND 1 and placed in culture for 7 d. Ovaries were treated daily with concentrations from $10^{-9}$ to $10^{-4} \text{M}$ estradiol, and nest breakdown, oocyte survival, and follicle development were analyzed after whole-mount immunohistochemistry. Control ovaries had approximately 90% single oocytes, whereas estradiol-treated ovaries had fewer single oocytes.
Prenatal organ culture triggers premature oocyte development that is rescued by estradiol treatment

Ovaries from 16.5 dpc fetuses were grown in organ culture in the absence of maternal hormones for 3 d, which would

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be equivalent to the day of birth. We found that the majority of nests had broken down (63% single oocytes) in fetal ovaries cultured for 3 d as compared with the equivalent in vivo time point of PND 1 (19.5 dpc) (only 16% single oocytes) (Fig. 8A). We also found that the number of oocytes was reduced to 28 per section, compared with 39 in vivo (Fig. 8B) and unassembled oocytes were reduced from 84 to 37% (Fig. 8C). In vivo, ovaries at PND 1 had some primordial follicles (16%) and no primary or secondary follicles. Cultured ovaries in the absence of maternal hormones had significantly more primordial follicles (57%) and some primary follicles (6%).

To further test the effects of hormone signaling on nest breakdown, estradiol, progesterone, or both hormones were added to the culture media daily for 3 d and nest breakdown rate and oocyte survival were compared with the ovaries grown without added hormone and to PND 1 ovaries. In estradiol-treated ovaries, nest breakdown was reduced with only 18% single oocytes, which is comparable with PND 1 in vivo (Fig. 8A). The number of oocytes was increased from 28 to 34 oocytes per section, although this is still lower than PND 1 in vivo (39 oocytes per section) (Fig. 8B). Most oocytes were unassembled (82%) and the number of all follicle types was reduced (17% primordial, 1% primary, and no secondary follicles), compared with ovaries cultured in media alone. Progesterone partially reduced nest breakdown, compared with ovaries cultured in medium without added hormone (29% single oocytes, compared with 63% in the cultured control ovaries) but did not completely rescue to in vivo numbers (16% single oocytes). Premature follicle formation was also reduced, resulting in a higher percentage of unassembled oocytes (71%, compared with 37% in the in vitro control) and did not increase the number of oocytes (Fig. 8, A and B). When estradiol and progesterone were both added, oocyte development was similar to added estradiol alone. Thus, premature oocyte development including nest breakdown is triggered by removal of the ovaries from the maternal hormonal environment, and this premature ovarian differentiation can be partially rescued by addition of estradiol or progesterone to organ culture supporting our model of ovarian differentiation.

Discussion

It is a challenge to control the maternal environment of the fetus and to directly observe oocyte development in the fetal and neonatal ovary. To facilitate an analysis of hormonal effects on fetal and neonatal oocyte development, we explored an in vitro organ culture system that has previously been used by others (22, 24–26). This permitted us to directly visualize the effect of hormone levels on nest breakdown and primordial follicle formation. Previous work showed that treatment of neonates with estrogenic compounds caused an increase in MOFs in the adult ovary (12–16). Genistein treatment inhibits nest breakdown supporting the idea that MOFs are nests that did not break down (20). Our results indicate
that genistein, estradiol, and progesterone inhibit nest break-
down and primordial follicle assembly in organ culture.

Oocytes from prenatal ovaries grown in culture begin nest 
breakdown prematurely, and this is partially prevented by 
addition of estradiol or progesterone to the culture medium. 
These findings support our current model of the role of 
estrogen in regulation of nest breakdown and oocyte apoptosis (20). At birth, estrogen and progesterone levels drop remov-
ing the inhibitory signal and nests begin to break apart. We 
do not understand why it is necessary for nests to break apart 
at birth. It may be that if this process occurs earlier, the 
oocytes are not yet in the right stage of meiosis. In a recent 
study, inhibition of synaptonemal complex protein-1 caused 
pregature arrival in the diplotene stage of meiosis and ac-
celeration of primordial follicle assembly, suggesting a link 
between cell cycle stage and primordial follicle development (28).

Recently progesterone and estrogen were found to affect 
neonatal oocyte development in rats (22). Neonatal progester-
tone treatment reduced primordial follicle assembly, whereas both progesterone and estrogen treatment reduced the 
primordial to primary follicle transition in the initial wave of follicles that begin to develop. However, nest break-
down was not examined, only whether primordial follicles 
had formed. Progesterone also reduced the amount of pro-
gramed cell death in the neonatal ovary but estrogen was not 
tested. In our studies, both progesterone and estrogen in-
hibited nest breakdown and primordial follicle assembly but 
not subsequent follicle development. In addition, neither 
hormone had an effect on oocyte survival in organ culture. 
One possibility for the observed differences between the two 
studies is that certain aspects of hormonal effects on oocyte 
development differ between rats and mice. Another possi-
bility is that different methods of analysis and counting re-
sulted in the apparent differences. One should note here that 
published counts of oocyte number in developing mouse 
ovaries vary widely, and care should be taken when com-
paring studies that use different methods (8, 29).

There are several possible ways that abnormal MOFs 
found in adult ovaries from mice that were treated with 
estrogenic compounds could form. Granulosa cells could 
improperly enclose more than one oocyte in a follicle or 
ovoce nest breakdown could be disrupted. Alternatively, 
two follicles could fuse. Studies with genistein treatment 
show that more unassembled oocytes after genistein 
treatment and more oocytes still in nests. In addition, inter-
cellular bridges still connected oocytes in genistein-treated 
mice, whereas none were apparent in control mice at PND 4 
(20), suggesting that MOFs in adult ovaries result from in-
complete breakdown of oocyte nests during neonatal devel-
opment leaving pregranulosa cells with multiple oocytes to 
surround. Our results here show that estradiol and progester-
one disrupt nest breakdown, further supporting the idea 
that MOFs in adult ovaries are oocyte nests that did not break 
apart.
Programmed cell death of oocytes occurs at the time of oocyte nest breakdown, but the relationship between these two processes is not known (8). Currently it is thought that programmed cell death is required for nests to break apart. Supporting this idea, mutants in the cell death regulator, Bcl-2-associated X protein, have more oocytes and a delay in nest breakdown (30). In addition to inhibiting nest breakdown, neonatal genistein treatment increased oocyte survival in vivo and reduced apoptosis (20). However, here in organ culture, genistein, estradiol, or progesterone treatment inhibited nest breakdown, but most concentrations of hormone had no effect on oocyte death. At the highest level of estradiol (10^{-8} M), there was an increase in oocytes surviving, whereas somatic cells appeared to be dying (data not shown). These responses may be due to which receptors are ex-
pressed in different cell types. This level of estradiol may have a toxic effect on the ovary and somatic cells are just more sensitive to estradiol than oocytes. The oocytes may have some protective mechanism so that they can survive an otherwise toxic insult. Ovaries did not survive the organ culture period when treated with $10^{-4}$ M genistein or progesterone, again suggesting that extremely high levels of hormones have a general toxic effect on the ovary. Interestingly, in vivo genistein injections protected more oocytes from dying, whereas there was no change in oocyte number in organ culture. This may reflect differences in the mechanism of action of genistein, depending on how the ovary is receiving the hormone. However, neither estradiol injections in vivo nor estradiol in culture altered the number of oocytes surviving through the nest breakdown period. Based on counting numbers of oocytes, estradiol does not influence oocyte survival. This suggests that programmed cell death is not the driving force causing nests to break apart or that the processes of nest breakdown and oocyte cell death can be separated at some level.

There are two mammalian estrogen receptors, ER$\alpha$ and ER$\beta$, with multiple roles in female reproduction (31). MOFs result from genistein treatment of ER$\alpha$ mutant mice but not ER$\beta$ mutant mice, suggesting that effects of genistein are mediated primarily through ER$\beta$ (16). However, genistein has higher affinity for ER$\beta$, whereas estradiol has equal affinity for ER$\alpha$ or ER$\beta$ (32). Using neonatal ovary culture and in vivo injections, we find that estradiol, like genistein, inhibits nest breakdown and primordial follicle assembly. Although it is likely that maternal estradiol maintains oocytes in nests through ER$\beta$, there are possible alternative mechanisms. Estradiol may exert its effects through ER$\alpha$ or both ER$\alpha$ and ER$\beta$. Another possibility is that estradiol may not exert its effects through either ER. Recently steroid hormones have been found to mediate effects through membrane bound receptors (33). ER$\beta$ knockout mice have reduced fertility with fewer overall litters and fewer pups per litter (19). Mutant adult ovaries have more atretic follicles and fewer corpora lutea, suggesting that more oocytes may be dying. However, immature ER$\beta$ knockout mice (PND 28–30) do not have fewer primordial follicles than wild-type mice (34). ER$\alpha$ mutants are sterile with ovaries lacking corpora lutea and containing cystic and hemorrhagic follicles (35). Nest breakdown has not been examined in either ER mutant.

Several genes have mutant phenotypes, suggesting potential involvement in nest breakdown. For example, mice lacking bone morphogenetic factor 15 or growth differentiation factor 9 have more MOFs than wild-type mice as well as other defects of ovarian differentiation (36). The increased number of MOFs in these mutant animals suggests that these proteins function in nest breakdown. Both proteins are members of the TGF$\beta$ superfamily; they are oocyte-secreted factors expressed early in ovarian differentiation (37). Another TGF$\beta$ family member, activin, has also been implicated in early oocyte differentiation. Interfering with the activin signaling pathway results in abnormal ovarian development including ovaries with MOFs (38, 39). Furthermore, treatment of neonatal mice with estradiol or diethylstilbestrol reduces activin mRNA and protein levels, supporting the idea that activin is a target of estrogen signaling (40). Lunatic fringe (Lfng) mutants are infertile and also have MOFS (41). Lfng is a member of the fringe family of proteins that function by either stimulating or inhibiting Notch signaling (42, 43). Lfng is expressed in granulosa and theca cells of developing follicles (41). Notch signaling may play a role in nest breakdown or follicle assembly. Interestingly, in Drosophila, fringe mutants have follicles with more than one oocyte (44). Estrogen may affect nest breakdown by regulating some of these genes. Estrogen may also up-regulate cell adhesion genes that would keep oocytes in nests. In addition, somatic cells need to migrate around oocytes to form follicles, and

![Figure 8](https://academic.oup.com/endo/article-lookup/1488/2501757)
estrogen signaling may inhibit genes involved in this migration. 

It remains unclear what cell types respond to estrogen during ovarian differentiation. Estrogen could signal directly to the oocytes to regulate nest breakdown or to somatic cells that could in turn signal the oocytes. For example, cultured primordial germ cells are stimulated to proliferate by treatment with estrogen but only in the presence of somatic gonadal cells, suggesting that estrogen can mediate this effect on germ cells indirectly by signaling through somatic cells (45). Using immunocytochemistry, we detect ERβ in oocytes at birth (Pepling, M. E., unpublished data). However, ERβ is expressed in granulosa cells by PND 5, continuing into adulthood, and has been detected as early as PND 1 by RNase protection in whole ovaries (46). ERα is expressed in theca cells in adult ovary and is also detected in neonatal ovaries (46). Cell-specific knockouts of estrogen receptors will be required to determine which receptor and which cells types are required for oocyte development.

Our model of ovarian differentiation is that in the developing mouse, maternal estrogen and progesterone keep oocytes in nests and that after birth the drop in these hormones causes nests to break apart and follicles to assemble. However, in humans, follicle formation occurs at about 4.5 months gestation, not at birth (47–49). It may be that humans do not use the same mechanism as rodents. However, another possibility is that even though total progesterone and estrogen levels are high late in human pregnancy, the amount that fetal tissues are exposed to is reduced at this time. This interpretation is supported by a study in monkeys, which showed progesterone was reduced in fetal tissues during late pregnancy (50). It is possible that steroid hormone binding proteins such as α-fetoprotein, which binds estrogen, could sequester hormones causing the levels in ovarian tissue to drop (27).

We have shown that estradiol, progesterone, and genistein disrupt nest breakdown and primordial follicle formation. The mechanism by which these hormones act on the ovary is still not known. Investigation into how these signals are received and what cell types receive these signals is ongoing. The basic mechanisms underlying normal primordial follicle formation as well as how disorders disrupt these normal processes are not well understood. Elucidation of the processes involved in establishment of the primordial follicle pool will provide a better understanding of oocyte development. This understanding will give insight into premature ovarian failure, reproductive life span, menopause, and ovarian cancer and contribute to potential treatments of female infertility. In addition, this knowledge will aid in the understanding of toxic effects of exogenous estrogen exposure.

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Address all correspondence and requests for reprints to: Melissa Pepling, Department of Biology, Syracuse University, 130 College Place, Syracuse, New York 13244. E-mail: mepeplin@syr.edu.

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