

Estrogen can signal through multiple pathways to regulate oocyte cyst breakdown and primordial follicle assembly in the neonatal mouse ovary

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Abstract

During mouse embryonic development, oocytes develop in germline cysts, formed by several rounds of cell division followed by incomplete cytokinesis. Shortly after birth, cysts break down and individual oocytes are enclosed by granulosa cells to form primordial follicles. At the same time, two-thirds of the oocytes die by apoptosis with only one-third surviving. We have previously shown that the steroid hormones, estradiol (E_2), and progesterone as well as the phytoestrogen genistein can inhibit cyst breakdown and primordial follicle assembly. However, the mechanisms by which steroid hormones regulate oocyte cyst breakdown and selective oocyte survival are unknown. Here, we confirmed the expression of estrogen receptor (ER) mRNA and protein in neonatal mouse ovaries using reverse transcriptase-PCR, western blotting, and immunocytochemistry. We then used ER-specific agonists and antagonists to understand the mechanism of estrogen signaling. 4,4',4''-(4-propyl-[1H]-pyrazole-1,3,5-triyl)

trisphenol, an ER α -selective agonist, and 2,3-bis(4-hydroxyphenyl)-propionitrile, an ER β -selective agonist, both inhibited cyst breakdown in organ culture, suggesting that E_2 can signal through both the receptors to regulate cyst breakdown. ICI 182,780, an ER antagonist, completely blocked E_2 's action. 1,3-bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1H-pyrazole dihydrochloride, an ER α -specific antagonist, fully blocked E_2 's effect on oocyte cyst breakdown and primordial follicle assembly and (R,R)-5,11-diethyl-5,6,11,12-tetrahydro-2,8-chrysenediol, an ER β -specific antagonist, partially blocked E_2 , further supporting the idea that both receptors are involved in estrogen signaling in neonatal oocyte development. E_2 conjugated to BSA, which can only exert effects at the membrane, was able to inhibit cyst breakdown, implying that E_2 could also function through a membrane-bound ER to regulate cyst breakdown.

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Introduction

Formation of functional gametes is essential for reproduction. In the mouse, primordial germ cells (PGCs) migrate to the gonad during embryonic development (Bendel-Stenzel *et al.* 1998). In the female, after the PGCs arrive at the gonad, they proliferate until 13.5 days post coitum (dpc) when they initiate meiosis (Pepling 2006). During this time, cysts are formed because each round of mitosis is followed by incomplete cytokinesis leaving the oogonia connected by intercellular bridges (Pepling & Spradling 1998). Shortly after birth, the cysts undergo breakdown, accompanied by apoptosis of about two-thirds of the germ cells (Pepling & Spradling 2001). Then, each individual germ cell associates with somatic (granulosa) cells and becomes enclosed in a primordial follicle. Apoptosis is thought to be the primary mechanism by which the multicellular cysts break apart into single oocytes (Pepling & Spradling 2001, Greenfield *et al.* 2007). However, it is unknown how cyst breakdown is regulated or coordinated with neonatal development.

Recent research has implicated estrogen signaling in the cyst breakdown process. Neonatal exposure to genistein, an estrogen-like compound from soy, induces multiple oocyte follicles (MOFs) in the mature mouse ovary (Jefferson *et al.* 2002). During neonatal ovary development, genistein-treated mice had significantly more oocytes still in cysts compared with control mice (Jefferson *et al.* 2006). These results support the idea that MOFs observed in the genistein-treated adult ovaries result from incomplete breakdown of oocyte cysts during neonatal oocyte development. In our previous work, we have found that estradiol (E_2) inhibited cyst breakdown and primordial follicle assembly both *in vitro* and *in vivo* (Chen *et al.* 2007).

Estrogens function via nuclear receptors, which are members of the steroid hormone receptor superfamily. There are at least two estrogen receptors (ERs) in mammals namely ER α and ER β (Pettersson & Gustafsson 2001) that are also listed as ESR1 and ESR2 in the MGI Database. In addition to acting as nuclear hormone receptors, there is evidence that ERs can be translocated to and act at the plasma

membrane (Pedram *et al.* 2006). Recent studies have also provided evidence that estrogen may also signal through membrane-bound receptors including a G-coupled membrane receptor called GPR30 (Revankar *et al.* 2005). Like normal wild-type mice, *Er α* -deficient mice treated with genistein also have MOFs. However, *Er β* knockout mice treated with genistein do not have MOFs, implying that *Er β* is the major ER in the breakdown process (Jefferson *et al.* 2002).

The importance of estrogen signaling in female fertility has been investigated in ER knockout mice. *Er α* knockout females are infertile, do not ovulate, and lack corpora lutea (Lubahn *et al.* 1993, Dupont *et al.* 2000). Instead, there are a high number of abnormal hemorrhagic or atretic antral follicles. *Er β* knockout female mice are subfertile with fewer and smaller litters (Krege *et al.* 1998). In contrast to *Er α* knockout mice, *Er β* knockout mice have some corpora lutea although fewer than wild-type. Loss of both receptors in the double knockout mice leads to female infertility and ovaries that lack corpora lutea similar to ER α mutants. However, in contrast to *Er α* knockouts, the double mutants have Sertoli-like cells arranged into structures resembling seminiferous tubules, suggesting that the two ERs are required for the maintenance of ovarian cell identity (Couse *et al.* 1999). It is not known whether mutation of either or both ERs affects the process of cyst breakdown.

ERs are expressed in a variety of tissues. Both receptors are expressed in the adult ovary (Enmark & Gustafsson 1999). ER α protein is localized primarily in the theca cells in adult mice (Enmark & Gustafsson 1999), while in fetal ovaries, it is localized to a few stromal cells and the epithelium (Nielsen *et al.* 2000). ER β is expressed in the granulosa cells starting from postnatal day (PND) 5 (Jefferson *et al.* 2000). Although ER β RNA has been detected earlier at PND 1 by reverse transcriptase (RT)-PCR, it is unknown whether ER β protein is present in the neonatal ovaries, and if so what cell type it is localized to.

In this study, we examined the expression of ERs in the neonatal mouse ovary using RT-PCR, western-blot analysis, and immunocytochemistry. Both ER mRNAs are present in the neonatal ovary, suggesting that estrogen could signal through either receptor to regulate cyst breakdown and primordial follicle formation. We then utilized ER-specific agonists and antagonists to determine the role of each ER. We found that both ER-specific agonists inhibit cyst breakdown, while receptor-specific antagonists block the effects of estrogen in the ovary. We also provide evidence supporting the role of a membrane ER (mER) in neonatal oocyte development.

Materials and Methods

Animals

Adult cluster of differentiation-1 female mice were obtained from Charles River Laboratories (Wilmington, MA, USA) and bred to male mice of the same strain. Vaginal plug

detection was considered to be day 0.5 of pregnancy. Pregnant mice delivered pups at 19.5 dpc, which were designated PND 1. Pregnant mice were housed under controlled lighting (12 h light:12 h darkness cycles) and temperature (21–22 °C) conditions. All animal procedures complied with the Syracuse University Institutional Animal Care and Use Committee.

In vitro ovary organ culture

Ovaries were collected at PND 1 and placed into culture for 7 days. Ovaries were cultured in drops of media on 0.4 μ M floating filters (Millicell-CM, Millipore Corp., Bedford, MA, USA) in 0.4 ml DMEM–Ham's F-12 media supplemented with penicillin–streptomycin, 5 \times ITS-X (Life Technologies, Inc.), 0.1% BSA, 0.1% albumax, and 0.05 mg/ml L-ascorbic acid in four-well culture plates. Ovaries were grown in culture medium alone or in the presence of estrogen agonists or antagonists as noted, which was added daily. The ovaries were randomly distributed to different treatment groups. The data for control ovaries for the E₂-BSA studies were collected in a separate experiment.

Chemicals used in the *in vitro* studies were as follows: (E₂, Sigma Chemical Company); 4,4',4''-(4-propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT, Tocris Cookson Inc., Ellisville, MO, USA); 2,3-bis(4-hydroxyphenyl)-propionitrile (DPN, Tocris Cookson Inc.); 1,3-bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinyloxy)phenol]-1H-pyrazole dihydrochloride (MPP, Tocris Cookson Inc.); (R,R)-5,11-diethyl-5,6,11,12-tetrahydro-2,8-chrysenediol ((R,R)-THC, Tocris Cookson Inc.); 7 α ,17 β -[9]([4,4,5,5,5-pentafluoropentyl)sulfinyl]nonyl]estra-1,3,5(10)-triene-3,17-diol (ICI 182,780, Tocris Cookson Inc.); albumin-FITC conjugate (BSA-FITC, Sigma-Aldrich Inc.); and β -E₂ 6-(O-carboxy-methyl)oxime: BSA-FITC conjugate (E₂-BSA-FITC, Sigma-Aldrich Inc). All chemicals were dissolved in DMSO at a concentration of 0.1 M 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. All chemicals were added at the start of organ culture except for antagonist studies using MPP, R,R-THC, and ICI 182,780 where the antagonist was added first and E₂ was added 6 h later.

Whole-mount immunohistochemistry and fluorescence microscopy

Ovaries collected after *in vitro* experiments or for ER localization studies were fixed in 5% electron microscopy grade paraformaldehyde (Ted Pella, Inc., Redding, CA, USA) in PBS overnight at 4 °C followed by several washes in 0.1% Triton X-100 in PBS and then incubated with 5% BSA in 0.1% Triton X-100 in PBS to block nonspecific binding. Whole ovaries were immunostained as previously described (Murphy *et al.* 2005). The STAT3 (C20) antibody (Santa Cruz Biotechnology, La Jolla, CA, USA) was used at

a dilution of 1:500 (Murphy *et al.* 2005). The secondary antibody anti-rabbit Alexa 488 (Molecular Probes, now part of Invitrogen) was used at a dilution of 1:200. Propidium iodide (Molecular Probes, now part of Invitrogen) was used to label nuclei. Samples were imaged on a Zeiss Pascal Confocal microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY, USA). For analysis of ER expression in neonatal cluster of differentiation-1 ovaries, tissues were treated as above except the primary antibodies used were ER α (MC20) at 1:100 (Santa Cruz Biotechnology) and ER β (311) at 1:250 (Affinity Bioreagents, Golden, CO, USA).

Analysis of oocyte cyst breakdown, primordial follicle assembly, and follicle development

Whole ovaries were labeled with an antibody against STAT3, a specific marker for germ cells. Ovaries were examined for percent single oocytes relative to the total number of oocytes to assess oocyte cyst breakdown (Jefferson *et al.* 2006). The number of individual oocytes relative to the number of oocytes in cysts was determined by examining eight optical regions per ovary. 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 of these regions, a stack of 10 sections, 1 μm apart centered around the single confocal 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 each oocyte in the single section that appeared not to be associated with other oocytes, sections above and below were examined to confirm that the oocyte was not in a cyst. 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 that the oocytes were associated. Follicles were classified as follows: primordial (oocyte surrounded by several flattened granulosa cells), primary (oocyte surrounded by one layer of cuboidal granulosa cells), or secondary (oocyte surrounded by at least two layers 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, USA) was used to calculate the least-squares means and test-specific hypotheses for effects. *P* value <0.05 was considered significant.

RNA isolation from mouse ovaries

Ovaries were dissected in PBS and kept on ice. Twenty microliters of Trizol (Invitrogen) per ovary were added and ovaries were then homogenized by hand. The homogenates were pooled until 40 ovaries were collected. The homogenate was phase separated with 20 μl of 1 mg/ml glycogen and 160 μl of chloroform. The mixture was centrifuged at 15 000 *g*. for 15 min at 4 °C and the top phase was kept. The RNA was washed with 400 μl of isopropanol and then with 70% ethanol. The RNA pellet was air dried and dissolved in 25 μl of PCR grade water. It was incubated at 55 °C for 10 min and then stored at -80 °C until further use.

Reverse transcriptase PCR

RT (Invitrogen) was used to create cDNA, primed with a poly(T)-containing primer (3' race primer (Invitrogen)), from Trizol-extracted RNA. The mixture of cDNA thus produced, representing mRNA, was then subjected to PCR or nested PCR using primers specific to the gene of interest. Specifically for ER α and ER β , cDNA was subjected to the first round of PCR for 20 cycles: 94 °C for 30 s, 55 °C for 30 s, and then 72 °C for 1 min. Then the resulting DNA product was subjected for the second round of nested PCR for 30 cycles: 94 °C for 30 s, 55 °C for 30 s, and then 72 °C for 1 min. Primers for GAPDH forward (5'-TGCMTCCCTGCACCACCAACT-3') and reverse (5'-YGCCCTGCTTCACCACCTTC-3') where Y=T or C and M=A or C were used as control. Primers for the following genes, with sequences, were used: ER α forward primer (5'-AATTCTGACAATCGACGCAG-3') and reverse (5'-GTGCTTCAACATTCTCCCTCCTC-3'); ER α nested forward primer (5'-GAGAAAGGAAACATGATCATGGA-3') and nested reverse (5'-TTCATCATGCCACTTGGTAAC-3'); ER β forward primer (5'-ACGAAGTAGGAATGGTCAAGTGT-3') and reverse (5'-ATGCCAAAATTTCCAGAATC-3'); ER β nested forward (5'-AAAGCCAAGAGAACCAGTGGGCAC-3') and nested reverse (5'-GCCAATCATGTGCACCAGTTCCTT-3'). As a negative control, PCR was also performed on the RNA samples without reverse transcription and confirmed that the bands detected in the reverse transcribed samples were not amplified from contaminating genomic DNA (data not shown).

Western-blot hybridization

Ovaries were homogenized in sample buffer (2% SDS, 10% glycerol, 25 mM Tris pH 6.8, 0.00005% bromophenol blue, and 0.025% mercaptoethanol) plus mini complete protease inhibitor (Sigma). PND 1, 4, and 7 ovaries were

homogenized in 10 μ l sample buffer/ovary. Adult ovaries were homogenized in 200 μ l/ovary. For each sample, 20 μ l solubilized protein extract (equivalent to approximately two ovaries) was mixed with 1/10 volume of mercaptoethanol, heated to 95 °C for 3 min, separated on 10% SDS-polyacrylamide gels and electroblotted onto Immobilon-P membranes (Millipore). The blots were incubated with a blocking solution containing 5% nonfat milk powder in PBST (PBS/0.05% Tween 20) overnight at 4 °C and incubated with primary antibodies in blocking solution for 1 h at room temperature. The primary antibodies used were ER α (MC20) at 1:500 (Santa Cruz Biotechnology) and ER β (311) at 1:500 (Affinity Bioreagents). Following three washes in blocking solution, membranes were incubated with HRP-conjugated secondary IgG (1:25 000) in blocking solution at room temperature for 1 h, washed in PBST thrice, and signal visualized using the Supersignal kit (Pierce) on films. Blots were reprobed for GAPDH as a loading control.

Results

Er α and Er β mRNAs and protein are expressed in neonatal ovaries

We have previously shown that neonatal E₂ treatment disrupts normal oocyte development by inhibiting cyst breakdown and primordial follicle formation (Chen *et al.* 2007). Previous research has validated the expression of ERs in ovaries of adult mice, with ER α primarily in theca cells and ER β in granulosa cells (Jefferson *et al.* 2000). To determine when ER α and ER β were expressed in developing ovaries, we used RT-PCR and western blotting. Both ER α and ER β mRNAs were detected in ovaries at PND 1, 4, 7, and 42 (Fig. 1A). ER α protein was detected by western blotting in ovaries at PND 1, 4, 7, and 92 (Fig. 1B). In PND 92 ovaries, the full-length 66 kDa protein was detected while a slightly smaller protein was detected at PND 4, PND 7, and very weakly at PND 1. Several smaller isoforms of ER α have been observed by others in tissue culture cells that lack part of the N-terminal of the protein (Heldring *et al.* 2007). ER β protein was also detected by western blotting in ovaries at PND 1, 4, 7, and 92 (Fig. 1C). At all ages examined, proteins at two molecular masses were detected, one at about 50 kDa and the other at about 30 kDa. As for ER α , several splice variants of ER β have been observed in other tissues (Heldring *et al.* 2007).

To determine what cell types express ER α and ER β protein in developing neonatal gonads, whole-mount immunocytochemistry was used at PND 1, 4, and 7. These time points were chosen at the span time of cyst breakdown. ER α was detected starting at PND 1 in somatic cells (data not shown). At PND 4 and 7, ER α was expressed more strongly in the granulosa cells as they surrounded the oocytes and formed primordial follicles (Fig. 2A–C). ER α was also expressed weakly in the cytoplasm of the oocytes. ER β protein was detected at 18.5 dpc and PND 1 in the nuclei

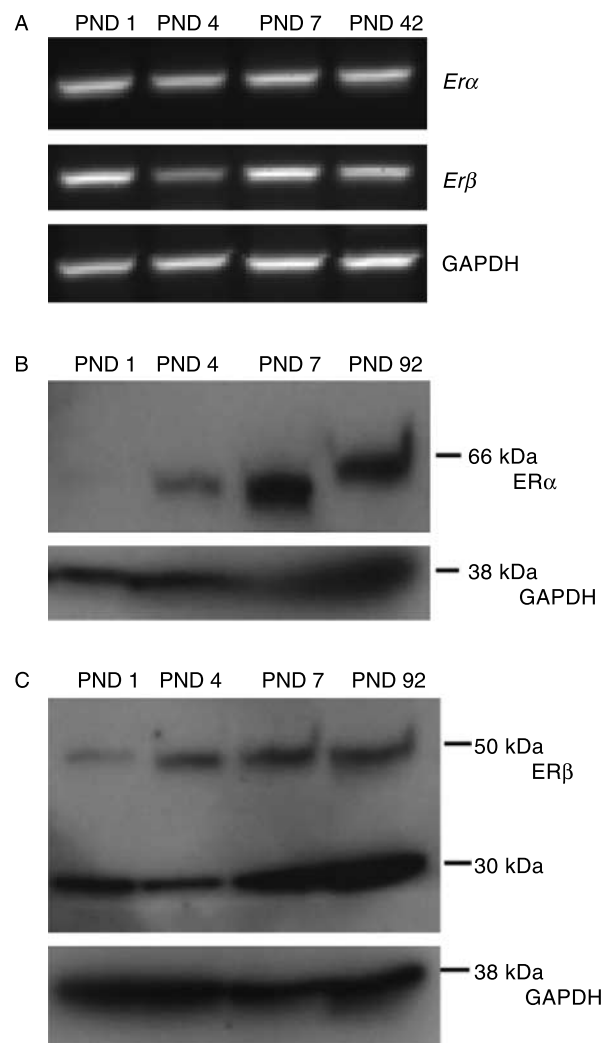


Figure 1 Estrogen receptor mRNA and protein expression in neonatal mouse ovaries. (A) mRNA was obtained from PND 1, 4, 7, and 42 ovaries and RT-PCR analysis was performed. Primers specific for GAPDH were used as a loading control. ER α and ER β mRNAs were detected in neonatal as well as adult ovaries. (B) Western-blotting analysis of ER α . Mouse tissue extracts from PND 1, 4, 7, and 92 ovaries were probed with an ER α antibody. (C) Western-blotting analysis of ER β . Mouse tissue extracts from PND 1, 4, 7, and 92 ovaries were probed with an ER β antibody. Blots were reprobed with GAPDH (38 kDa) as a loading control.

of a subset of oocytes but not at PND 4 or 7 (Fig. 2D–F). Thus, both ERs are present during cyst breakdown and primordial assembly.

Selective ER agonists disrupt oocyte development in vitro

We utilized ER-specific agonists to determine the involvement of each ER in neonatal oocyte development. Previously, it was shown that the effects of genistein on the ovary were mediated through ER β (Jefferson *et al.* 2002). We expected

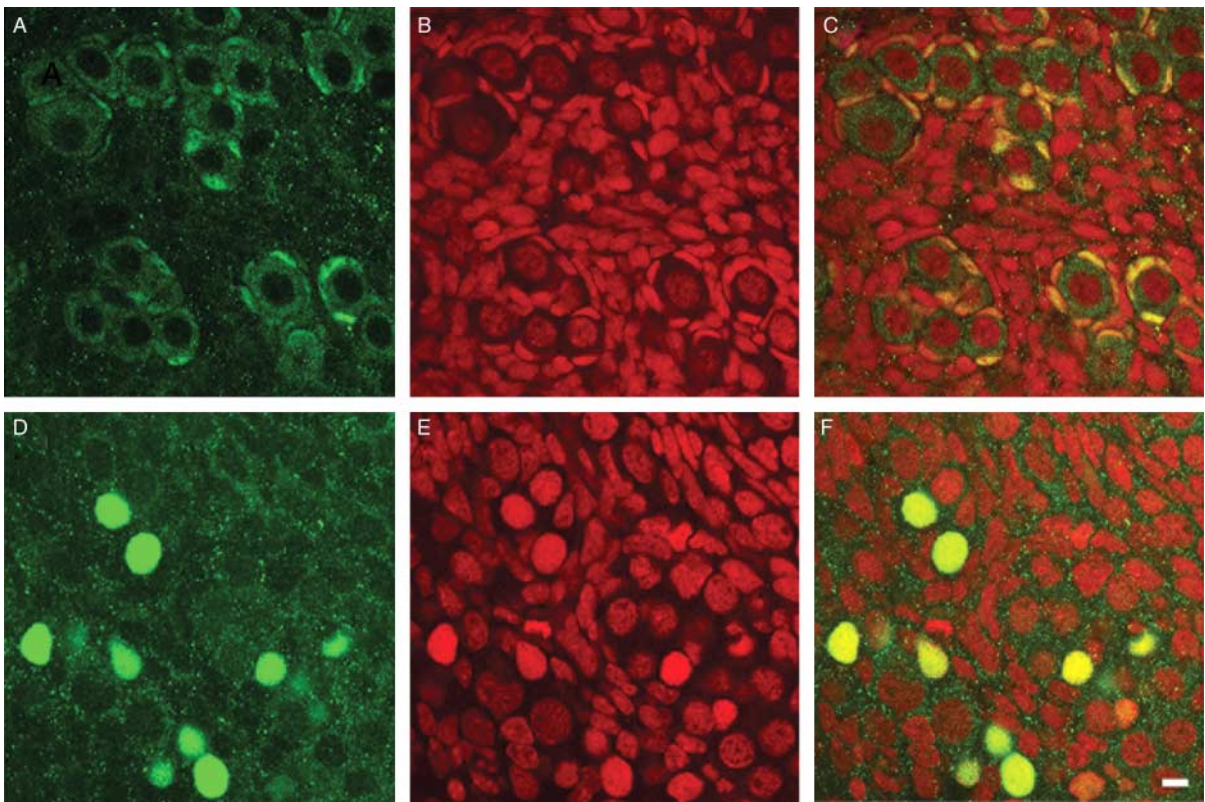


Figure 2 Expression of estrogen receptor proteins in whole-neonatal mouse ovaries. (A–C) Confocal section of a PND 7 mouse ovary labeled with an ER α antibody (A), propidium iodide (B), and overlay (C). (D–F) Confocal section of a PND 1 mouse ovary labeled with an ER β antibody (D), propidium iodide (E), and overlay (F). Estrogen receptor antibodies (green) and propidium iodide to visualize nuclei (red). Scale bar = 10 μ m. Full colour version of this figure available via <http://dx.doi.org/10.1677/JOE-09-0109>.

that the effects of E₂ on the ovary would also be mediated through ER β . DPN is an ER β -specific agonist, displaying 70-fold higher binding affinity for ER β than ER α (Meyers *et al.* 2001). DPN is 170 times more potent as a transcriptional activator through ER β than ER α . A dose–response curve was determined for DPN by examining the ability of DPN to stimulate gene expression from a promoter with an estrogen response element (ERE) and a 50% response using 10^{−9} M was obtained with ER β (Harrington *et al.* 2003). To test for the involvement of ER β in the regulation of cyst breakdown, ovaries were treated for 7 days starting at PND 1 with 10^{−8} M DPN. The ovaries were analyzed for cyst breakdown and primordial follicle formation by determining the number of single oocytes relative to the number of oocytes in cysts. During this phase of ovarian development, cysts break apart and oocytes become enclosed by granulosa cells, forming primordial follicles. Although cyst breakdown and primordial follicle formation may be distinct events, currently we have no way to separate the two processes because they occur concurrently. Therefore, the percent of single oocytes is a measure for both these processes. DPN-treated ovaries had fewer single oocytes compared with controls (only 58% compared with almost 90%, Fig. 3A). Large cysts were still

present in the agonist-treated ovaries but not in control ovaries after 7 days in culture (Fig. 3D and E).

During normal cyst breakdown, approximately two-thirds of the oocytes die. Previously, we showed that *in vivo* genistein treatment inhibited this death (Jefferson *et al.* 2006). However, E₂ treatment *in vivo* or in ovary organ culture did not affect oocyte death (Chen *et al.* 2007). Here, oocyte number was also analyzed, and no significant difference was found between the control and DPN-treated ovaries (Fig. 3B). We also examined the follicle development. Normally, in the neonate, some primordial follicles are activated and begin to develop immediately. This first group of developing follicles is sometimes referred to as the first wave of developing follicles. We assessed follicle activation and development, and found that DPN did not affect primordial follicle activation or subsequent development (Fig. 3C).

The effect of PPT, an ER α -selective agonist (Kraichely *et al.* 2000, Stauffer *et al.* 2000), was also tested. PPT displays 410-fold higher binding affinity for ER α than ER β . Similar to E₂, PPT is a potent agonist acting through ER α in transactivation assays performed in human endometrial cancer (HEC) cells. A 50% transactivation response was obtained with 10^{−9} M PPT and an ERE containing

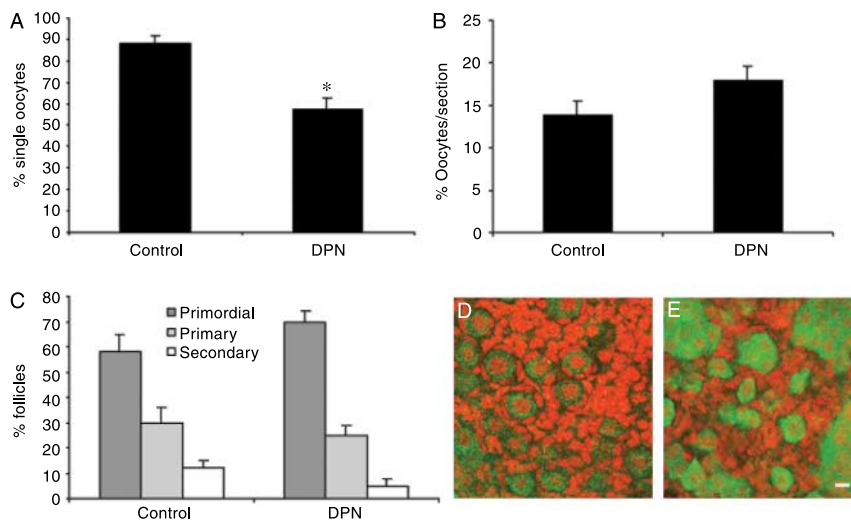


Figure 3 The ER β -specific agonist, DPN, inhibits cyst breakdown and primordial follicle assembly in organ culture. Percent single oocytes (A), number of oocytes per confocal section (B), and percent primordial, primary, and secondary follicles relative to the total number of follicles (C) in newborn ovaries after 7 days of culture in control ovaries and ovaries treated with 10^{-8} M DPN. Data are presented as the mean \pm s.e.m. *Indicates a significant difference between control and treated ovaries (ANOVA, $P < 0.05$). $n = 3-5$ ovaries per group. Confocal section of a control ovary (D) and an ovary cultured in 10^{-8} M DPN (E) grown in organ culture for 7 days labeled with STAT3 antibody to visualize oocytes (green) and propidium iodide to visualize nuclei (red). Scale bar = 10 μ m. Full colour version of this figure available via <http://dx.doi.org/10.1677/JOE-09-0109>.

promoter using ER α (Harrington *et al.* 2003). PPT is completely inactive in transactivation assays through ER β even at very high concentrations. To test the effects of PPT, ovaries were collected from cluster of differentiation-1 mice on PND1 and cultured for 7 days. Ovaries were treated daily with 10^{-8} M PPT. At the end of culture, ovaries were analyzed for cyst breakdown, oocyte survival, and follicle development by confocal microscopy following whole-mount immunocytochemistry. Unexpectedly, after 7 days in culture, control ovaries had nearly 90% single oocytes, while the PPT-treated ovaries had significantly fewer single oocytes (68%, Fig. 4A), showing that PPT inhibited cyst breakdown. Representative confocal images in Fig. 4D and E illustrate the effects of PPT on cyst breakdown. Although there was a slight increase in oocyte number in the PPT-treated ovaries (Fig. 4B), the difference was not significant. Follicle activation and development were not affected by PPT (Fig. 4C). Since DPN is an ER β -specific agonist and PPT is an ER α -specific agonist, and both PPT and DPN inhibit cyst breakdown, we conclude that E $_2$ can signal through either ER α or ER β to inhibit oocyte cyst breakdown and primordial follicle assembly in the neonatal mouse ovary.

ER antagonists can block the effects of estrogen

ICI 182,780 is an estrogen antagonist that can bind to both ER α and ER β with very high affinity and is able to completely antagonize the effects of estrogen through both

receptors (Wakeling *et al.* 1991). ICI 182,780 displays no agonist activity on estrogen target tissues. We used ICI 182,780 to block E $_2$ signaling through ER α and ER β in developing mouse ovaries. Ovaries were collected from cluster of differentiation-1 mice on PND 1 and cultured for 7 days. Ovaries were treated daily with DMSO as control, with 10^{-7} M E $_2$ alone, with 10^{-7} M E $_2$ and 10^{-6} M ICI 182,780, or with 10^{-7} M E $_2$ and 10^{-5} M ICI 182,780. At the end of culture, ovaries were stained and analyzed for cyst breakdown.

As expected, E $_2$ alone inhibited cyst breakdown, causing large cysts to persist and appearance of significantly fewer single oocytes (only 54% compared with 79% in control, Fig. 5A). ICI 182,780 alone had no effect on cyst breakdown, because single oocyte remained the same as the control (Fig. 5A). When E $_2$ and ICI 182,780 were added to the ovary in combination, inhibition of E $_2$ on cyst breakdown was blocked by ICI 182,780 in a dosage-dependent manner (Fig. 5A). When ICI 182,780 was present at tenfold in the concentration of E $_2$, the percent of single oocytes increased slightly to 62% and was further improved to 69% when ICI 182,780 was present at 100-fold. Thus, blocking ER α and ER β resulted in inhibition of E $_2$ action on cyst breakdown.

ER-specific antagonists were used to further assess the role of each receptor in the ovary. First, to specifically inhibit ER α , methyl-piperidino-pyrazole (MPP) was used as an ER α -selective antagonist (Sun *et al.* 2002). MPP has a 200-fold higher binding affinity for ER α over ER β , but does not

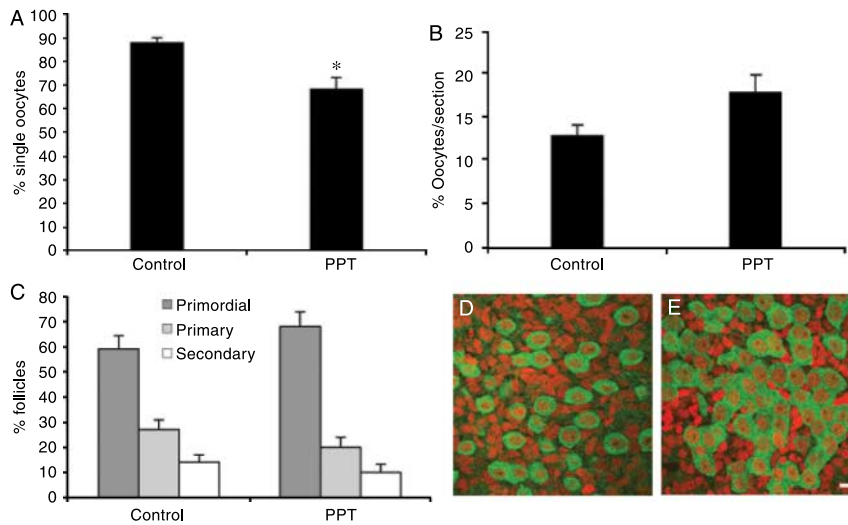


Figure 4 The ER α -specific agonist, PPT, inhibits cyst breakdown and primordial follicle assembly in organ culture. Percent single oocytes (A), number of oocytes per confocal section (B), and percent primordial, primary, and secondary follicles (C) in newborn ovaries after 7 days of culture in control ovaries and ovaries treated with 10^{-8} M PPT. Data are presented as the mean \pm s.e.m. *Indicates a significant difference between control and treated ovaries (ANOVA, $P < 0.05$). $n = 6$ ovaries per group. Confocal section of a control ovary (D) and an ovary cultured in 10^{-8} M PPT (E) grown in organ culture for 7 days labeled with STAT3 antibody to visualize oocytes (green) and propidium iodide to visualize nuclei (red). Scale bar = 10 μ m. Full colour version of this figure available via <http://dx.doi.org/10.1677/JOE-09-0109>.

activate transcription of reporter constructs in HEC cells through either receptor. In cotransfection experiments, MPP antagonizes the action of E₂ through ER α but not ER β . PND 1 ovaries from cluster of differentiation-1 mice were cultured for 7 days. Ovaries were treated daily with 10^{-7} M E₂ alone, or 10^{-7} M E₂ and 10^{-6} M MPP or 10^{-7} M E₂ and 10^{-5} M MPP. At the end of culture, ovaries were analyzed for cyst breakdown.

Consistent with our previous results, E₂ alone inhibited cyst breakdown, as shown by persistence of large cysts and fewer single oocytes (only 38% compared with 87% in the control, Fig. 5B). When MPP was added, no significant effect was observed when present at a tenfold higher concentration than E₂ (Fig. 5B). However, when MPP concentration was 100-fold more than E₂, the inhibition of E₂ on cyst breakdown was blocked resulting in fewer large cysts, similar to control ovaries, and an increase in single oocyte percent to 76% (Fig. 5B). The ER α -selective antagonist, MMP, was able to block the effect of E₂ on cyst breakdown in the neonatal ovary.

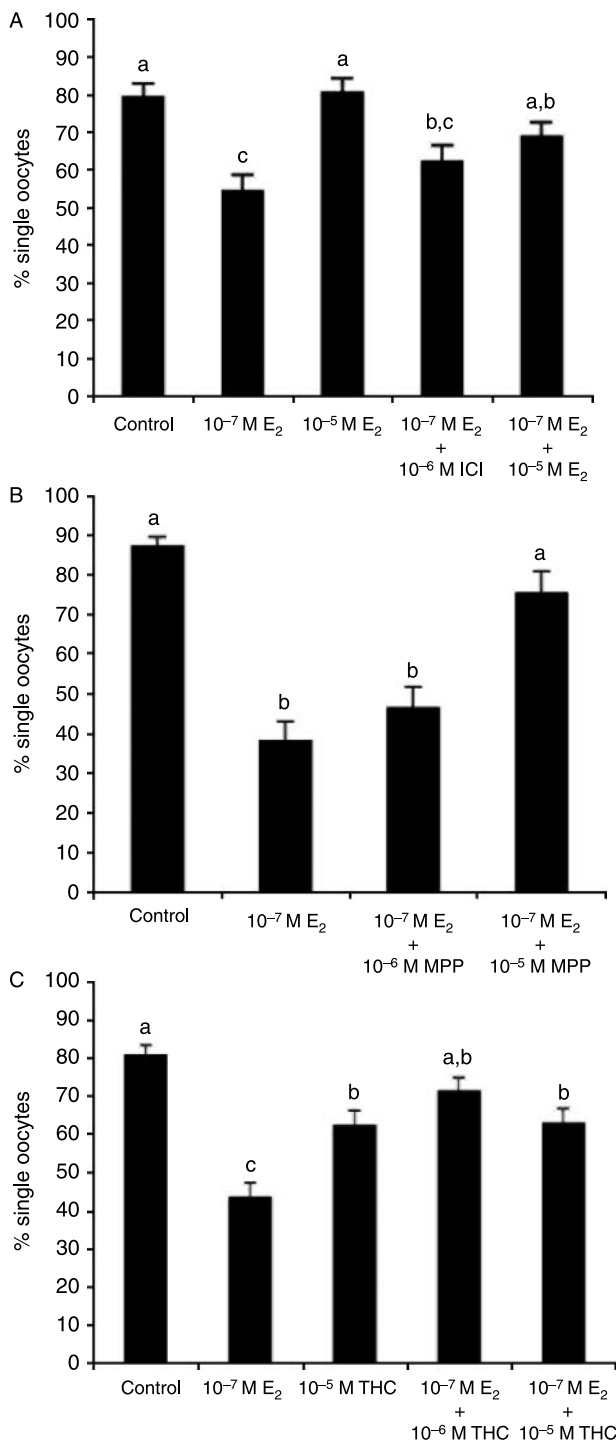
R,R-tetrahydrochrysenone (R,R-THC) is an ER β -selective antagonist, which retains partial ER α -binding affinity (Sun *et al.* 1999). R,R-THC is able to bind to both ER α and ER β , but has a sevenfold higher binding affinity for ER β (Sun *et al.* 1999). In transactivation assays, R,R-THC was able to fully suppress E₂ stimulation of ER β but not ER α (Sun *et al.* 1999). In addition to its ability to antagonize ER β , R,R-THC was able to act as an agonist through ER α , but had no effect through ER β (Sun *et al.* 1999). Similar to our previous

experiments, we found that 10^{-7} M E₂ inhibited cyst breakdown and primordial follicle formation, causing large cysts to persist in the cultured ovaries, and single oocytes to drop to 44% compared with 81% in the control ovaries (Fig. 5C). Since R,R-THC can act as an agonist through ER α , it is not surprising that we found R,R-THC alone inhibited cyst breakdown similar to estrogen, although to a lesser extent, with 62% single oocytes (THC) compared with 44% (E₂) (Fig. 5C). When ovaries were treated with both E₂ and R,R-THC, the effect of E₂ on cyst breakdown was partially suppressed. More large cysts were observed in the ovaries and single oocyte percentage was higher (71% and 63% single oocytes) though not completely restored to control levels (80% single oocytes; Fig. 5C). Thus, R,R-THC acted as an antagonist of the effects of E₂ on cyst breakdown through ER β .

Estrogen can signal through the membrane to regulate oocyte cyst breakdown

We wondered whether nonclassical estrogen signaling could be involved in neonatal oocyte development. In order to test whether an mER could be involved in the cyst breakdown process, we utilized BSA conjugated to E₂ (E₂-BSA). The size of BSA renders E₂ unable to pass through the cell membrane and bind to ERs located in the nucleus. BSA conjugated to E₂ has been widely used to test for E₂ signaling through membrane receptors (Berthois *et al.* 1986). Ovaries were collected from cluster of differentiation-1 mice on PND 1 and

cultured for 7 days. Ovaries were treated daily with DMSO as a control, 10^{-6} M BSA, or with 10^{-6} M E_2 -BSA. At the end of culture, ovaries were harvested and analyzed for cyst breakdown, oocyte survival, and follicle development by whole-mount immunocytochemistry and confocal microscopy.



As illustrated in Fig. 6, BSA alone had no significant effect on cyst breakdown or primordial follicle assembly, because the percent of single oocytes remained the same (84% compared with 89% in control ovaries, Fig. 6A). E_2 -BSA significantly inhibited cyst breakdown and primordial follicle assembly to an even greater extent than E_2 alone. Treated ovaries exhibited large cysts (Fig. 6F) and decreased numbers of single oocytes (only 39% single oocytes compared with 89% in the control, Fig. 6A), while in control or BSA-treated ovaries most oocytes were single (Fig. 6D and E). The number of primordial and primary follicles relative to the total number of follicles was similar in control, BSA-treated, and E_2 -BSA-treated ovaries but there were fewer secondary follicles in the E_2 -BSA-treated ovaries (Fig. 6C). Neither BSA alone nor E_2 -BSA had any significant effect on oocyte number (Fig. 6B). This suggests the involvement of a mER in the process of cyst breakdown and primordial follicle assembly.

Discussion

The establishment of the primordial follicle pool is important for female fertility. In turn, cyst breakdown is a prerequisite for primordial follicle assembly. In our previous research, we have shown that neonatal treatment with genistein, E_2 , or progesterone inhibits cyst breakdown and primordial follicle formation (Chen *et al.* 2007). Our working model is that maternal estrogen inhibits cyst breakdown in the developing fetus before birth. After birth, estrogen levels in the neonate drop dramatically, allowing cysts to break down and individual oocytes to become enclosed in primordial follicles. Here, we investigated the mechanism of estrogen signaling using receptor-specific agonists and antagonists. According to our data, estrogen can signal through $ER\alpha$ or $ER\beta$. Both $ER\alpha$ and $ER\beta$ are expressed in neonatal ovaries supporting the idea that they play an important role in cyst breakdown.

We found $ER\alpha$ in granulosa cells at PND1, 4, and 7 and $ER\beta$ in oocytes at 18-5 dpc and PND1. Our results differ from a previous study where $ER\alpha$ was found in interstitial cells at PND 1, 5, 12, 19, and 26, and $ER\beta$ was found in

Figure 5 Estrogen receptor antagonists blocked the effects of E_2 on cyst breakdown in organ culture. (A) Percent single oocytes in newborn ovaries after 7 days of culture in control ovaries and ovaries treated with 10^{-7} M E_2 , 10^{-5} M ICI 182,780, 10^{-7} M E_2 and 10^{-6} M ICI 182,780, or 10^{-7} M E_2 and 10^{-5} M ICI 182,780. $n=6-8$ ovaries per group. (B) Percent single oocytes in newborn ovaries after 7 days of culture in control ovaries and ovaries treated with 10^{-7} M E_2 , 10^{-7} M E_2 and 10^{-6} M MPP, or 10^{-7} M E_2 and 10^{-5} M MPP. $n=4-5$ ovaries per group. (C) Percent single oocytes in newborn ovaries after 7 days of culture in control ovaries and ovaries treated with 10^{-7} M E_2 , 10^{-5} M R,R-THC, 10^{-7} M E_2 and 10^{-6} M R,R-THC, or 10^{-7} M E_2 and 10^{-5} M R,R-THC. $n=6-9$ ovaries per group. Data are presented as the mean \pm s.e.m. Different letters indicate a significant difference between groups (one-way ANOVA, $P<0.05$).

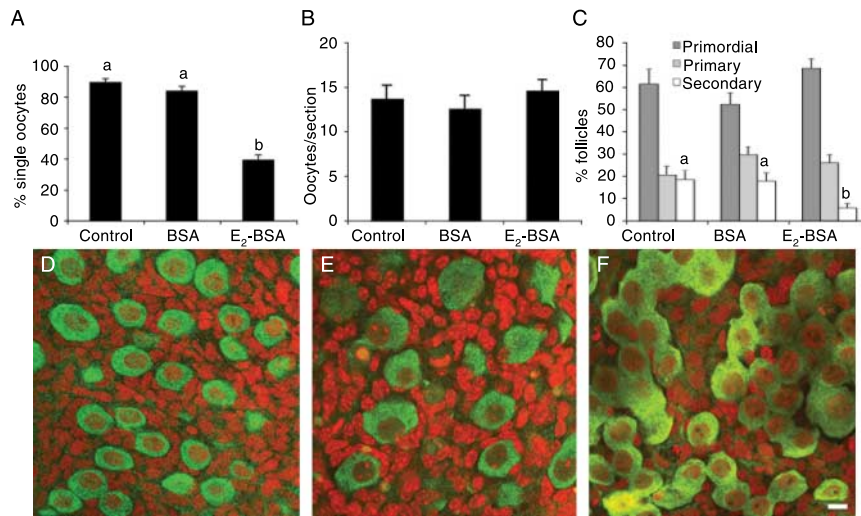


Figure 6 E₂-BSA inhibits cyst breakdown and follicle development in organ culture. Percent single oocytes (A), number of oocytes per confocal section (B), and percent primordial, primary, and secondary follicles (C) in newborn ovaries after 7 days of culture in control ovaries and ovaries treated with 10⁻⁶ M BSA or 10⁻⁶ M E₂-BSA. Data are presented as the mean ± s.e.m. Different letters indicate a significant difference between groups (ANOVA, *P* < 0.05). *n* = 5–10 ovaries per group. Confocal section of a control ovary (D), an ovary cultured in 10⁻⁶ M BSA (E), and an ovary cultured in 10⁻⁶ M E₂-BSA (F) grown in organ culture for 7 days labeled with STAT3 antibody to visualize oocytes (green) and propidium iodide to visualize nuclei (red). Scale bar = 10 μm. Full colour version of this figure available via <http://dx.doi.org/10.1677/JOE-09-0109>.

granulosa cells at PND 5, 12, 19, and 26 (Jefferson *et al.* 2000). There are several isoforms of each ER and it is possible that each study is detecting different isoforms. Supporting this idea, each study used different antibodies generated against different epitopes of the receptors. For ERβ, our studies here found the protein only in some oocytes and only at 18.5 dpc and PND 1 and may not have been detected previously.

Previous research has implicated ERβ in the process of cyst breakdown. If mice are exposed neonatally to genistein, an estrogenic compound from soy plants, MOFs are induced in the adult ovary. However, genistein failed to induce MOFs in *Erβ* knockout mice (Jefferson *et al.* 2002). In the studies described here, using ER-specific agonists, we found that estrogen signaling through both ERα and ERβ can affect ovarian differentiation. This discrepancy can be explained by the preferential binding affinity of genistein. Genistein binds to ERβ much more strongly than ERα and is a more potent activator of ERβ (Morito *et al.* 2001).

Analysis of ER knockout mice has shed some light on the roles of ERα and ERβ in the ovary. Single- and double-knockout mice appear to have normal early oocyte development. Although some normal primordial follicles and growing follicles can be seen in the adult ovary, they have problems in later follicle development or ovulation (Lubahn *et al.* 1993, Krege *et al.* 1998, Couse *et al.* 1999). No defects in neonatal ovary development have been described in mutants of either nuclear ER. However, cyst breakdown and

primordial follicle assembly have not been characterized in the knockout mice.

Using BSA-conjugated E₂, we also detected the involvement of a mER in the process of cyst breakdown. It is possible that there could be a small amount of free E₂ in the E₂-BSA preparation, but it is unlikely that it is present in a high enough concentration to affect our results. Several cell lines have been shown to possess high-affinity E₂-binding sites on their plasma membranes (Berthois *et al.* 1986). The identity of the mER is still under debate (reviewed in Warner & Gustafsson 2006). It has been suggested that ERα can translocate to the cell membrane and function to activate the nongenomic pathway (Razandi *et al.* 2003). On the other hand, there is also evidence that GPR30, a G-protein-coupled receptor, is the novel functional mER (Revankar *et al.* 2005, Filardo *et al.* 2007). However, GPR30 mutants have recently been generated and are fertile with no reproductive abnormalities detected (Otto *et al.* 2009).

Oocyte death accompanies cyst breakdown. Only one-third of all the oocytes in cysts will be enclosed in primordial follicles with the others undergoing apoptosis. Although these two processes are closely related, the exact relationship between them is unclear. Recent evidence has suggested that oocyte death is required for cyst breakdown because mutants without Bax protein, an apoptosis regulator, have more oocytes and cyst breakdown is delayed (Greenfeld *et al.* 2007). In addition, *in vivo* genistein treatment of neonates not only

inhibits cyst breakdown, but also the results in fewer oocytes dying than untreated neonates again linking the processes of cyst breakdown and oocyte death (Jefferson *et al.* 2006). However, we have recently found that neonatal estrogen treatment, *in vitro* or *in vivo*, or genistein treatment *in vitro* had no effect on oocyte death. The difference of effects on cell death depending on the mode of delivery may reflect differences in the mechanism of action of genistein depending on how the ovary receives it. However, premature oocyte death that can be triggered by prenatal *in vitro* culture is rescued by the addition of estrogen (Chen *et al.* 2007). It may be that cell death is triggered when the mice are born and are no longer exposed to maternal estrogen, and the addition of estrogen after birth is not able to reverse the cell death unlike its action with cyst breakdown. Here, similar to E₂, neither ER-specific agonist had an effect on oocyte number. This illustrates the underlying complexity of the estrogen-signaling pathway. The gene encoding the BCL2 protein, important in the apoptosis pathway, possesses an ERE in its promoter region (Klinge 2001). Therefore, the BCL2 protein could be the potential link between the estrogen pathway and the apoptosis pathway. One simple model is that estrogen signaling only regulates the apoptosis pathway before birth. Supporting this hypothesis, as mentioned above, estrogen can rescue the premature oocyte loss in the prenatal culture. In fetal ovaries, estrogens inhibit apoptosis and maintain the integrity of cysts. The dramatic drop of estrogen levels at birth triggers the oocyte death process and induces cyst breakdown. After birth, the link between estrogen-signaling pathway and apoptosis is turned off and estrogen can no longer affect the cell death process. This could be achieved by substituting ER co-activators with co-repressors or by changing the phosphorylation status of apoptosis target proteins so that they become inactive.

Several possible downstream targets of estrogen signaling have been suggested. Activin was originally isolated based on its ability to stimulate the synthesis and secretion of FSH (de Kretser & Robertson 1989). Recently, it has been shown that activin promotes the establishment of the primordial follicle pool because neonatal activin treatment significantly increases the number of primordial follicles (Bristol-Gould *et al.* 2006). Activin expression and downstream signaling are suppressed in neonatal ovaries exposed to estrogen (Kipp *et al.* 2007). It is possible that estrogen inhibits cyst breakdown by suppressing activin activity before birth.

Both the ER α - and ER β -specific agonists were able to inhibit cyst breakdown, suggesting that estrogen could signal through either receptor to affect cyst breakdown. We expected that when ovaries were exposed to a receptor-specific antagonist in combination with E₂, E₂ would still have an effect on cyst breakdown by functioning through the other receptor. Instead, we observed a complete block of E₂ on cyst breakdown inhibition by the ER α antagonist and a partial block by the ER β antagonist. One possibility is that ER is functioning as a heterodimer of ER α and ER β . There is evidence that ER α and ER β can act as heterodimers in some

tissues (Cowley *et al.* 1997, Pace *et al.* 1997), but it is unclear whether the ERs are functioning as heterodimers in the neonatal ovary. However, this possibility is unlikely as ER α and ER β are expressed in different cell types.

More work needs to be done to fully understand the mechanisms behind cyst breakdown and oocyte death. Elucidation of how estrogen normally regulates these processes and how neonatal exposure to excess environmental estrogenic compounds disrupts them will give us insight into female infertility and possible treatments.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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