

Diethylstilbestrol Alters the Expression of Activins in the Neonatal Mouse Ovary *In Vitro*

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Abstract. *Background/Aim:* Perinatal diethylstilbestrol (DES) treatment induces the polyovular follicle containing two or more oocytes in a follicle of mouse ovary through estrogen receptor (ER) β . The aim of the study was to investigate the direct effects of DES on the neonatal mouse ovary and the gene expression of activins. *Materials and Methods:* Ovaries from neonatal wild-type (WT) or ER β -knockout (ER β KO) mice were organ-cultured in a serum-free medium with or without DES, and polyovular follicle induction and expression of activin signaling related genes were examined. *Results:* The polyovular follicle and cyst incidence in DES-treated organ-cultured ovaries from WT mice, but not from ER β KO mice, was significantly higher than that of control non-treated cultures. DES altered inhibin (Inh) α , Inh β and Inhbb expression in organ-cultured ovaries from C57BL/6J mice, while no change in Inh α and an increase of Inhbb were observed by DES, in both WT and ER β KO mice. *Conclusion:* Alterations in activin signaling are involved in the polyovular follicle induction by DES.

The synthetic estrogen, diethylstilbestrol (DES), had been prescribed for pregnant women from the 1940s to 1970s to prevent miscarriage, however, daughters born from DES-exposed mothers developed vaginal clear cell carcinoma and other reproductive abnormalities (1). In mice, neonatal exposure to DES causes various abnormalities in female reproductive organs, skeletal tissue and muscle (2-4). In the ovary, several morphological changes are detected, including the absence of corpora lutea, hypertrophy of interstitial tissue, and the appearance of hemorrhagic cysts (5-7). The

polyovular follicle, which contains two or more oocytes per follicle, was also induced in the ovaries of mice perinatally exposed to DES (8). Ovaries of newborn mice organ-cultured with serum-free medium containing 1 μ g/ml DES for 5 days and then transplanted into ovariectomized host mice have polyovular follicles (9, 10). These data indicate that DES acts directly on the mouse ovary to induce polyovular follicles. Actions of estrogen are mediated by estrogen receptors (ERs), ER α and ER β . DES can bind to both ER α and ER β , and its affinity is higher than 17 β -estradiol (E2) (11). Studies in ER α knockout (ER α KO) or ER β knockout (ER β KO) mice, have revealed that DES induces polyovular follicles through ER β (12).

The critical time for polyovular follicle induction by DES is postnatal day 3 (13), suggesting that DES can affect ovarian folliculogenesis during the neonatal period and induce polyovular follicles. In rodents, folliculogenesis begins soon after birth. Follicles are not yet formed in the prenatal mouse ovary, which consists only of germ cell cysts and somatic cells (14). Germ cell cysts are cell clusters formed by proliferation of female germ cells (14). After birth, cyst breakdown, involving a series of events such as apoptosis of oocytes, remodeling of the basal membrane and infolding of pre-granulosa cells occurs, and approximately two thirds of oocytes are lost by apoptosis (15). Oocytes in germ cell cysts undergo cell death, and somatic cells invade into the cysts, and oocytes are subsequently surrounded by an adequate number of pre-granulosa cells for initiation of the primordial follicle formation. Primordial follicles develop into primary follicles, which consist of an oocyte surrounded by cuboidal granulosa cells. Neonatal DES exposure significantly decreases oocyte apoptosis in ovaries of 2-day-old wild-type (WT) and C57BL/6J mice but not in the ovaries of ER β KO mice (6), indicating that DES suppresses oocyte apoptosis *via* ER β and results in polyovular follicle induction. However, the effects of DES on ER β KO mouse ovaries *in vitro* are not clear.

Activin, a member of the TGF β superfamily, and its functional antagonist inhibin were originally isolated from gonadal sources, based on their ability to stimulate (activin) or

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Table I. Sequences of oligonucleotides used as primers for real-time RT-PCR

Gene	Forward sequence (5' → 3')	Reverse sequence (3' → 5')
<i>Inha</i>	CTGCTCTCAATATCTCCTTCCAAGAG	CCATGGCAGTAGTGGAAGATGATGAA
<i>Inhba</i>	ATCATCACCTTTGCCGAGTCA	TTCTGCACGCTCCACTACTGA
<i>Inhbb</i>	CCATCCAGGCCTTGTTTGA	AGCTGTCACTGCACATCCA
<i>Fst</i>	TCTTCTGGCGTGCTTCTTGAA	TCCGAGATGGAGTTGCAAGAT
<i>Ppia</i>	AGGTCCTGGCATCTTGTCAT	CCATCCAGCCATTCACTCTTG

suppress (inhibin) the synthesis and secretion of follicle-stimulating hormone (FSH) (16-18). Activin consists of a dimer of two β -subunits, βA or βB , to form activin A ($\beta A\beta A$), activin B ($\beta B\beta B$), or activin AB ($\beta A\beta B$), whereas inhibin is a heterodimer of a unique inhibin α ($\text{inh}\alpha$) with either of the two β -subunits to form inhibin A ($\alpha\beta A$) or inhibin B ($\alpha\beta B$) (19). Activin binding to its type II receptor leads to recruitment and the subsequent phosphorylation of its type I receptor (20). Inhibin suppresses activin signaling by binding to activin type II receptors *via* its β -subunits without stimulation of type I receptor phosphorylation (21). Betaglycan (the type III TGF β receptor) binds to inhibin to facilitate antagonism of activin (22). During folliculogenesis, activin subunits and receptors are expressed in the germ cells and somatic cells of the neonatal mouse ovary (23). In addition, administration of recombinant human activin A increases the number of primordial follicles in neonatal mouse ovary (23), indicating that activin facilitates follicle formation during the neonatal period. DES suppresses activin signals and delays follicle formation and follicle development *in vivo* (6, 24), therefore, DES may affect folliculogenesis through activin signaling in the neonatal mouse ovary. DES also increases the expression of *Inha* in the neonatal ovary (6), and mice over expressing the *Inha* gene show polyovular follicles (25), suggesting that an increase of *Inha* may be involved in polyovular follicle induction. However, whether DES can increase *Inha* through ER β in the neonatal mouse ovary has not yet been elucidated.

This study aimed to examine the effects of DES on polyovular follicle induction through ER β in the neonatal ovary *in vitro*. Ovaries from 0-day-old WT or ER β KO mice were organ-cultured on collagen gels for 5 days with or without DES, and histological changes and the expression of activin-related genes were subsequently examined.

Materials and Methods

Animals. Wild-type (WT) and ER β knockout (ER β KO) mice (26), and C57BL/6J Jcl mice (C57BL/6J, CLEA Japan, Tokyo, Japan) were kept at 23°C with a 12 h light/12 h dark cycle caused by artificial illumination (lights on 0800-2000). They were fed a commercial diet (MF, Oriental Yeast, Tokyo, Japan) and had tap water *ad libitum*. ER β KO mice were obtained from Professor Pierre Chambon at Institut de Génétique et de Biologie Moléculaire et

Cellulaire and they were backcrossed to C57BL/6J mice for more than ten generations. Pup genotypes were determined by multiplex PCR and WT mice were used as a control for ER β KO mice. The day of birth was regarded as day 0 of age. All animals were maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and all experiments were approved by the Institutional Animal Care Committee of the Yokohama City University (No. H-A-16-002).

Organ culture system. An organ culture system for neonatal ovary was performed according to previous reports (9, 10). Eight volumes of Cellmatix type I-A (Nitta Gelatin, Osaka, Japan) were mixed with 1 volume 10x Waymouth MB 752/1 medium (Sigma Chemical, St. Louis, MO, USA) and then 1 volume 0.34 N NaOH was added to the mixture. This cold gelation mixture (300 μ l) was poured into the well of a 4-well plate and allowed to form a gel at 37°C. Ovaries of 0-day-old C57BL/6J, WT and ER β KO mice were dissected and placed on collagen gels. Since ER β KO mice were a mixed C57BL6/129sv background, WT mice were regarded as the control for ER β KO mice. Ovaries were organ-cultured in a humidified incubator at 37°C and 5% CO₂ with a serum-free medium (SF) for 1, 2, 3, or 5 days. For SF, Dulbecco's modified Eagle's medium/ Ham's F-12 medium (Thermo Fisher Scientific, Waltham, MA, USA) containing 100 units of penicillin and 100 μ g/ml streptomycin (Thermo Fisher Scientific) was supplemented with 10 μ g/ml insulin (Sigma), 10 μ g/ml transferrin (Sigma), 10 μ g/ml epidermal growth factor (EGF, Sigma), 10 ng/ml cholera toxin (Sigma), and 5 mg/ml bovine serum albumin fraction V (BSA, Sigma). The medium contained 1 μ g/ml diethylstilbestrol (DES, Sigma) or ethanol alone as a control. One ovary was organ-cultured with SF containing ethanol and the other was organ-cultured with SF containing DES from the same mouse. Ten μ l of medium was added to each ovary every other day or at day 3. Ovaries cultured for 5 days were fixed in Bouin's solution overnight (n=8-22), embedded in paraffin, serially sectioned at 6 μ m and stained with hematoxylin and eosin (HE). The numbers of follicles, cysts, primordial, primary and polyovular follicles per section of each ovary were counted in every 7th section. A polyovular follicle was defined as a follicle which contained more than two oocytes surrounded by granulosa cells within the basement membrane.

Grafting. C57BL/6J mouse ovaries organ-cultured for 5 days with SF containing 1 μ g/ml DES or ethanol, were transplanted under the renal capsule of 2- to 3-month-old C57BL/6J mice. At the time of grafting, all host mice were ovariectomized. Animals were sacrificed 20 days after grafting to harvest the grafted ovaries (n=4-9 host mice). Grafted ovaries were fixed in Bouin's solution overnight, embedded in paraffin, serially sectioned at 8 μ m and stained with HE. The numbers of follicles and polyovular follicles of each ovary were counted on every 13th section.

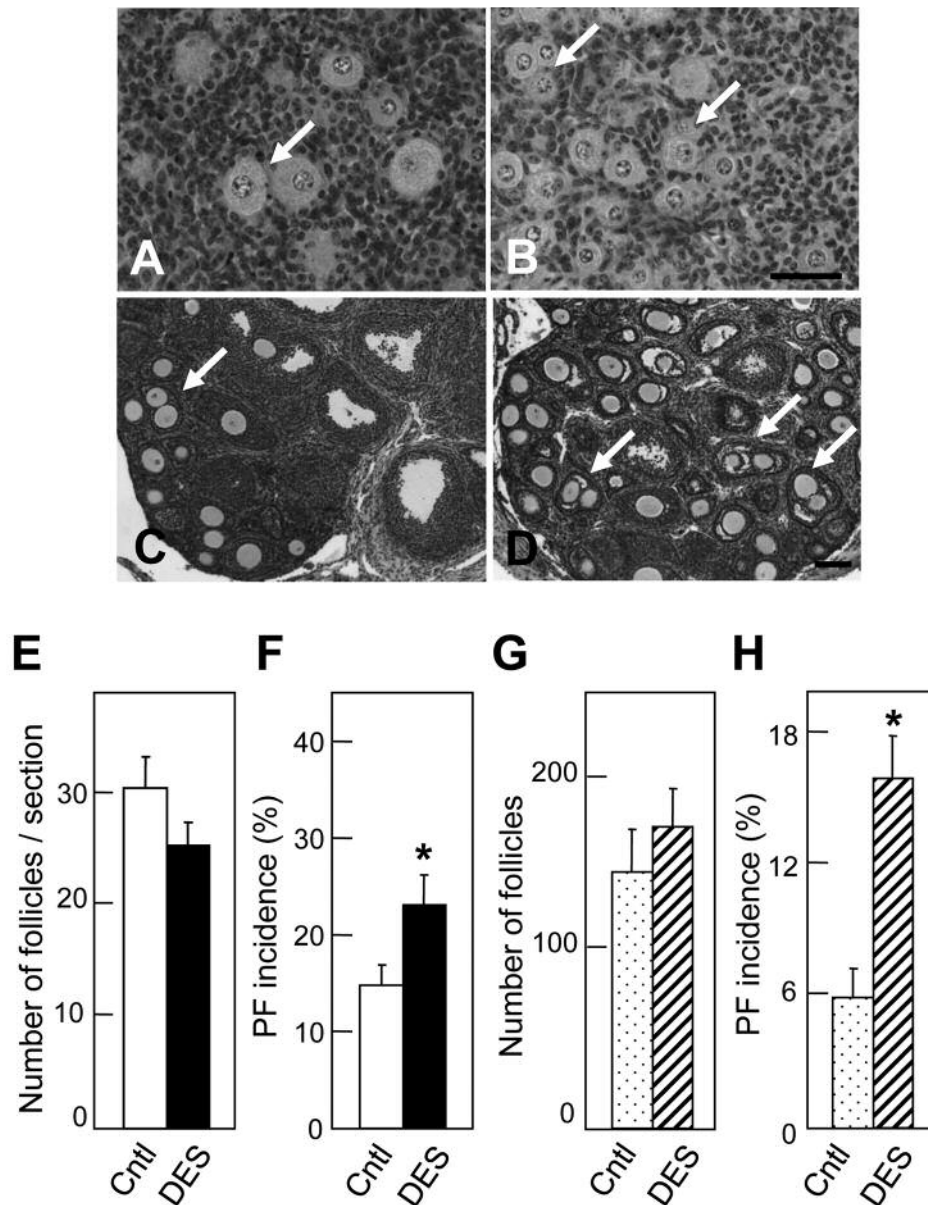


Figure 1. Ovaries organ-cultured with a serum-free medium (SF) containing ethanol (A) or 1 $\mu\text{g/ml}$ DES (B) for 5 days and grafted ovaries grown in vitro with SF containing ethanol (C) or 1 $\mu\text{g/ml}$ DES (D) for 5 days, respectively. Number of follicles per section (E) and polyovular follicle incidence (F) of organ-cultured ovaries with (DES) or without (control, Cntl) 1 $\mu\text{g/ml}$ DES for 5 days. Number of follicles (G) and polyovular follicle incidence (H) of grafted ovaries grown in vitro with (DES) or without (Cntl) 1 $\mu\text{g/ml}$ DES for 5 days. * $p < 0.05$, compared with the Cntl group; White arrows=polyovular follicles; Scale bar in B=100 μm ; Scale bar in D=200 μm .

RNA isolation and real-time RT-PCR. C57BL/6J, WT or ER β KO mouse ovaries organ-cultured for 5 days with or without 1 $\mu\text{g/ml}$ DES were homogenized in TRIzol (Thermo Fisher Scientific) and total RNA was purified with a RNeasy total RNA kit (Qiagen, Hilden, Germany). Total RNA was reverse transcribed into cDNA using Super Script II reverse transcriptase (Thermo Fisher Scientific) with 0.05 mM oligo (dT) primer (Thermo Fisher Scientific). Real-time RT-PCR was carried out using an Applied Biosystems StepOnePlus Real Time PCR System with Fast SYBR

Green Master Mix (Thermo Fisher Scientific). Relative mRNA expression of *Inha*, βA (*Inhba*) subunit, βB (*Inhbb*) subunit and *follistatin* (*Fst*; Table I) was determined by the standard curve method. Peptidylprolyl isomerase A (*Ppia*) was chosen as an internal standard to control for variability in amplification due to differences in the starting mRNA concentrations. Melting-curve analysis showed a single peak for all samples. Organ-cultured ovaries of 10 mice were pooled for each point, and three independent experiments were carried out for each study.

Follicle count and Statistical analysis. The numbers of total follicles, primordial follicles, primary follicles, cysts and polyovular follicles per section of each ovary were counted in every 7th section. The data were analyzed by two-way analysis of Student's t-test after application of Levene's test for comparison of two mean values. For multiple comparisons, differences were estimated using ANOVA followed by Tukey HSD *post-hoc* test. Data were expressed as the mean \pm standard error. $p < 0.05$ was considered to denote a statistically significant difference.

Results

Polyovular follicle induction in the neonatal mouse ovary *in vitro*. Many primordial and primary follicles were observed in organ-cultured ovaries with SF (Figure 1A and B), showing that follicles in ovaries organ-cultured with SF were well developed. The number of follicles per section was not changed by treatment with 1 μ g/ml DES (Figure 1E), but the incidence of polyovular follicles was significantly increased in organ-cultured ovaries (Figure 1B and F).

Follicles at different stages, including secondary follicles and antral follicles, were observed in all grafted ovaries grown *in vitro* for 5 days (Figure 1C and D). The number of total follicles was not changed by treatment with 1 μ g/ml DES (Figure 1G), but polyovular follicles were frequently observed in grafted ovaries grown *in vitro* (Figure 1D). DES treatment significantly increased the polyovular follicle incidence of grafted ovaries grown *in vitro* (Figure 1H).

Effects of DES on organ-cultured ovaries from 0-day-old WT and ER β KO mice. To examine the involvement of ER β in polyovular follicle induction by DES *in vitro*, ovaries from 0-day-old WT and ER β KO mice were organ-cultured in SF with or without 1 μ g/ml DES and histological analysis was performed (Figure 2). Germ cell cysts, primordial, primary and polyovular follicles were found in ovaries of both WT and ER β KO mice organ-cultured in SF regardless of DES treatment (Figure 2). In ER β KO mouse ovaries organ-cultured in SF with or without DES, the numbers of primordial follicles and primary follicles per section were not changed compared with those in WT mouse ovaries (Figure 3A). In WT mouse ovaries organ-cultured in SF with DES treatment, the numbers of follicles and primordial follicles per section were significantly lower than those organ-cultured in SF without DES (Figure 3A). However, the number of primary follicles per section was not changed by DES treatment (Figure 3A). In ER β KO organ-cultured mouse ovaries, the numbers of follicles, primordial follicles and primary follicles per section were not changed by DES treatment (Figure 3A). In WT mouse ovaries organ-cultured in SF with DES, the incidences per section of polyovular follicles+cysts, cysts, and polyovular follicles were significantly increased compared with those in SF without DES treatment (Figure 3B). In contrast, the incidences of

polyovular follicles+cysts, cysts, and polyovular follicles were not changed compared with those in control medium in organ-cultured ER β KO mouse ovaries (Figure 3B).

Changes in mRNA expression of organ-cultured ovaries. In our previous report, neonatal DES exposure increased the percentage of primordial follicles and decreased the percentage of primary follicles in 5-day-old C57BL/6J mice, but not in WT mice (27). Therefore, gene expression was examined in the organ-cultured ovaries of C57BL/6J, WT and ER β KO mice. The mRNA expression of *Inha*, *Inhba* and *Inhbb* subunits and *Fst* was determined by real-time RT-PCR in organ-cultured C57BL/6J, WT and ER β KO mouse ovaries with or without 1 μ g/ml DES treatment. DES significantly increased *Inha* and *Inhbb* expression in organ-cultured ovaries, while the expression of *Inhba* was significantly decreased and *Fst* remained unchanged (Figure 4). In WT mouse organ-cultured ovaries, the expression of *Inha* tended to increase by DES, however, it was not changed in ER β KO mouse organ-cultured ovaries (Figure 4). The expression of *Inhbb* was significantly increased by DES treatment both in WT and ER β KO mouse organ-cultured ovaries (Figure 4). The expression of *Fst* was significantly decreased by DES in WT mouse organ-cultured ovaries, while it was increased in the absence of DES in ER β KO mouse organ-cultured ovaries (Figure 4).

Discussion

Reduced estrogen and progesterone levels at birth may be a primary signal to initiate cyst breakdown (28). Polyovular follicles are induced in the ovaries of mice exposed perinatally to DES (8), therefore, impaired folliculogenesis may result in polyovular follicle induction. In this study, we used an organ culture system to examine the effects of DES on gene expression *via* ER β . Follicles in ovaries organ-cultured with SF were well developed and addition of DES significantly increased the polyovular follicle incidence both in organ-cultured and grafted ovaries that had been cultured *in vitro* (9, 10). Similarly, DES significantly increased the polyovular follicle incidence both in organ-cultured and grafted ovaries in this study.

Our previous report showed that the percentage of primary follicles in the ovaries of neonatally DES-treated WT and ER β KO mice was not changed compared with that of oil-treated control mice (27). In this study, the number of primary follicles per section in WT and ER β KO mouse ovaries organ-cultured in SF was not changed by DES. Thus, DES treatment cannot inhibit the development of follicles from the primordial follicle stage to the primary follicle both *in vivo* and *in vitro* regardless of ER β . The polyovular follicle and cyst incidence per section in WT mouse ovaries organ-cultured in SF with DES was significantly higher than that in control medium, whereas it was not changed in organ-cultured ER β KO mouse

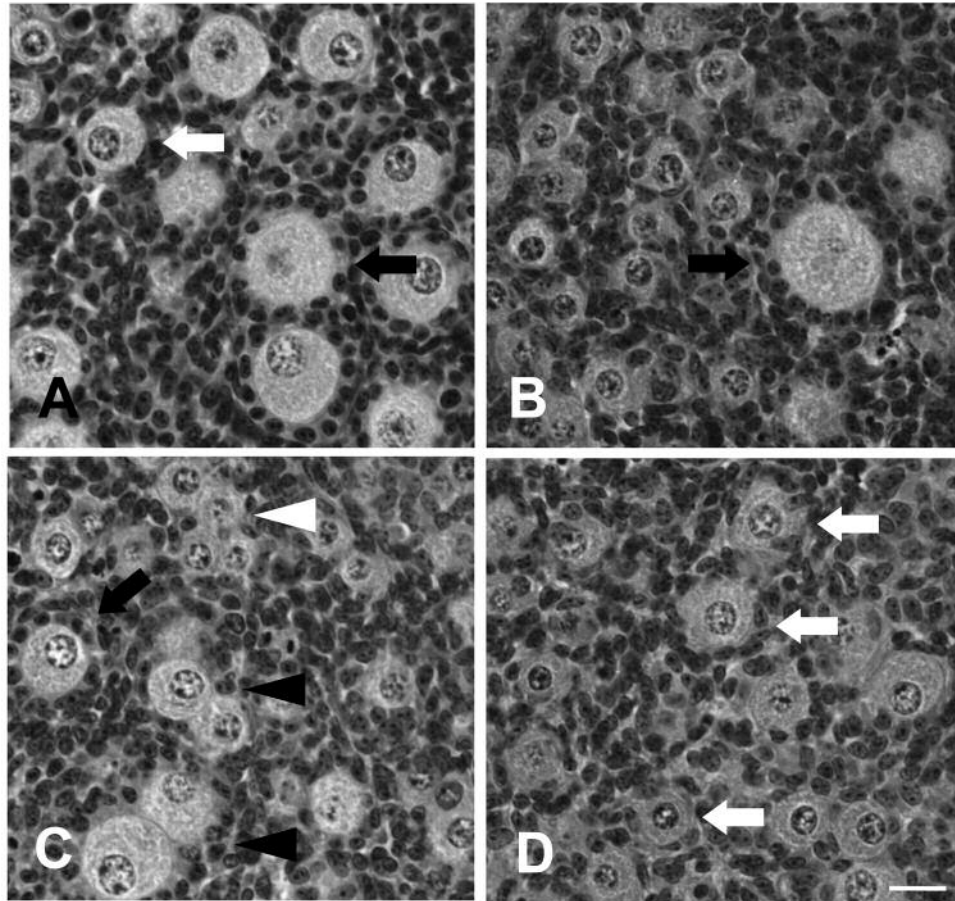


Figure 2. Histology of organ-cultured ovaries from 0-day-old wild-type (WT) (A and C) and $ER\beta$ knockout ($ER\beta$ KO, B and D) mice in SF with 1 μ g/ml DES (C and D) or ethanol alone (A and B) for 5 days. White arrowheads indicate a cyst, black arrowheads indicate polyovular follicles, white arrows indicate primordial follicles and black arrows indicate primary follicles. Scale bar=20 μ m.

ovaries. These results suggest that DES can induce polyovular follicles through ovarian $ER\beta$ directly.

Neonatal DES exposure increases the expression of *Inha* in the ovary (6), however, the roles of inhibin on polyovular follicle induction are not clear. The expression of *Inha* and *Inhbb* was increased whereas the expression of *Inhba* was decreased by DES treatment in organ-cultured ovaries, similar to changes in the ovaries of neonatally DES-treated mice (6). This fact suggests that DES directly alters activin signaling in the ovary. In addition, DES altered *Inha*, *Inhba* and *Inhbb* expression in organ-cultured ovaries of C57BL/6J mice. No change of *Inha* and an increase of *Inhbb* by DES were observed in organ-cultured ovaries of $ER\beta$ KO mice. In WT mouse ovaries, the expression of *Inha* tended to increase by DES, but this increase was not statistically significant. Thus, the expression of *Inha* could be affected by DES through $ER\beta$. Changes in numbers of primordial and primary follicles are induced by DES in C57BL mice at 5 days of age, but not in WT mice (27), suggesting that the response

of WT mouse ovaries to DES is not exactly the same as that of C57BL mouse ovaries. The incidence of polyovular follicles is different among the mouse strains (29), therefore, changes in the gene expression after DES treatment may be different in each mouse strain.

The expression of *Inha* and *Inhba* was relatively high, but *Inhbb* was low *in vitro* as well as *in vivo* (data not shown), indicating that activin A and inhibin A may be dominant in the neonatal ovary. An increase of *Inhbb* can alter the ratio of activin A and B, and inhibin A and B following DES treatment. Since activin A increases the number of primordial follicles in the neonatal mouse ovary (22), it is possible that DES treatment can suppress follicle formation through the inhibition of activin A signaling. In addition, an increase of *Inhbb* by DES treatment in organ-cultured ovaries may also result in an increase of activin B and activin AB. Activin B increases both the expression of *Inha* and *Inhba* *in vitro* in the isolated secondary follicles from the immature mouse ovary, however, activin B does not stimulate follicle growth

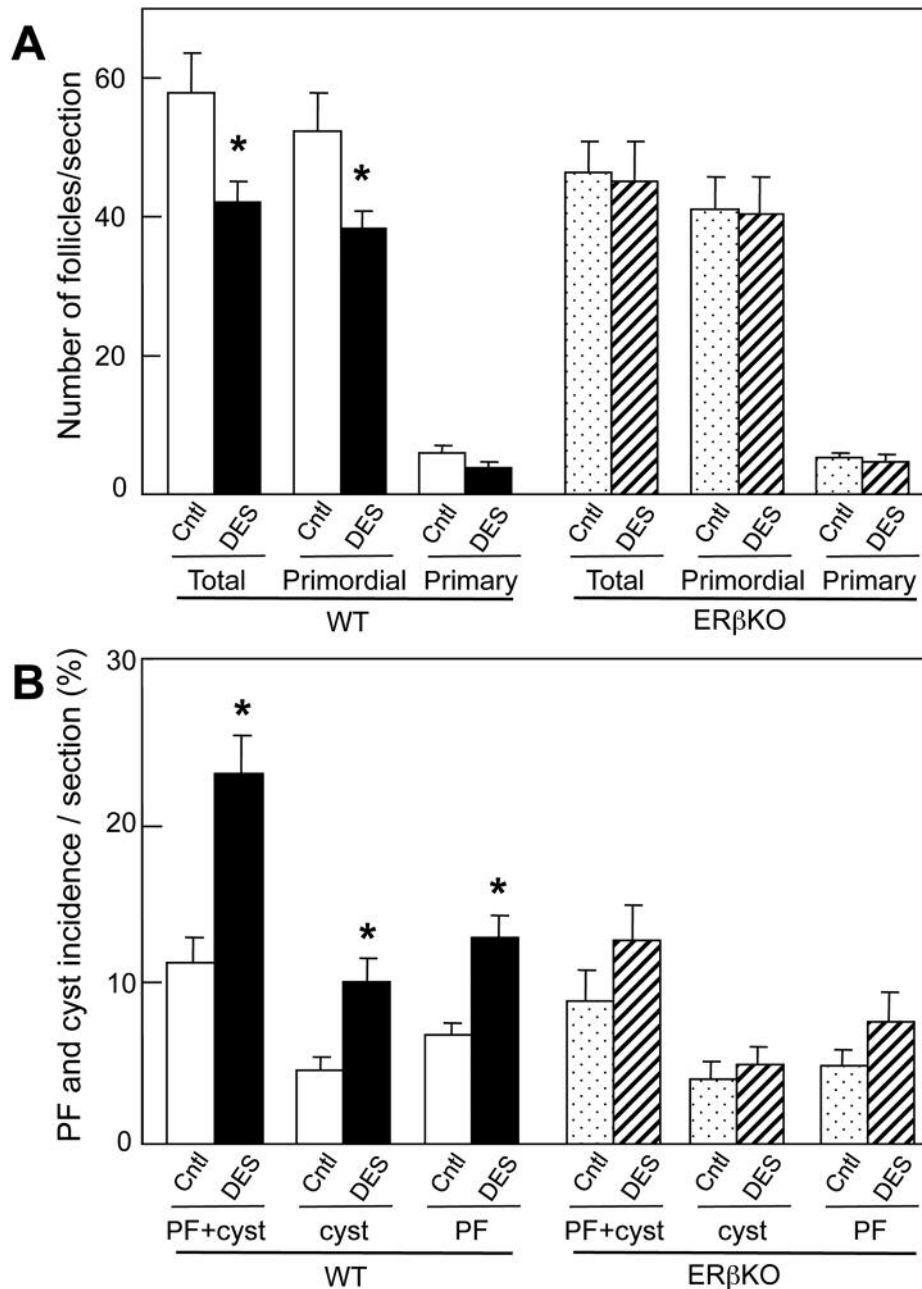


Figure 3. The numbers of total follicles, primordial follicles and primary follicles per section of organ-cultured ovaries from 0-day-old WT and $ER\beta$ KO mice in SF with (DES) or without (Cntl) 1 μ g/ml DES for 5 days (A). The incidence of polyovular follicles+cysts, cysts and polyovular follicles per section of organ-cultured ovaries from 0-day-old WT and $ER\beta$ KO mice in SF with (DES) or without (Cntl) 1 μ g/ml DES for 5 days (B). * $p < 0.05$, compared with Cntl group.

(30). Indeed, the numbers of total and primordial follicles were reduced by DES treatment in the organ-cultured ovary of WT mice, but not in $ER\beta$ KO mouse ovaries. Since the number of follicles in organ-cultured ovaries was less than that in 5-day-old mouse ovaries regardless of DES treatment (6), the effects of DES on follicle numbers may not be

evident. Kipp *et al.* (24) have shown that DES treatment suppresses both *Inhba* and *Inhbb* expression and reduces follicle numbers in ovaries at days 6 and 19. In immature rat granulosa cells, 10 μ M E2 increases the expression of *Inha* and *Inhbb*, whereas *Inhba* is not altered (31), similar to our results. Thus, the effects of estrogens on activin signaling in

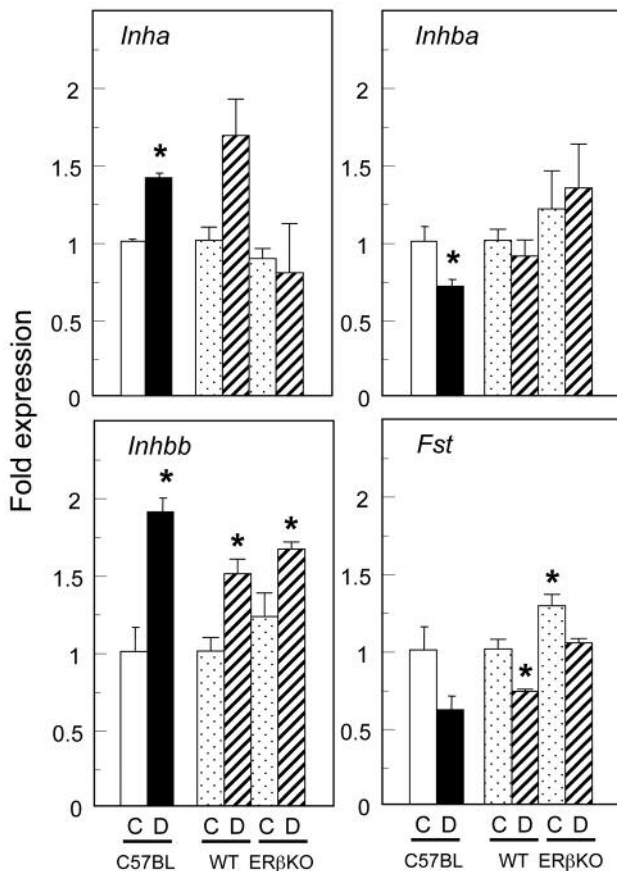


Figure 4. The expression of Inhibin α (*Inha*), *Inhba*, *Inhbb* and follistatin (*Fst*) in organ-cultured ovaries of C57BL/6J (C57BL), WT and ER β KO mice with 1 μ g/ml DES (D) or without DES (C). Peptidylprolyl isomerase A was chosen as an internal standard. Data were expressed relative to mRNA expression of organ-cultured C57BL or WT mouse ovaries in SF for 5 days (=1.0). * $p < 0.05$, compared to C57BL or WT C group.

the ovary may be different by age and estrogenic potency.

Administration of recombinant human activin A does not alter oocyte apoptosis but increases both granulosa and germ cell proliferation, resulting in an increase of the number of primordial follicles (23). Activin A increases preantral follicle diameters in immature mice, but not in adult mice (32). In the fetal mouse ovary, FSH stimulates primordial follicle formation accompanied with pre-granulosa cell proliferation and an increase of *Inhba* (33). These results suggest that activins play important roles in folliculogenesis through the regulation of somatic cell proliferation. Thus, dysregulation of the gene expression of each subunit may affect primordial follicle formation. Further study is needed to clarify the actions of activins and inhibins in polyovular follicle induction.

In conclusion, DES can directly induce polyovular follicles through the ovarian ER β *in vitro* as well as *in vivo*.

The expression of *Inha* could be affected by DES through ER β , whereas that of *Inhbb* was not. Alterations in activin signaling, as well as other events during cyst breakdown, may be involved with polyovular follicle induction and suppression of follicle formation by DES.

Conflicts of Interest

There are no conflicts of interest regarding this study.

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Authors' Contributions

SO, SK and TS designed and performed research; SO, SK and TS analyzed data; SM provided ER β knockout mice; SO, SK TI and TS wrote the paper.

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