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# Effects of 2,3-*Bis*(4-hydroxyphenyl)-propionitrile on Induction of Polyovular Follicles in the Mouse Ovary

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**Abstract.** *Background/Aim: Neonatal diethylstilbestrol (DES) treatment induces polyovular follicles (PFs), which contain more than two oocytes in a follicle, through estrogen receptor (ER) β, not ERα. 2,3-Bis(4-hydroxyphenyl)-propionitrile (DPN) is a specific ERβ agonist; the effects of neonatal DPN exposure on PF induction and gene expression in the mouse ovary were examined. Materials and Methods: Histological analysis and real-time reverse transcription-polymerase chain reaction were performed. Results: The PF incidence was significantly high in the ovary of neonatally DPN-exposed mice compared to that in oil-exposed mice. The gene expression of growth differentiation factor 9 (Gdf9), Mullerian-inhibiting substance, steroidogenic factor 1 (Sf1) and steroidogenic acute regulatory protein (Star) in the ovary was significantly increased in the mice neonatally exposed to 40 μg DPN compared to oil-treated mice. Conclusion: Since SF1 is an important transcription factor of several genes involved in ovarian function, up-regulation of Sf1 expression by neonatal exposure to DPN, through ERβ, might affect expression of Gdf9, Mis and Star, resulting in increased PFs in mouse ovary.*

Two types of estrogen receptors (ER), ERα and ERβ are widely found in mammals including mouse, rat and human. In the mouse ovary, ERα is localized in the interstitial and thecal cells, whereas ERβ is mainly localized in the granulosa cells (1, 2). ERβ-knockout mice are subfertile due to fewer oocytes even after superovulation, therefore ERβ is important for follicle maturation from the antral stage (3). However, the role of ERβ in the neonatal ovary is still unclear. It is reported that neonatal treatment with genistein or diethylstilbestrol (DES) induces polyovular follicles (PFs), which contain more than two oocytes in a follicle, through ERβ, not ERα (1, 4). These findings indicate that ERβ in the neonatal mouse ovary can be physiologically activated by ERβ ligand.

2,3-*Bis*(4-hydroxyphenyl)-propionitrile (DPN) is a specific ERβ agonist and exhibits 70-fold greater binding affinity for ERβ than ERα (5). DPN also exhibits the highest transactivation activity with mouse ERβ at $10^{-9}$ M and no significant estrogenic activity for mouse ERα. Neonatal exposure to 25 μg DPN induced PFs in the mouse ovary at 30 days of age (6). Surprisingly, neonatal exposure to 4,4',4''-(4-propyl-[1*H*]-pyrazole-1,3,5-triyl) triphenol (PPT), a specific ERα agonist, also induces PFs in the mouse ovary at 30 days, although not having significant estrogenic activity on mouse ERβ in *in vitro* transactivation assay (6). These results suggest that both ERα and ERβ are involved in PF induction, however, ERβ is essential for PF induction because a lack of ERβ results in no PF induction in the ovary even after neonatal exposure to DES (4).

PFs are found in the ovary of growth differentiation factor 9 (*Gdf9*)$^{+/-}$ bone morphogenetic protein 15 (*Bmp15*)$^{-/-}$ mice, both are oocyte-secreted growth factors (7), and transgenic mice expressing the rat inhibin α (*Inha*) subunit gene (8). These facts indicate that PF induction can be accompanied with changes in expression of those genes. However, no significant changes in the expression of *Gdf9* and *Bmp15* are found in the ovary of mice neonatally exposed to DES at 5, 20 and 30 days, but only *Inha* is increased (4, 9). Since DES can bind and transactivate both ERα and ERβ (10, 6), it is possible that changes in gene expression include all of the genes downstream of ERα and ERβ. Downstream signaling *via* ERβ and PF has not been elucidated *in vivo*.

This study examined the effects of neonatal exposure to DPN on the mouse ovary. To study the mechanism of PF induction, the effects of DPN on the mRNA expression of genes possibly involved with PF induction were studied in C57BL/6J mice.



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*Key Words:* Polyovular follicle, diethylstilbestrol, estrogen receptor β, ovary.

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

| Gene | Encoded proteins | Forward sequence (5' → 3') | Reverse sequence (5' → 3') |
|---|---|---|---|
| *Bmp15* | Bone morphogenetic protein 15 | TCCTTGCTGACGACCCTACATT | GGTCAGCCGAACGATGGTATAA |
| *Gdf9* | Growth differentiation factor 9 | TGGTGGACCTGCTGTTTAACCT | CCAGAAGACATGGCCTCCTTTA |
| *Inha* | Inhibin alpha subunit | CTGCTCTCAATATCTCCTTCCAAGAG | CCATGGCAGTAGTGGAAGATGATGAA |
| *Mis* | Mullerian inhibiting substance | CCTACATCTGGCTGAAGTGATATGG | GAGGCTCTTGGAACTTCAGCAA |
| *Sf1* | Steroidogenic factor 1 | TTGCCTCCTGAAAGCCACTCT | CGCGAAAGCAGGAAAGACA |
| *Star* | Steroidogenic acute regulatory protein | GCTGCCGAAGACAATCATCA | GATGGACAGACTTGCAGGCTT |
| *Cyp11a1* | Cholesterol side chain cleavage (P450SCC) | GTGAATGACCTGGTGCTTCGT | TCGACCCATGGCAAAGCTA |
| *Peptidylprolyl isomerase A* | Cyclophilin A | AGGTCCTGGCATCTTGTCCAT | CCATCCAGCCATTCAGTCTTG |

## Materials and Methods

*Animals*. Female 20-day-old, 4- and 8-month-old C57BL/6J mice (mean weights 10, 25 and 32 g, respectively) (CLEA Japan, Tokyo, Japan) were kept under 12 h light/12 h dark by artificial illumination (lights on 0800-2000) at 23-25˚C. They were fed commercial diet (MF; Oriental Yeast Co., Ltd, Tokyo, Japan) and tap water *ad libitum*. All animals were maintained in accordance with the National Institutes of Health guide for the care and use of laboratory animals. All experiments were approved by the Institutional Animal Care Committee of the Yokohama City University (YCU-A605). The day of birth was regarded as day 0 of age. Female pups were injected subcutaneously with 20, 40 or 60 μg DPN (Tocris Biosciens, Ellisville, MO, USA), 3 μg DES (Sigma Chemical Co., St Louis, MO, USA) dissolved in 0.02 ml sesame oil, or the vehicle alone from day 0 to day 4 (5 days).

*Histological analysis*. Ovaries of 20-day-old C57BL/6J mice treated neonatally with oil, DPN or DES were fixed overnight in Bouin's solution at room temperature. Ovaries were embedded in paraffin, serially sectioned at 8 μm and stained with hematoxylin and eosin (HE) stain. Every 13th section of 20-day-old mouse ovaries were observed and the incidence of PFs (%) was estimated by counting the number of PFs having more than two oocytes per follicle larger than 50 μm in diameter per mouse, as described previously (11). The number of mice with PFs in 20-day-old C57BL/6J mice treated neonatally with 20, 40 or 60 μg DPN, DES or oil vehicle was expressed as the frequency of PFs. Four to five C57BL mice exposed to oil, DPN or DES were used for counting the follicles. Ovaries of 4- and 8-month-old C57BL/6J mice treated neonatally with oil or 20, 40 or 60 μg DPN were analyzed histologically. Three to eight mice were used for each data point.

*Real-time reverse transcription-polymerase chain reaction (RT-PCR)*. Total RNA was isolated from ovaries of the 20-day-old C57BL/6J mice treated neonatally with oil or 40 μg DPN, and reverse transcribed into cDNA by Super Script II reverse transcriptase (Life Technologies, Carlsbad, CA, USA) using 0.05 mM oilgo dT primer (Life Technologies). Real-time PCR was carried out by a Smart Cycler II System (Takara, Ohtsu, Japan) with SYBR Premix Ex Taq™ (Takara). Relative mRNA expression of *Bmp15, Gdf9, Inha, müllerian-inhibiting substance (Mis), steroidogenic factor-1 (Sf1), steroidogenic acute regulatory protein (Star) and cholesterol side chain cleavage (Cyp11a1)* (Table I) was determined by second derivative method. *Peptidylprolyl isomerase A* was chosen as an internal standard to control for variability in amplification due to differences in starting mRNA concentration. Melting curve analysis showed a single peak for all samples. Five to ten mice were used for each group and three independent experiments were carried out for each study.

*Statistical analysis*. Data are expressed as the mean±standard error. For multiple comparisons, treatment groups were compared using analysis of variance (ANOVA) followed by Dunnett's *post hoc* test. Two-tailed Student's *t*-test was used for single comparisons. Fisher's exact probability test was used to examine the significance of the association between the two kinds of classification. A statistically significant difference was defined as that with $p<0.05$.

## Results

In the ovary of mice neonatally exposed to 20, 40 or 60 μg DPN, all of the stages of follicles developed similar l to that of control mice at 20 days of age (Figure 1A and B). PFs were found in the ovary of mice neonatally exposed to 40 or 60 μg DPN (Figure 1D). The total number of follicles of mice neonatally exposed to DPN was not changed compared with that of the exposed to oil or DES (Figure 2A). The PF incidence was significantly higher in the ovary of mice neonatally exposed to 40 or 60 μg DPN compared with that in oil-exposed mice, as well as that in the ovary of mice neonatally exposed to DES (Figure 2B). However, the PF incidence in the ovary of mice exposed to 40 or 60 μg DPN was significantly lower than that in the ovary of mice exposed to DES.

Since PFs were significantly induced in the ovary of mice neonatally exposed to 40 μg DPN, changes in the gene expression were examined by real-time RT-PCR. In the ovary of these mice, the expression of *Gdf9, Mis, Sf1* and *Star* was significantly increased compared with that of oil-treated mice at 20 days (Figure 3). The expression of *Bmp15, Inhα* and *Cyp11a1* in the ovary was not changed by exposure to 40 μg DPN.

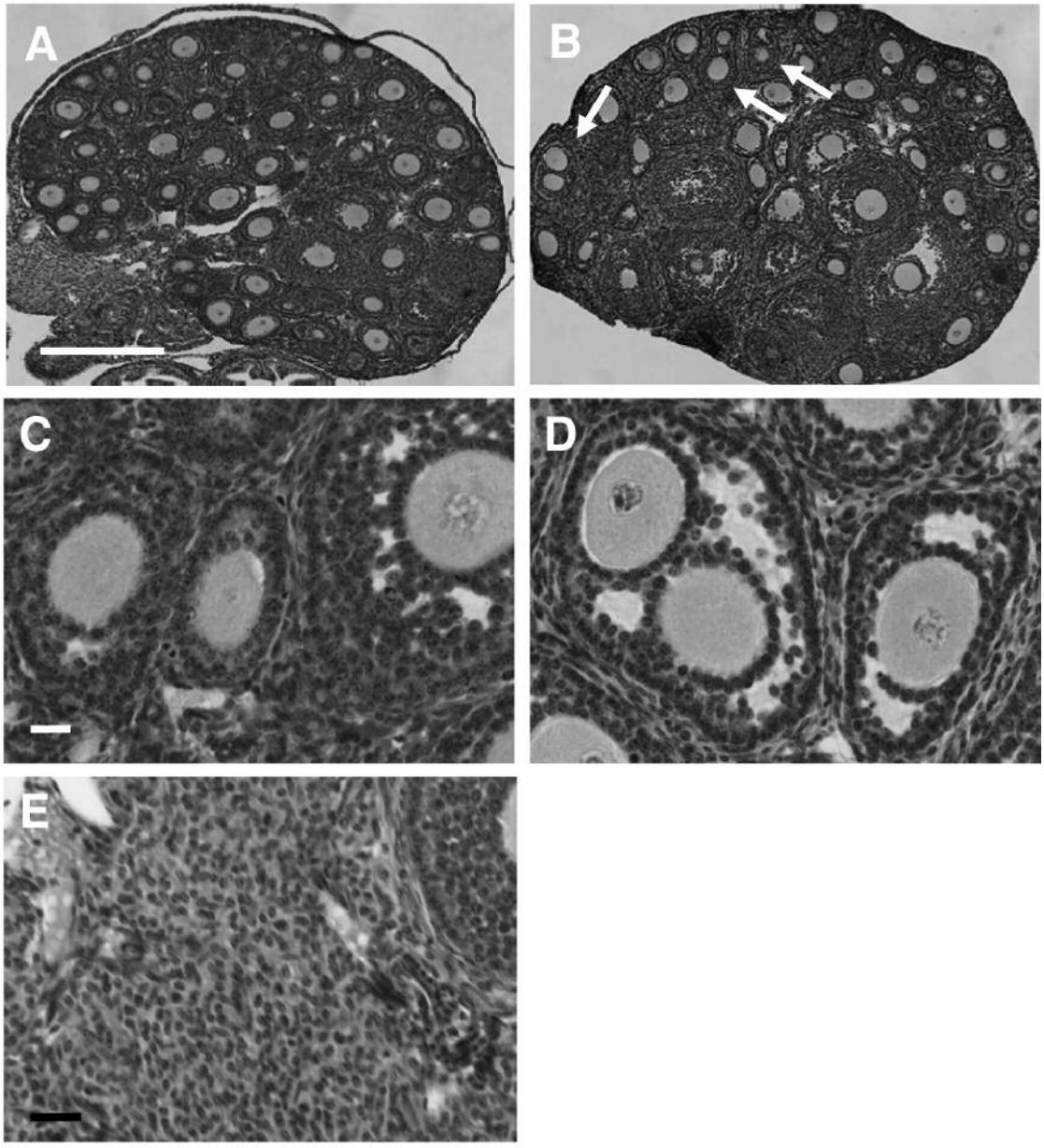


Figure 1. *Histology of 20-day-old C57BL/6J mouse ovaries exposed neonatally to oil (A, C), or 40 μg 2,3-bis(4-hydroxyphenyl)-propion itrile (DPN) (B, D), and ovaries of 4-month-old C57BL/6J mouse exposed neonatally to 60 μg DPN (E). Arrows: Polyovular follicles. Scale bar: A,B: =200 μm, C-E: 25 μm.*

In the adult ovary, several antral follicles and *corpora lutea* (CL) were found. At 4 months of age, the ovary of mice neonatally exposed to 20, 40 or 60 μg DPN showed medullary tubule-like structures, but differences in incidence was not significant. At 8 months, no CL was found in the ovary of mice neonatally exposed to 60 μg DPN and all mouse ovaries showed medullary tubule-like structures (Figure 1E, Table II).

## Discussion

Neonatal DES exposure causes several morphological changes in the ovary including PFs, absence of CL, hypertrophy of the interstitial tissue and appearance of hemorrhagic cysts (12). This study demonstrated that DPN induces PFs accompanied with changes in expression of genes possibly involved with PF induction in the mouse

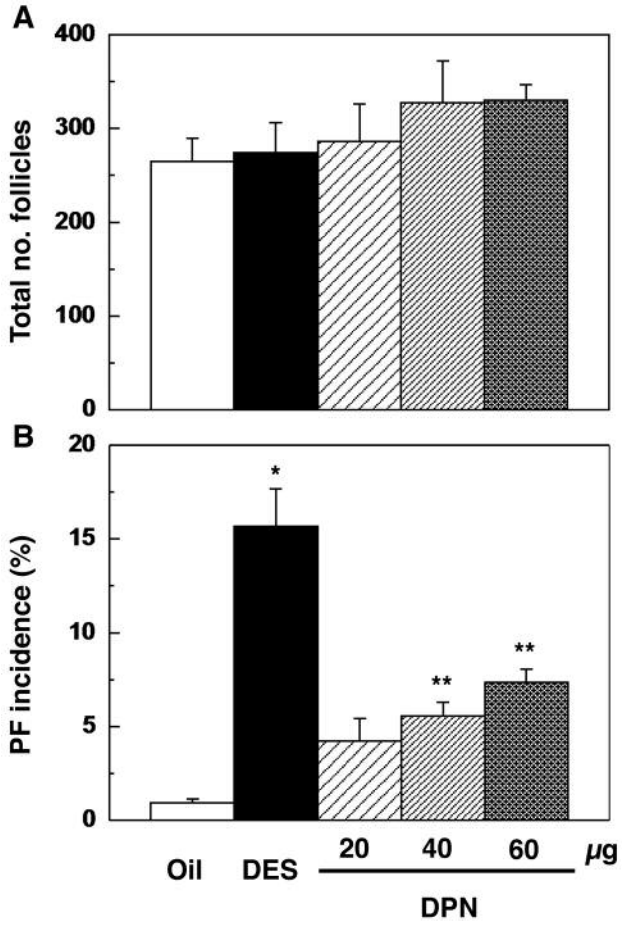


Figure 2. *The total number of follicles (A) and incidence of polyovular follicles (PF) (B) in ovaries of 20-day-old C57BL/6J mice exposed neonatally to oil, diethylstilbestrol (DES) or 20 μg, 40 μg, or 60 μg 2,3-bis(4-hydroxyphenyl)-propionitrile (DPN). Significantly different at *p<0.05 compared to oil controls, and **p<0.05 compared to mice exposed to DES.*

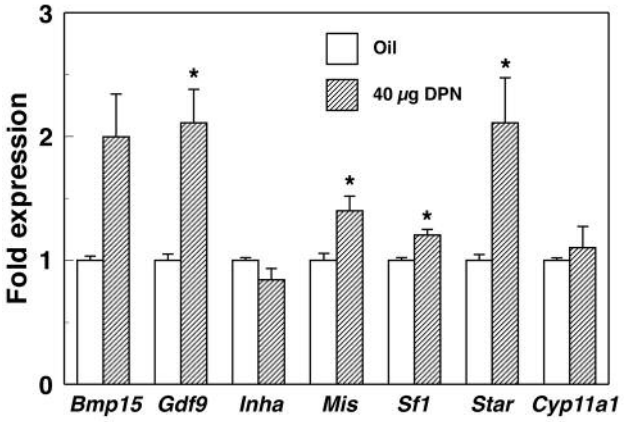


Figure 3. *Relative mRNA expression of genes possibly associated with induction of polyovular follicles (PF) in ovaries of 20-day-old C57BL/6J mice exposed neonatally to oil or 40 μg 2,3-bis(4-hydroxyphenyl)-propionitrile (DPN). *Significantly different at p<0.05 compared with oil-exposed mice. Bmp15: Bone morphogenetic protein 15; Gdf9: growth differentiation factor 9; Inha: inhibin-α; Mis: müllerian-inhibiting substance; Sf1: steroidogenic factor-1; Star: steroidogenic acute regulatory protein; Cyp11a1: cholesterol side chain cleavage.*

ovary. DPN is a specific ERβ agonist with high transactivation activity to mouse ERβ (5), therefore changes in gene expression can be induced through ERβ. However, exposure to high doses of DPN also led to the formation of medullary tubule-like structures in the interstitial cells of the ovary. Absence of CL and medullary tubule-like structures are due to the alterations of gonadotropin levels, induced by neonatal exposure to DES through ERα (13, 14). Indeed, DPN can transactivate mouse ERα at $10^{-7}$-$10^{-8}$ M *in vitro* (6, 15). Therefore, no CL and an appearance of medullary tubule-like structures in the interstitial cells of the ovary may be an indicator of the effects of DPN *via* ERα. Consequently, ovary of neonatally 40-μg-DPN-exposed mice was further analyzed for gene expression.

In the ovary of mice neonatally exposed to 40 μg DPN, the expression of *Gdf9, Mis, Sf1* and *Star* was significantly increased, indicating that these genes are affected by neonatal exposure to DPN. GDF9 is a member of oocyte-derived BMP family which regulates the function of granulosa cells during follicle growth and ovulation as well as BMP15 (16). *Gdf9*-null mice exhibit an absence of normal follicles caused by the loss of granulosa cell proliferation at the end of the primary stage (17). In addition, *KIT proto-oncogene receptor tyrosine kinase (Kit)* ligand in the granulosa cells and *Inha* in the primary follicles are highly expressed in *Gdf9*-null mice (18), suggesting that *Gdf9* is involved with cell proliferation in the granulosa cells and down-regulation of *Inha* gene expression during early follicle growth. Indeed, early follicle growth in *Inha*/*Gdf9* double-null ovary is normal (19). Although neonatal exposure to DES cannot alter the expression of *Gdf9* even at 2 days of age (4, 9), dysfunction of granulosa cells by neonatal exposure to DPN through ERβ may result in an increase of *Gdf9*. However, although the expression of *Inhα* is increased by DES (4, 9), it was not changed by neonatal exposure to 40 μg DPN. Thus, an increase of *Gdf9* may suppress the expression of *Inha* even if DPN induces an increase of *Inha*.

MIS is found in granulosa cells and involved in the entry of primordial follicles into the growing pool (20). SF1 is an essential transcription factor of endocrine development and function, and *Mis* is a target gene of SF1 (21-23). Although our previous report showed no significant change in the expression of *Mis* and *Sf1* in ovaries of mice neonatally exposed to DES (4), neonatal exposure to estradiol benzoate increases *Mis* mRNA and protein, with inhibition of follicle growth in the rat

Table II. *Effects of neonatal exposure of DPN on the ovary of 4- or 8-month-old mice.*

| Treatment (μg/pup) | Age (months) | No. of mice examined | BW (g) | Ovarian weight (mg/20 g BW) | No. of mice with | |
|---|---|---|---|---|---|---|
| | | | | | *Corpora lutea* | Medullary tubule-like structures |
| Oil | 4 | 5 | 24.7±0.82 | 3.36±0.324 | 5 | 0 |
| 20 DPN | 4 | 5 | 24.0±0.56 | 3.99±0.373 | 5 | 1 |
| 40 DPN | 4 | 8 | 26.0±1.37 | 3.25±0.148 | 7 | 1 |
| 60 DPN | 4 | 7 | 24.9±0.68 | 3.08±0.400 | 4 | 4 |
| Oil | 8 | 5 | 31.4±1.44 | 2.31±0.334 | 5 | 0 |
| 20 DPN | 8 | 6 | 29.6±1.93 | 2.34±0.280 | 5 | 4* |
| 40 DPN | 8 | 7 | 31.3±1.55 | 2.82±0.259 | 5 | 2 |
| 60 DPN | 8 | 3 | 30.5±2.17 | 2.81±0.564 | 0* | 3* |

BW: Bodyweight. *Significantly different at $p<0.05$ compared to age-matched control mice (Fisher's exact probability test).

at days 6 and 14 (24). In this study, the expression of *Sf1* was also increased in the ovary of mice exposed to DPN, therefore, an increase of *Mis* may be due to the changes in the *Sf1* expression. Neonatal exposure to DPN possibly plays a role in the function of granulosa cells through ERβ.

MIS is highly expressed in the cuboidal granulosa cells of primary and secondary follicles and decreases in later stages of follicle development (24). In *Mis* null mice, primordial follicle recruitment is more stimulated (20, 25), therefore, an increase of MIS may be correlated with the inhibition of follicle growth. In contrast, neonatal exposure to DES suppresses both primary and secondary follicle progression *via* ERα without change in expression of *Mis* (4, 26). In this study, follicle growth in the ovary of mice exposed to DPN is similar to that of control mice, suggesting that an increase of *Mis* is not necessarily associated with the inhibition of follicle growth. Indeed, mice exposed to DPN were able to ovulate and deliver litters (data not shown). Vitt *et al*. reported that *in vivo* treatment with GDF9 stimulated early follicle progression in the rat ovary (27), therefore, an increase in expression of both of *Mis* and *Gdf9* may result in normal follicle development. Several BMPs, including BMP15, significantly induce the expression of *Mis* in human granulosa cells at mRNA and protein levels, but GDF9 does not (28), suggesting that changes in *Mis* and *Gdf9* expression may not affect each other.

Similarly to *Mis*, *Star* expression was also increased in the ovary of mice exposed to DPN, however, localization of these two proteins is different. STAR is mainly localized in the interstitial cells, and both STAR and $P450_{SCC}$ are also decreased in the theca and interstitial cells of rats neonatally exposed to estradiol benzoate (EB) (29). A decrease of expression of these genes is mediated by ERα in the theca and interstitial cells (29), therefore, it is possible that DPN directly affects the expression of *Star* through ERα. However, *Star* is also a direct target gene of SF1 (23, 30), it is also considered that an increase of *Star* expression may be due to an increase of *Sf1*, as well as *Mis*.

SF1 is found in the thecal, interstitial, granulosa and luteal cells in the ovary (23). Neonatal exposure to EB also reduces *Sf1* mRNA and protein in the rat through days 6 to 21 (24), however, the expression of *Sf1* in mice neonatally exposed to DES is not altered through days 10 to 30 (4). *Sf1* mRNA is clearly expressed in granulosa cells of most large preantral follicles following treatment with estradiol (31), suggesting that *Sf1* in granulosa cells is regulated by estrogen. In this study, the expression of *Sf1* significantly increased in the ovary at 20 days of age in mice exposed to DPN. Although the localization of increased *Sf1* expression is not clear, this indicates that the regulation of *Sf1* is mediated *via* ERβ.

In conclusion, neonatal exposure to DPN alters the expression of *Sf1* through ERβ, and changes in *Sf1* expression may affect expression of other genes such as *Gdf9, Mis* and *Star*. These changes may be involved with PF induction mediated by ERβ. Since several endocrine disruptors such as bisphenol-A can induce PFs (32), further analysis of signaling molecules downstream of ERβ is important.

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