Journal of Reproduction and Development, Vol. 55, No. 6, 2009

—Full Paper—

Effects of Maternal Exposure to Low Doses of DES on Testicular Steroidogenesis and Spermatogenesis in Male Rat Offspring

Tetsuo KOBAYASHI[1)], Mitsuyuki SHIRAI[2)], Motoharu SAKAUE[1)], Masaru MURAKAMI[3)], Hideharu OCHIAI[4)], Kazuyoshi ARISHIMA[1)] and Masako YAMAMOTO[1)]

[1)]Department of Anatomy II, [2)]Department of Veterinary Pharmacology and [3)]Department of Molecular Biology, School of Veterinary Medicine and [4)]Research Institute of Biosciences, Azabu University, Kanagawa 229-8501, Japan

Abstract. Our previous studies have demonstrated that prenatally administered diethylstilbestrol (DES) impairs testicular endocrine function in male offspring. The present study examined whether maternal DES treatment influences testicular steroidogenesis and spermatogenesis. DES was injected subcutaneously at 0.5 or 1.5 μg/kg/day (DES 0.5 and 1.5 groups, respectively) into pregnant SD rats on days 7–21 of gestation. Male offspring in the DES 0.5 and 1.5 groups were autopsied at 1, 3, 6 and 15 weeks after birth. At 1 week, DES treatment did not lead to a change in the volume of P450scc-positive cells (Leydig cells), suggesting that DES has no inhibitory effect on the development of Leydig cells. DES administration disrupted luteinizing hormone receptor (LHr) expression and exerted inhibitory effects on signal transduction from LHr to steroidogenic acute regulatory protein (StAR) in testicular steroidogenesis ($P<0.05$), although there were no changes in the mRNA expression levels of steroidogenic enzymes, such as P450scc, 3β-hydroxysteroid dehydrogenase (3β-HSD) and $P450_{17\alpha}$, which may have caused a decrease in the plasma testosterone level. DES treatment did not disrupt the cycle of spermatogenesis but did upregulate the expression levels of androgen receptor (AR) mRNA in both DES groups at 15 weeks ($P<0.05$). These results indicate that maternal DES treatment disrupts steroidogenesis but induces a high level of AR mRNA expression to counteract the low levels of testosterone during spermatogenesis.

Key words: Diethylstilbestrol (DES), Rat, Spermatogenesis, Steroidogenesis, Testis

(**J. Reprod. Dev. 55:** 629–637, 2009)

During embryonic development, rat primordial germ cells appear in the yolk sac on embryonic day 10, and the gonadal primordium begins to develop as the genital ridge around embryonic day 11. Primordial germ cells begin to reach the genital ridge on embryonic day 12 [1]; thereafter, Sertoli and Leydig cells in the male differentiate from genital ridge cells, leading to a series of differentiation events. The duration of pregnancy is generally divided into periods of embryonic development (first trimester), organogenesis (second trimester) and organ maturation (third trimester).

Diethylstilbestrol (DES), a synthetic non-steroidal estrogen, exhibits strong estrogenic activity by binding to estrogen receptors (ERs). It has been shown that the treatment of pregnant rats with DES dose-dependently (range: 10 to 300 μg/kg) suppresses testosterone levels in the blood and testes of male fetuses [2–4]. We administered doses of DES much lower (1.5 μg/kg) than those previously applied to pregnant rats at 7–21 days of gestation (in the second and third trimesters) and demonstrated that DES suppresses plasma testosterone levels in adolescent male offspring (6 weeks after birth) [5].

Androgen plays an important role in the development and function of the testis and male reproductive tract via the androgen receptor (AR). Similarly, since ERs have been found in these tissues [6–10], estrogen may be involved in development of the male reproductive system. Nevertheless, it is apparent that male ER- or aromatase-knockout mice initially develop a grossly normal testis and reproductive tract [6, 11–14]. As estrogen production is itself dependent on prior androgen production, it is difficult to separate androgenic from estrogenic effects in tissues in which both ERs and ARs are expressed [15]. Several studies have shown that interactions between the ARs and ERs are possible [16, 17]. It has been suggested that many of the adverse changes in the testis and reproductive tract induced by exposure to estrogens result from a combination of high estrogen and low androgen activity [18]. Indeed, in our previous study, a low dose of DES (1.5 μg/kg) induced not only suppression of plasma testosterone levels in male offspring (6 weeks after birth), but also promoted follicular maturation in female offspring [5]. The number of ARs is maximal in Sertoli cells at stages VII-VIII of spermatogenesis [19]. Administration of DES in the neonatal period has been reported to suppress both testosterone levels and AR expression [18, 20, 21] and to induce impaired spermatogenesis [15, 20, 22–26]; however, our previous study [27] demonstrated that a fetal DES treatment-induced low level of testosterone led to little modification of spermatogenesis at 15 weeks.

Although many studies involving DES administration during fetal and neonatal periods have been performed, no study employing DES administration during the fetal period alone has investigated hormone-secreting ability after maturation of newborns by measurement of only fetal testicular hormone-secreting ability, hormone levels and steroidogenic enzyme mRNA levels [28]. The only exception to this is a study in which DES was administered at a high dose (10 μg/kg/animal) to pregnant mice at

Accepted for publication: July 29, 2009
Published online in J-STAGE: September 7, 2009
Correspondence: M Yamamoto (e-mail: masako@azabu-u.ac.jp)

7, 10, 13 and 16 days of gestation and hormone levels were measured in progenies at 8 weeks of age [4]; however, no changes were noted in the plasma testosterone, testicular steroid hormone receptor or StAR mRNA expression levels. Thus, it is unclear how long-term administration of a low dose (1.5 μg/kg) of DES suppresses plasma testosterone levels at 6 weeks after birth and inhibits testicular steroidogenesis after birth, but induces little modification of spermatogenesis.

The aim of the present study was to elucidate the mechanism of the influence of DES on steroidogenesis and spermatogenesis in mature and immature male offspring of mothers that received low doses of DES during pregnancy by examining the mRNA expressions of steroidogenic enzymes, hormone receptors, spermatogenesis-regulating factors, sex hormone levels and stages in the cycle of the testicular seminiferous epithelium.

Materials and Methods

Animals and treatments

Sprague-Dawley rats (Japan SLC, Hamamatsu, Japan) were given a commercially available diet (CE-2, CLEA , Tokyo, Japan) and water, both *ad libitum*. Females were mated with males overnight and examined the next morning for the presence of sperm in a vaginal smear. The day on which sperm was detected was counted as day 0 of gestation. Pregnant rats were housed individually and maintained under a 12/12 h light-dark cycle at a room temperature of 21 ± 2 C and humidity of 55 ± 5%. The pregnant rats were divided into three groups. The rats in each group were treated daily subcutaneously with DES (Sigma Chemical, St. Louis, MO, USA) at 0.5 or 1.5 μg/kg/day dissolved in corn oil (tocopherol-stripped, ICN Biomedicals, Aurora, OH, USA) or corn oil alone (control group) on days 7–21 of gestation. The volume of a single dose ranged from 0.2–0.4 ml.

Four days after birth, each litters was adjusted so that it contained 4 males and females. There were 10 litters per group. The pups were kept with their biological dams until weaning on day 21. Male rats at postnatal weeks 1, 3, 6 and 15 were autopsied under ether anesthesia to collect individual blood samples. The testes of each pup were removed and weighed; the left testis was subjected to RNA extraction and the right was subjected to histological analysis.

This study was carried out in accordance with the Azabu University Animal Experiment Guidelines.

Extraction of intratesticular testosterone

At 6 weeks, samples of sliced testis were homogenized in 250 μl of PBS at room temperature. The homogenized samples were transferred to a glass tube, 2 ml of diethyl ether was added and the samples were vortexed for 2 min. The mixture was allowed to stand at room temperature for 20 min. The bottom of the glass tube was placed in liquid nitrogen to freeze precipitations, and the upper phase of diethyl ether was carefully removed. The aqueous phases were allowed to evaporate in a water bath and dissolved in 500 μl of PBS.

Hormone assay

The blood samples were centrifuged at 4 C, and the plasma was stored at –80 C until assay. The concentrations of plasma and intratesticular testosterone were measured by radioimmunoassay using a [^{125}I] total testosterone assay kit (Diagnostic Products, Los Angeles, CA, USA). The sensitivity was determined as 4 ng/dl. The concentrations of LH and FSH were measured using the rat luteinizing hormone [^{125}I] Biotrak assay system and rat follicle-stimulating hormone [^{125}I] Biotrak assay system, respectively (Amersham Biosciences, Buckinghamshire, UK). The sensitivities were determined as 0.1 and 0.9 ng/ml, respectively.

Morphological analysis of Leydig cells

At 1 week, testes were fixed in Bouin's solution, dehydrated, embedded in paraffin, and sectioned at 5 μm. The serially sectioned slides were immunostained with anti-P450scc serum (Chemicon International, Temecula, CA, USA) using the ABC method. The area of every fifth section of a series of P450scc-positive cells was measured with an image-analyzing apparatus (Olympus, Tokyo, Japan). The total volume (μm^3) = the sum of the areas of P450scc-positive cells (μm^2) × 25 μm (the distance between the sections).

Spermatogenesis

At 6 and 15 weeks, testes were fixed in Bouin's solution and sectioned at 4 μm. Sections were stained with periodic acid-Schiff and counterstained with hematoxylin. Seminiferous tubules were staged according to criteria previously published [29]. Five-hundred to one thousand tubules per testis were classified into the following stages: I, II/III, IV, V, VI, VII, VIII, IX, X, XI, XII, XIII and XIV. The number of seminiferous tubules at each stage was expressed as the proportion (%) of the total number of tubules.

Semiquantitative reverse transcription-PCR

Total RNA was extracted from the testis of male offspring at 6 and 15 weeks using Isogen (Nippon Gene, Toyama, Japan) according to the manufacturer's protocol. The subsequent isolation involved chloroform extraction, isopropanol precipitation and washing in 70% ethanol. The concentration of total RNA was determined by measuring the optimal density at 260 and 280 nm.

PCR amplification from reverse-transcribed cDNA was carried out with the following primers: LH receptor (LHr), steroidogenic acute regulatory protein (StAR), P450scc, 3β-HSD, 17α-hydroxylase + $C_{17\text{-}21}$ lyase ($P450_{17\alpha}$), 5α-reductase 1, P450arom (aromatase), AR, ERα, anti-Müllerian Hormone (AMH) and β-actin (Table 1). The reactions were performed according to the manufacturer's instructions for the SuperScript One-Step RT-PCR kit (Invitrogen, Carlsbad, CA, USA). The total volume, 50 μl, of the reaction mixture contained RNA, 200 nM of sense and antisense primers, Tag Mix and 1 X reaction mix. Synthesis of cDNA and pre-denaturation were performed using one cycle of 50 C for 30 min and 94 C for 2 min. Amplification was carried out in a thermal cycler (Bio-Rad, Hercules, CA, USA).

Following PCR, the amplified DNA was separated by electrophoresis in a 2.5% agarose gel with an appropriate molecular weight marker (Bio-Rad). Gels were stained with ethidium bro-

Table 1. PCR primers for detection of the various gene expressions used in this study

Gene	Direction	Sequence (5'–3')
LHr	Forward	CGGGCCATGGGGCGGCGAGTCCC
	Reverse	GTTTTTGGTGTTCTGGATCAG
StAR	Forward	AGGCAGGGGGATCTTTCTAA
	Reverse	TGCCTGACTAGGGTTTCGTT
P450scc	Forward	CTGAGGGAGAACGGAACACAC
	Reverse	AGGCAAAGCGGAATAGGTCATC
3β-HSD	Forward	GGTGACAATGTTAGAAGGAG
	Reverse	GCCACATTGCCTACATACAC
P450$_{17\alpha}$	Forward	GCCAGGGAGGTGCTCATCAAG
	Reverse	GGCGTGGACAGGTCTATGGACT
5α-reductase 1	Forward	TCCTGGTCACCTTTGTCTTGGC
	Reverse	GTTTCCCCTGGTTTTCTCAGATTC
P450arom	Forward	GCTTCTCATCGCAGAGTATCCGG
	Reverse	CAAGGGTAAATTCATTGGGCTTGG
AR	Forward	CCCATCGACTATTACTTCCCACC
	Reverse	TTCTCCTTCTTCCTGTAGTTTGA
ERα	Forward	AAGAGAAGGACCACATCCACC
	Reverse	GGAATGTGCTGAAGTGGAGC
AMH	Forward	TCCTACATCTGGCTGAAGTGATATGGGAGC
	Reverse	CTCAGGGTGGCACCTTCTCTGCTTGGTTGA
β-Actin	Forward	CAGCCTTCCTTCCTGGGTATG
	Reverse	TAGAGCCACCAATCCACACAG

Table 2. Effects of prenatal exposure to DES on body and testicular weights

	Group	1 week	3 weeks	6 weeks	15 weeks
Body weight	Control	13.8 ± 0.5	45.6 ± 1.6	189.9 ± 3.7	440.3 ± 10.4
(g)	DES 0.5	13.9 ± 0.6	45.0 ± 3.0	193.0 ± 2.3	475.8 ± 11.7
	DES 1.5	13.0 ± 0.6	46.5 ± 2.1	182.0 ± 3.6$^{\#}$	429.4 ± 13.1$^{\#}$
Testicular weight	Control	19.4 ± 0.8	215.4 ± 6.9	1,847 ± 44	3,399 ± 132
(mg)	DES 0.5	19.8 ± 0.4	229.1 ± 6.5	1,912 ± 46	3,418 ± 53
	DES 1.5	18.3 ± 0.6	216.9 ± 11.7	1,788 ± 39	3,208 ± 126
TW/BW × 100	Control	140.8 ± 4.2	473.9 ± 9.8	975.1 ± 25.4	770.9 ± 17.4
(mg/100 g)	DES 0.5	144.2 ± 5.6	529.5 ± 36.3	991.0 ± 21.7	720.9 ± 14.9*
	DES 1.5	142.7 ± 5.1	466.6 ± 13.2	988.9 ± 14.6	750.5 ± 28.1

n=10 for all cases. *: Significantly different from control values (P<0.05). $^{\#}$: Significantly different from the DES 0.5 group (P<0.05).

mide. The intensity of the ethidium bromide luminescence was measured using photographs taken with a Polaroid camera, and the band intensity was quantified using the NIH Image software. The signal intensities were measured in five individual animals, with two replications for each sample. Data were normalized for β-actin mRNA levels in each sample.

Statistical analyses

Levels of significance were analyzed by Student's *t*-test. All values are expressed as means ± standard error of the mean (SEM). Differences were considered significant at P<0.05.

Results

General observations

All pregnant rats in the DES 1.5, DES 0.5 and control groups gave birth to pups; however, one rat in the DES 1.5 group did not nurse its pups and bit them to death. The remaining pregnant rats in the three groups gave birth to pups and nursed them normally.

The body weighs of the rats at 6 and 15 weeks of age were significantly lower in the DES 1.5 group compared with the DES 0.5 group (P<0.05). The testis/body weight ratio of the rats at 15 weeks of age was significantly lower in the DES 0.5 group than in the control group (P<0.05; Table 2).

Hormone levels

Table 3 shows the results of the determination of plasma and intratesticular hormone concentrations. The plasma testosterone concentrations of offspring at 3 weeks after birth were below the detection limits. The testosterone levels in the DES 1.5 group at 6 weeks of age and DES 0.5 group at 15 weeks of age were significantly lower than those in the control group (P<0.05).

Table 3. Effects of prenatal exposure to DES on plasma hormone concentrations

	Group	3 weeks	6 weeks	15 weeks
Testosterone	Control	ND	57.4 ± 9.9 [9]	163.7 ± 27.5 [10]
(ng/dl)	DES 0.5	ND	61.7 ± 19.7 [10]	83.5 ± 13.1 [10] *
	DES 1.5	ND	26.0 ± 7.7 [10] *	131.6 ± 12.3 [10]
LH	Control	1.63 ± 0.07 [8]	1.61 ± 0.09 [8]	2.14 ± 0.14 [10]
(ng/ml)	DES 0.5	2.09 ± 0.18 [9] *	1.59 ± 0.14 [9]	1.88 ± 0.12 [8]
	DES 1.5	2.03 ± 0.15 [8] *	1.43 ± 0.08 [9]	2.26 ± 0.20 [9]
FSH	Control	9.74 ± 0.87 [5]	15.20 ± 0.82 [8]	9.56 ± 1.16 [8]
(ng/ml)	DES 0.5	9.24 ± 0.40 [7]	14.79 ± 0.66 [9]	8.48 ± 0.57 [9]
	DES 1.5	9.02 ± 0.48 [7]	14.69 ± 0.74 [9]	8.81 ± 0.59 [9]
Intratesticular	Control		57.1 ± 15.3 [8]	
testosterone (ng/dl)	DES 1.5		30.7 ± 6.0 [8]	

*: Significantly different from control values (P<0.05). [ ]: No. of samples. ND: Not detected.

Table 4. Changes in the total volume of anti-P450scc serum-positive cells in the testes

Age (weeks)	Group	n	Total volume of anti-P450scc-positive cells (mm^3)
1	Control	5	1.15 ± 0.29
	DES 0.5	5	0.94 ± 0.08
	DES 1.5	5	0.79 ± 0.09

Table 5. Effects of DES on the stage frequencies in the cycles of seminiferous tubules

	Spermatogenesis (percentage of seminiferous tubules)					
	6 Weeks (n=10)			15 Weeks (n=10)[a]		
Stage	Control	DES 0.5	DES 1.5	Control	DES 0.5	DES 1.5
I	17.2 ± 0.5	19.2 ± 0.6*	18.3 ± 0.9	20.3 ± 0.6	19.1 ± 1.0	19.9 ± 1.0
II/III	10.8 ± 0.5	11.9 ± 0.9	11.3 ± 0.7	11.0 ± 0.5	10.1 ± 0.6	10.6 ± 0.5
IV	3.6 ± 0.3	3.0 ± 0.2	4.1 ± 0.4[#]	2.1 ± 0.1	2.8 ± 0.3*	2.8 ± 0.4
V	3.7 ± 0.2	3.5 ± 0.3	3.9 ± 0.3	2.4 ± 0.1	3.1 ± 0.3*	2.7 ± 0.3
VI	7.5 ± 0.3	6.7 ± 0.4	6.7 ± 0.4	5.1 ± 0.4	5.3 ± 0.4	5.2 ± 0.5
VII	22.7 ± 0.6	22.7 ± 0.7	22.6 ± 0.8	24.6 ± 0.7	23.4 ± 0.7	24.3 ± 0.7
VIII	7.5 ± 0.5	6.2 ± 0.4	6.8 ± 0.4	6.9 ± 0.5	7.4 ± 0.6	7.3 ± 0.3
IX	3.0 ± 0.2	3.3 ± 0.2	2.8 ± 0.3	2.7 ± 0.3	2.8 ± 0.3	2.2 ± 0.2
X	3.4 ± 0.3	2.8 ± 0.1	3.5 ± 0.3	3.2 ± 0.3	3.5 ± 0.4	3.0 ± 0.3
XI	4.9 ± 0.2	4.9 ± 0.2	4.2 ± 0.3	4.3 ± 0.4	4.1 ± 0.4	3.6 ± 0.3
XII	8.9 ± 0.6	8.8 ± 0.4	8.2 ± 0.4	7.2 ± 0.5	6.4 ± 0.5	6.2 ± 0.6
XIII	3.4 ± 0.2	3.9 ± 0.4	4.0 ± 0.4	6.1 ± 0.5	7.4 ± 0.5	8.2 ± 0.6*
XIV	3.4 ± 0.3	3.3 ± 0.2	3.9 ± 0.3	4.2 ± 0.6	4.6 ± 0.6	4.2 ± 0.6

*: Significantly different from control values (P<0.05). [#]: Significantly different from the DES 0.5 group (P<0.05). [a] DES 1.5 group; n=9.

The LH levels in the DES 1.5 and DES 0.5 groups at 3 weeks of age were significantly higher than those in the control group (P<0.05). DES administration did not influence the plasma FSH levels in the male pups (P>0.05).

The intratesticular testosterone levels in the DES 1.5 group remained unchanged (P>0.05).

Leydig cells and spermatogenesisis

DES administration did not influence the total volume of P450scc-positive cells in the testis at 1 week after birth (Table 4).

No mature spermatozoa were present in the testicular seminiferous tubules of the three groups at 6 weeks of age. At 15 weeks of age, the presence of mature spermatozoa with elongated heads was confirmed in the lumens of the testicular seminiferous tubules in all three groups. When the seminiferous tubules were classified into the 13 stages of the spermatogenesis cycle (Table 5), the percentage of stage I seminiferous tubules at 6 weeks of age was significantly higher in the DES 0.5 group than in the control group (P<0.05). The percentage of stage IV seminiferous tubules was significantly higher in the DES 1.5 group than in the DES 0.5 group

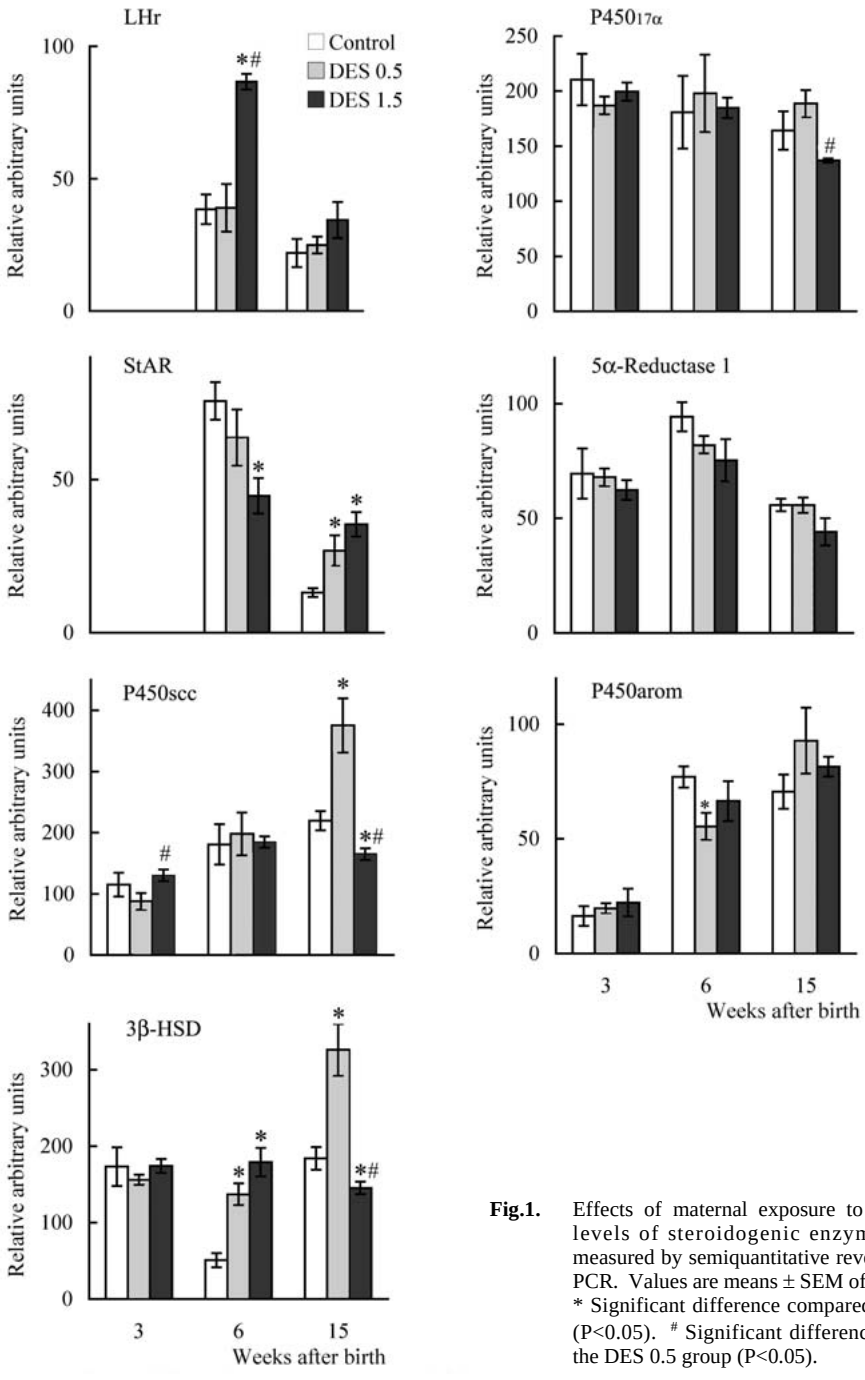

Fig.1. Effects of maternal exposure to DES on mRNA levels of steroidogenic enzymes in the testis measured by semiquantitative reverse transcription-PCR. Values are means ± SEM of 5 determinations. * Significant difference compared with the control ($P<0.05$). # Significant difference compared with the DES 0.5 group ($P<0.05$).

($P<0.05$). At 15 weeks of age, the percentage of stage XIII seminiferous tubules was significantly higher in the DES 1.5 group than in the control group ($P<0.05$). The percentages of stage IV and V seminiferous tubules were significantly higher in the DES 0.5 group than in the control group ($P<0.05$).

Expression levels of LHr, StAR, steroidogenic enzymes (P450scc, 3β-HSD, and P450$_{17\alpha}$), 5α-reductase 1 and P450arom mRNA

At 6 weeks of age, the LHr mRNA expression levels were significantly higher in the DES 1.5 group than in the other two groups ($P<0.05$; Fig.1).

At 6 weeks of age, the StAR mRNA expression levels were sig-

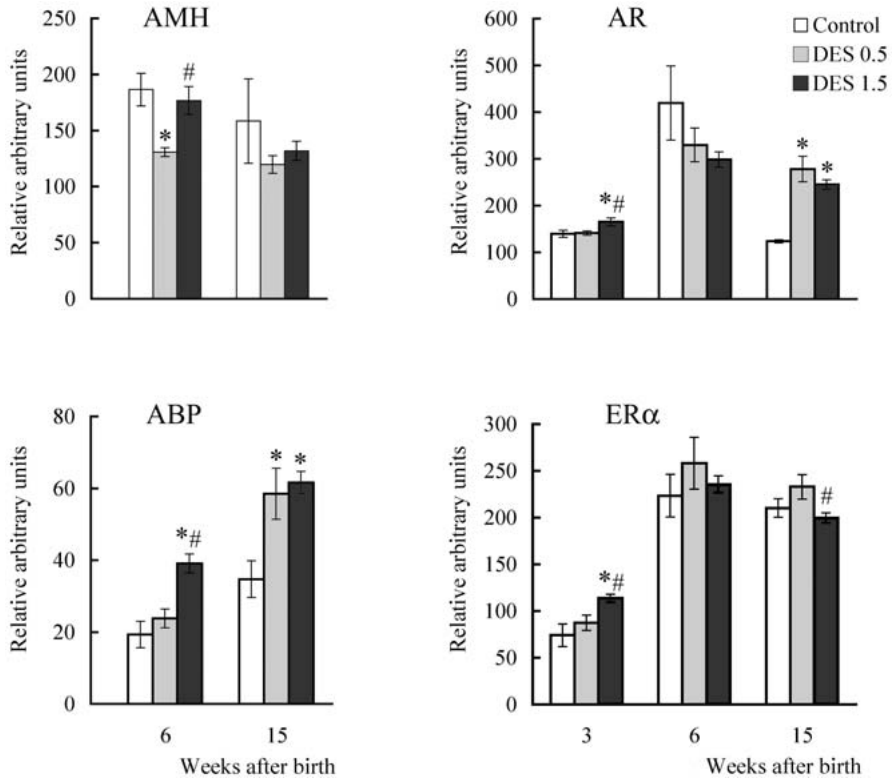

Fig. 2. Effects of maternal exposure to DES on mRNA levels of several factors in the testis measured by semiquantitative reverse transcription-PCR. Values are means ± SEM of 6 determinations. * Significant difference compared with the control (P<0.05). # Significant difference compared with the DES 0.5 group (P<0.05).

nificantly lower in the DES 1.5 group compared with the other two groups, but, at 15 weeks, these levels were significantly lower (P<0.05).

At 3 weeks of age, the P450scc mRNA expression levels were significantly higher in the DES 1.5 group than in the DES 0.5 group (P<0.05; Fig. 1). At 15 weeks of age, the P450scc mRNA expression levels were significantly lower in the DES 1.5 group than in the control group and significantly higher in the DES 0.5 group than in the control group (P<0.05).

The 3β-HSD mRNA expression levels were significantly higher in the DES 1.5 and 0.5 groups at 6 weeks of age than in the control group (P<0.05). At 15 weeks of age, the 3β-HSD mRNA expression levels were significantly lower in the DES 1.5 group than in the control group and significantly higher in the DES 0.5 group than in the control group (P<0.05).

At 15 weeks of age, the $P450_{17\alpha}$ mRNA expression levels were significantly lower in the DES 1.5 group than in the DES 0.5 group (P<0.05).

At all ages, the 5α-reductase mRNA expression levels remained unchanged after DES administration (P>0.05).

At 6 weeks of age, the P450arom mRNA expression levels were significantly lower in the DES 0.5 group than in the control group (P<0.05).

Expression of sex hormone receptor mRNAs

At 3 weeks of age, the AR mRNA expression levels were significantly higher in the DES 1.5 group than in the DES 0.5 and control groups (P<0.05). At 15 weeks of age, the AR mRNA expression levels were significantly higher in the DES 0.5 and DES 1.5 groups than in the control group (P<0.05; Fig. 2).

At 3 weeks of age, the ERα mRNA expression levels were significantly higher in the DES 1.5 group than in the DES 0.5 and control groups (P<0.05). At 15 weeks of age, the ERα mRNA expression levels were significantly lower in the DES 1.5 group than in the DES 0.5 group (P<0.05; Fig. 2).

Expression of spermatogenesis-regulating factor mRNAs

At 6 weeks of age, the AMH mRNA expression levels were significantly lower in the DES 0.5 group than in the DES 1.5 and control groups (P<0.05; Fig. 2).

At 6 weeks of age, the ABP mRNA expression levels were significantly higher in the DES 1.5 group than in the DES 0.5 and control groups (P<0.05). At 15 weeks of age, the ABP mRNA expression levels were significantly higher in the DES 1.5 and 0.5 groups than in the control group (P<0.05).

Discussion

In our previous studies [5, 27], we determined the effects of various levels of DES (15, 4.5, 1.5 and 0.5 μg/kg/day) considering Boylan's results [30], achieved by administering various doses of DES s.c. to SD rats, showing that 1.5 μg/kg/day is approximately the maximum possible dose. In the present study, the testis/body weight ratio was lower in the DES 0.5 group than in the control group. This was probably due to promotion of an increase in body weight rather than inhibition of testicular growth in the DES 0.5 group.

The testosterone levels in the DES 1.5 group at 6 weeks of age and DES 0.5 group at 15 weeks of age were significantly lower than that in the control group. Some studies have suggested that the DES dose response can be described by a U-shaped curve. Specifically, administration of a high level of DES during the fetal or neonatal period dose-dependently inhibits the development of male genitalia, while the inhibitory effects of DES at a low level are similar to those of DES at the highest level and appear after maturation of animals [23, 31]. Therefore, the line describing the changes between DES at a low dose (0.5 μg/kg/day) and the changes in the plasma testosterone level observed in this experiment may be U-shaped.

Decreased testosterone secretion induces pituitary secretion of LH, which promotes synthesis of testosterone by testicular Leydig cells. However, in this study, despite the decreased testosterone level in the DES 1.5 group at 6 weeks and in the DES 0.5 group at 15 weeks of age, there was no increase in the level of LH. Although the testosterone assay kit used in this study could not detect testosterone in pups at 3 weeks of age, the LH levels were increased in both DES groups. Therefore, testosterone secretion may have been inhibited in the DES groups at 3 weeks of age. These results suggest that DES administration altered pituitary function at 6 weeks of age, although it did not influence the pituitary at 3 weeks of age. Since it has been reported that administration of more than 100 times the dose of DES used in the present study to rats on days 13, 15 and 17 of gestation reduces the plasma testosterone level at 20 days of gestation, but does not change the LH content of the pituitary [2], further studies are necessary to investigate how the pituitary is involved in the postnatal decrease in testosterone after prenatal administration of DES.

LH binds to the testicular Leydig cell LHr and promotes StAR expression mainly via the cAMP-stimulated extracellular signal-regulated kinase, thereby activating steroid synthesis [32]. Androgen treatment enhances expression of AR and LHr mRNA in progenitor Leydig cells [19]. In this study, the level of LHr mRNA expression in the DES 1.5 group at 6 weeks of age was about two times higher than those of the other two groups, despite the markedly reduced plasma testosterone and unaltered plasma LH levels. This low level of testosterone may not directly up-regulate LHr mRNA expression. In addition, StAR expression in the DES 1.5 group did not increase with the rise in LHr, but actually decreased markedly. Testosterone has been reported to directly inhibit StAR transcription [33]. However, StAR expression was inhibited despite the low level of testosterone in the DES 1.5 group, suggesting that DES administration disrupts LHr expression, exerts inhibitory effects on signal transduction from LHr to StAR and, moreover, directly inhibits StAR expression.

It has been reported that DES administration in the fetal and newborn periods increases expression levels of mRNA for the steroid hormone-synthesizing enzymes P450scc and $P450_{17\alpha}$ (17α-hydroxylase + $C_{17\text{-}21}$ lyase) and that prenatal DES administration reduces $P450_{17\alpha}$ in the testis after birth [34]. In addition, another study has reported that when the rat embryonic testis at 14 days of gestation is cultured in the presence of DES, the level of testosterone secretion decreases after 75 h [35]. These observations suggest that the decrease in the plasma testosterone levels in the DES groups observed in the present experiment was due to the direct action of the administered DES on the embryonic testis to influence its ability to synthesize testosterone. In the present study, no changes were observed in enzyme mRNA expression that could account for the decrease in the testosterone levels in the DES 1.5 group at 6 weeks of age and the DES 0.5 group at 15 weeks of age. These results suggest that factors other than steroid hormone-synthesizing enzymes affect the testosterone levels, such as, changes in the activity of testosterone-metabolizing cytochrome P450s (especially CYP2Bs) in the liver.

Our laboratory has previously demonstrated that administration of two polychlorobiphenyls (PCB126 and PCB169) with different structures to pregnant rats during the same period of pregnancy as in the present study causes postnatal decreases in the level of testosterone and percentage of testicular Leydig cells in pups [36]. However, in the present study, DES administration did not influence the total volume of Leydig cells, suggesting that it has no inhibitory effect on their development.

Anti-Müllerian hormone (AMH) expression is inversely proportional to the serum concentration of testosterone in males after birth, *in vitro* AMH can lower testosterone production by mature rat Leydig cells and AMH-mediated down-regulation of expression of $P450_{17\alpha}$ and P450scc contributes to this [37, 38]. In the present study, the AMH mRNA expression level decreased in the DES 0.5 group at 6 weeks of age. However, the expression levels of $P450_{17\alpha}$ and P450scc mRNA and the plasma testosterone level of this group did not change in the manner described above; therefore, AMH is not likely to be directly involved in the decrease in the testosterone level. The decrease in the testosterone level induced by DES treatment may be caused by the activation of aromatase, which catalyzes testosterone to estrogen and/or 5α-reductase, which then converts testosterone to dihydrotestosterone. However, the changes in P450arom, ERα and 5α-reductase mRNA expression in the present study did not parallel the decrease of testosterone.

In immature animals, testosterone is an important factor regulating the differentiation of Leydig cells in an autocrine manner. When immature Leydig cells are cultured in the presence of androgen and LH, synthesis of testosterone is promoted [39]. On the other hand, in the mature testis, androgen itself is considered to inhibit steroid hormone-synthesizing enzymes, thereby limiting synthesis of androgen [40, 41]. In the DES groups at 15 weeks of age, the expression levels of AR mRNA increased. The testosterone level clearly differed between the DES groups, but the levels of both groups were lower than that in the control group, suggesting that low levels of testosterone induced AR expression. However,

we believe that the AR level increased to maintain spermatogenesis in the testis rather than to regulate testosterone synthesis.

In our previous study [27], DES administration disrupted the spermatogenic cycle at 15 weeks after birth in comparison with 6 weeks of gestation, but only five 15-week-old pups were used to examine spermatogenesis, although ten 6-week-old pups were employed in other analyses. Hess *et al*. [42] employed the testes of 10 pups and demonstrated that classification of more than 200 seminiferous tubules per pup provided sufficiently reliable data. Thus, in the present study, to more closely examine spermatogenesis, we observed more than 500 seminiferous tubules per animal and demonstrated that DES administration at 15 weeks of age had little or no effect on spermatogenesis. Some rat-based studies have demonstrated that Sertoli cells require high levels of testicular testosterone to support spermatogenesis [43–45]. Testosterone is known to preferentially induce conversion of round spermatids between stages VII and VIII [46–49], and this coincides with the maximal immunoexpression of AR protein in Sertoli cells [50]. In the present study, the percentage of stage IV seminiferous tubules was increased in the DES 1.5 group at 6 weeks of age. Although the percentages of stages VII and VIII seminiferous tubules remained unchanged, the testosterone levels were decreased, suggesting that the rate of transition from stages VII to VIII was reduced, resulting in an increase in the number of stage IV seminiferous tubules. The cause of the increase in the percentages of stage IV and V seminiferous tubules in the DES 0.5 group at 15 weeks of age was similar to that described above.

Transgenic mice overexpressing rat androgen-binding protein in their testes show progressive abnormalities of spermatogenesis, eventually leading to infertility [51, 52]. ABP-transgenic mice have increased total intratesticular testosterone concentrations but decreased free testosterone levels, suggesting that the decrease in the availability of free testosterone lowers germ cell numbers and reduces fertility [53]. Furthermore, in the present experiment, the ABP mRNA expression level was significantly higher in the DES 1.5 group at 6 weeks of age compared with the other groups and in the DES 0.5 and 1.5 groups at 15 weeks of age compared with the control group. In the DES 1.5 group at 6 weeks of age, the total plasma testosterone level decreased, but the total intratesticular testosterone level did not. These results are in agreement with those of Jeyaraj *et al*. (2005). Therefore, we speculate that the level of intratesticular free testosterone, which was not measured in this experiment, was decreased, but not to a level that would disrupt spermatogenesis. The ABP mRNA expression level increased in both DES groups at 15 weeks of age, but there was a less marked change in spermatogenesis at 15 weeks than at 6 weeks of age. On histological observation, DES treatment did not alter the number of germ cells in any stage of spermatogenesis. We speculate that, although the level of intratesticular free testosterone was also decreased at 15 weeks of age, the marked increase in AR mRNA expression may have inhibited disruption of spermatogenesis. In the immature testis, ARs are more abundantly expressed in Leydig cells than in Sertoli cells, but they are more abundantly expressed in the Sertoli cells in the mature testis; thus, androgens exert their action on spermatogenesis through activation of ARs in Sertoli cells [54, 55]. If the AR increase observed in this experiment was mainly due to the rise in ARs in Sertoli cells, it might explain why no significant change in spermatogenesis occurred despite the decrease in the plasma cholesterol level in the DES 0.5 group. Administration of DES at an about 10-fold higher dose from 12 days of gestation till weaning (20 days after birth) reduces the number of Sertoli cells per testis at 3 weeks of age [56]. In the testis, AR expression is enhanced in Leydig cells during the immature period (21 days of age) but is enhanced in Sertoli cells after maturation (90 days of age) [54]. Combining these with our findings, the overall testicular AR expression level was not changed at 6 weeks of age and increased at 15 weeks of age, although the number of Sertoli cells decreased, suggesting that the AR expression level per Sertoli cell may have increased.

References

1. **Kemper CH, Peters PW**. Migration and proliferation of primordial germ cells in the rat. *Teratology* 1987; 36: 117–124.
2. **Haavisto T, Nurmela K, Pohjanvirta R, Huuskonen H, El-Gehani F, Paranko J**. Prenatal testosterone and luteinizing hormone levels in male rats exposed during pregnancy to 2,3,7,8-tetrachlorodibenzo-p-dioxin and diethylstilbestrol. *Mol Cell Endocrinol* 2001; 178: 169–179.
3. **Haavisto TE, Adamsson NA, Myllmaki SA, Toppari J, Paranko J**. Effect of 4-tert-octylphenol, 4-tert-butylphenol, and diethylstilbestrol on prenatal testosterone surge in the rat. *Reprod Toxicol* 2003; 17: 593–605.
4. **Warita K, Sugawara T, Yue ZP, Tsukahara S, Muto K, Hasegawa Y, Kitagawa H, Hoshi N**. Progress of the dose-related effects of estrogenic disruptors, an important factor in declining fertility, differs between the hypothalamic-pituitary axis and reproductive organs of male mice. *J Vet Med Sci* 2006; 68: 1257–1267.
5. **Yamamoto M, Shirai M, Sugita K, Nagai N, Miura Y, Mogi R, Yamamoto K, Tamura A, Arishima K**. Effects of maternal exposure to diethylstilbestrol on the development of the reproductive system and thyroid function in male and female rat offspring. *J Toxicol Sci* 2003; 28: 385–394.
6. **Couse JF, Lindzey J, Grandeien K, Gustagsson JA, Korach KS**. Tissue distribution and quantitative analysis of estrogen receptor-α (ERα) and estrogen receptor-β (ERβ) messenger ribonucleic acid in the wild-type and ERα-knockout mouse. *Endocrinology* 1997; 138: 4613–4621.
7. **Jefferson WN, Couse JF, Banks EP, Korach KS, Newbold RR**. Expression of estrogen receptor is developmentally regulated in reproductive tissue of male and female mice. *Biol Reprod* 2000; 62: 310–317.
8. **Saunders PTK, Maguire SM, Gaughan J, Millar MR**. Expression of estrogen receptor β (ERβ) in multiple rat tissues. *J Endocrinol* 1997; 154: R13–R16.
9. **Saunders PTK, Fisher JS, Sharpe RM, Millar MR**. Expression of oestrogen receptor beta[ERβ] occurs in multiple cell types, inducing some germ cells, in the rat testis. *J Endocrinol* 1998; 156: R13–R17.
10. **van Pelt AMM, de Rooij DG, van der Burg B, van der Saag PT, Gustafsson J-Å, Kuiper GJM**. Ontogeny of estrogen receptor-β expression in rat testis. *Endocrinology* 1999; 140: 478–483.
11. **Eddy EM, Washburn TF, Bunch DO, Goulding EH, Gladen BC, Lubahn DB, Korach KS**. Targeted disruption of the estrogen receptor gene in male mice causes alteration of spermatogenesis and infertility. *Endocrinology* 1996; 137: 4796–4805.
12. **Fisher CR, Granes KH, Parlow AF, Simpson ER**. Characterization of mice deficient in aromatase(ArKO) because of targeted disruption of the cyp19 gene. *Proc Natl Acad Sci USA* 1998; 95: 6965–6970.
13. **Hess RA, Bunick D, Lee K-H, Bahr J, Taylor JA, Korach KS, Lubahn DB**. A role for estrogens in the males reproductive system. *Nature* 1997; 390: 509–512.
14. **Robertson KM, O'Donnell L, Jones ME, Meachem SJ, Boon WC, Fisher CR, Graves KH, McLachlan RI, Simpson ER**. Impairment of spermatogenesis in mice lacking a functional aromatase (cyp19) gene. *Proc Natl Acad Sci USA* 1999; 96: 7986–7991.
15. **Sharpe RM**. The roles of oestrogen in the male. *Trends Endocrinol Metab* 1998; 9: 371–377.
16. **Panet-Raymond V, Gottlieb B, Beitel LK, Pinsky L, Trifiro MA**. Interactions between androgen and estrogen receptors and the effects on their transactivational properties. *Mol Cell Endocrinol* 2000; 167: 139–150.
17. **Yeh S, Miyamoto H, Shima H, Chang CH**. From estrogen to androgen receptor: a new pathway for sex hormones in prostate. *Proc Natl Acad Sci USA* 2000; 95: 5527–5532.
18. **Williams K, McKinnell C, Saunders PTK, Walker M, Fisher JS, Turner KJ, Atanass-**

ova N, Sharpe M. Neonatal exposure to potent and environmental oestrogens and abnormalities of the male reproductive system in the rat: evidence for importance of the androgen-oestrogen balance and assessment of the relevance to man. *Hum Reprod Update* 2001; 7: 236–247.

19. **Shan LX, Hardy JF, Catterall MP**. Effects of luteinizing hormone (LH) and androgen on steady state levels of messenger ribonucleic acid for LH receptors, androgen receptors, and steroidogenic enzymes in rat Leydig cell progenitors *in vivo*. *Endocrinology* 1995; 136: 1686–1693.
20. **McKinnell C, Atanassov N, Williams JS, Fisher JS, Walker M, Turner KJ, Saunders TK, Sharpe RM**. Suppression of androgen action and the induction of gross abnormalities of the reproductive tract in male rats treated neonatally with diethylstilbestrol. *J Androl* 2001; 22: 323–338.
21. **Prins GS, Birch L**. The developmental pattern of androgen receptor expression in rat prostate lobes is altered after neonatal exposure to estrogen. *Endocrinology* 1995; 136: 1303–1314.
22. **Atanassova N, McKinnell C, Walker M, Turner KJ, Fisher JS, Morley M, Millar MR, Groome NP, Sharpe RM**. Permanent effects of neonatal estrogen exposure in rats on reproductive hormones levels, Sertoli cell number, and the efficiency of spermatogenesis in adulthood. *Endocrinology* 1999; 140: 5364–5373.
23. **Atanassova N, McKinnell C, Turner KJ, Walker M, Fisher JS, Morley M, Millar MR, Groome NP, Sharpe RM**. Comparative effects of neonatal exposure of male rats to potent and week (environmental) estrogens on spermatogenesis at puberty and relationship to adult testis size and fertility: Evidence for stimulatory effects of low estrogen levels. *Endocrinology* 2000; 141: 3898–3907.
24. **Goyal HO, Braden TD, Mansour M, Willimas CS, Kamaleldin A, Srivastava KK**. Diethylstilbestrol-treated adult rats with altered epididymal sperm numbers and sperm motility parameters, but without alterations in sperm production and sperm morphology. *Biol Reprod* 2001; 64: 927–934.
25. **Goyal HO, Robateay A, Braden TD, Williams CS, Srivastava KK, Ali K**. Neonatal estrogen exposure of male rats alters reproductive functions at adulthood. *Biol Reprod* 2003; 68: 2081–2091.
26. **Sharpe RM, Fisher JS, Millar MM, Jobling S, Sumpter JP**. Gestational and lactational exposure of rats to xenoestrogens results in reduced testicular size and sperm production. *Environ Health Perspect* 1995; 103: 1136–1143.
27. **Yamamoto M, Shirai M, Tamura A, Kobayashi T, Kohara S, Murakami M, Arishima K**. Effects of maternal exposure to a low dose of diethylstilbestrol on sexual dimorphic nucleus volume and male reproductive system in rat offspring. *J Toxicol Sci* 2005; 30: 7–18.
28. **Guyot R, Odet F, Leduque P, Forest MG, Magueresse-Battistoni BL**. Diethylstilbestrol inhibits the expression of the steroidogenic acute regulatory protein in mouse fetal testis. *Mol Cell Endocrinol* 2004; 220: 67–75.
29. **Hess RA**. Quantitative and qualitative characteristics of the stages and transitions in the cycle of the rat seminiferous epithelium: light microscopic observations of perfusion-fixed and plastic-embedded testis. *Biol Reprod* 1990; 43: 525–542.
30. **Boylan ES**. Morphological and functional consequences of prenatal exposure to diethylstilbestrol in the rat. *Biol Reprod* 1978; 19: 854–863.
31. **vom Saal FS, Timms BG, Timms M, Montano TM, Palanza P, Tyayer KA, Nagel SC, Dhar MD, Ganjam VK, Parmigiani S, Welshons WV**. Prostate enlargement in mice due to fetal exposure to low doses of estradiol or diethylstilbestrol and opposite effects at high doses. *Proc Natl Acad Sci USA* 1997; 94: 2056–2061.
32. **Gyles SL, Burns CJ, Whitehouse BJ, Sugden D, Marsh PJ, Persaud SJ, Jones PM**. ERKs regulate cyclic AMP-induced steroid synthesis through transcription of the steroidogenic acute regulatory (StAR) gene. *J Biol Chem* 2001; 276: 34888–34895.
33. **Houk CP, Pearson EJ, Martinelle N, Donahoe PK, Teixeira J**. Feedback inhibition of steroidogenic acute regulatory protein expression *in vitro* and *in vivo* by androgens. *Endocrinology* 2004; 145: 1269–1275.
34. **Majdic G, Sharpe RM, O'Shaughnessy PJ, Saunders PT**. Expression of cytochrome P450 17alpha-hydroxylase/C17-20 lyase in the fetal, rat testis is reduced by maternal exposure to exogenous estrogens. *Endocrinology* 1996; 137: 1063–1070.
35. **Lassurguére J, Livera G, Habert R, Jegou B**. Time- and dose-related effects of estradiol and diethylstilbestrol on the morphology and function of the fetal rat testis in culture. *Toxicol Sci* 2003; 73: 160–169.
36. **Yamamoto M, Narita A, Kagohata M, Shirai M, Akahori F, Arishima K**. Effects of maternal exposure to 3,3′,4,4′,5-Pentachlorobiphenyl (PCB126) or 3,3′,4,4′,5,5′-Hexachlorobiphenyl (PCB169) on testicular steroidogenesis and spermatogenesis in male offspring rats. *J Androl* 2005; 26: 205–214.
37. **Lyet L, Louis F, Forest MG, Josso N, Behringer RR, Vigier B**. Ontogeny of reproductive abnormalities induced by deregulation of anti-müllerian hormone expression in transgenic mice. *Biol Reprod* 1995; 52: 444–454.
38. **Trbovich AM, Sluss PM, Laurich VM, O'Neill FH, MacLaughlin DT, Donahoe PK, Teixeira J**. Müllerian inhibiting substance lowers testosterone in luteinizing hormone-stimulated rodents. *Proc Natl Acad Sci USA* 2001; 98: 3393–3397.
39. **Hardy MP, Kelce WR, Klinefelter GR, Ewing LL**. Differentiation of Leydig cell precursors *in vitro*: a role for androgen. *Endocrinology* 1990; 127: 488–490.
40. **Darney KJ.Jr, Zirkin BR, Ewing LL**. Testosterone autoregulation of its biosynthesis in the rat testis: inhibition of 17 alpha-hydroxylase activity. *J Androl* 1996; 7: 137–142.
41. **Ruiz de Galarreta CM, Fanjul LF, Meidan R, Hsueh AJ**. Regulation of 3 beta-hydroxysteroid dehydrogenase activity by human chronic gonadotropin, androgens, and anti-androgens in cultured testicular cells. *J Biol Chem* 1983; 58: 10988–10996.
42. **Hess RA, Schaeffer DJ, Eroschenko VP, Keen JE**. Frequency of the stages in the cycle of the seminiferous epithelium in the rat. *Biol Reprod* 1990; 43: 517–524.
43. **Wright WW, Frankel AI**. Endogenous androgen concentrations in nuclei isolated from seminiferous tubules of mature rat testes. *J Steroid Biochem* 1979; 10: 633–640.
44. **Rommerts FFG**. How much androgen is required for maintenance of spermatogenesis? *J Endocrinol* 1998; 116: 7–9.
45. **Veldscholte J, Berrevoets CA, Ris-Stalpers C, Kuiper GG, Jenster G, Trapman J, Brinkmann AO, Mulder E**. The androgen receptor in LNCaP cells contains a mutation in the ligand binding domain which affects steroid binding characteristics and response to antiandrogens. *J Steroid Biochem Mol Biol* 1992; 41: 665–669.
46. **O'Donnell L, McLachlan RI, Wreford NG, Robertson DM**. Testosterone promotes the conversion of round spermatids between stages VII and VIII of the rat spermatogenic cycle. *Endocrinology* 1994; 135: 2608–2614.
47. **O'Donnell L, McLachlan RI, Wreford NG, de Kretser DM, Robertson DM**. Testosterone withdrawal promotes stage-specific detachment of round spermatids from the rat seminiferous epithelium. *Biol Reprod* 1996; 55: 895–901.
48. **Sharpe RM, Maddocks S, Miller M, Saunders PTK, Kerr JB, McKinnell C**. Testosterone and spermatogenesis : identification of stage-dependent, androgen-regulated proteins secreted by adult rat seminiferous tubules. *J Androl* 1992; 13: 172–184.
49. **Kerr JB, Miller M, Maddocks S, Sharpe RM**. Stage-dependent changes in spermatogenesis and Sertoli cells in relation to the onset of spermatogenic failure following withdrawal of testosterone. *Anat Rec* 1993: 235; 547–559.
50. **Bremner WJ, Miller MR, Sharpe RM, Saunders PT**. Immunohistochemical localization of androgen receptors in the rat testis: evidence for stage-dependent expression and regulation by androgens. *Endocrinology* 1994; 135: 1227–1234.
51. **Reventos J, Sullivan PM, Joseph DR, Gordon JW**. Tissue-specific expression of the rat androgen-binding protein/sex hormone-binding globulin gene in transgenic mice. *Mol Cell Endocrinol* 1993; 96: 69–73.
52. **Joseph DR, O'Brien DA, Sullivan PM, Becchis M, Tsuruta JK, Petrusz P**. Overexpression of androgen-binding protein/sex hormone-binding globulin in male transgenic mice: tissue distribution and phenotypic disorders. *Biol Reprod* 1997; 56: 21–32.
53. **Jeyaraj DA, Grossman G, Petrusz P**. Altered bioavailability of testosterone in androgen-binding protein-transgenic mice. *Steroids* 2005; 70: 704–714.
54. **Shan LX, Bardin CW, Hardy MP**. Immunohistochemical analysis of androgen effects on androgen receptor expression in developing Leydig and Sertoli cells. *Endocrinology* 1997; 138: 1259–1266.
55. **Griswold MD**. The central role of Sertoli cells in spermatogenesis. *Semin Cell Dev Biol* 1998; 9: 411–416.
56. **Fielden MR, Halgren RG, Fong CJ, Staub C, Johnson L, Chou K, Zacharewski TR**. Gestational and lactational exposure of male mice to diethylstilbestrol causes long-term effects on the testis, sperm fertilizing ability *in vitro*, and testicular gene expression. *Endocrinology* 2002 ; 143: 3044–3059.