

EXPERIMENTAL STUDIES ON MORPHOLOGICAL CHANGES OF THE EPIDIDYMIS AND TESTIS IN NEONATALLY ESTROGENIZED MICE REVISITED

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Abstract Our primary concern in this review is to give possible explanations and pose new questions on the phenomena concerning effects of neonatal estrogen exposure on the development and functions of reproductive organs and immune mechanisms particularly in male animals, in the light of new findings and concepts. At first, our own experimental results on the pathological changes in the epididymis and testis in neonatally estrogenized mice have been presented. The results of our first experiment showed that neonatal single injection of 10 μ g of estradiol benzoate induced marked inflammatory changes in the epididymis and testis, in which the initial pathological change occurred concomitantly with the appearance of sperms in the epididymal duct. The second experiment showed that such epididymo-testicular changes did not occur when the testis was removed or neonatal estrogen was followed by testosterone. From these results, a possibility was suggested that a hormone-related autoimmune mechanism might participate in such changes. Relating to these, various reports concerning effects of neonatal estrogen treatment, either alone or with concomitant treatment with androgen, on the development and functions of reproductive organs and immune mechanisms in the adolescent or adult have been reviewed. In addition, advances in the related areas, such as estrogen-related mechanisms in the development and functions of reproductive organs and immune mechanisms including autoimmune disease mechanisms, gender differences and the role of CD25⁺ CD4⁺ T lymphocytes have also been reviewed briefly.

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Key words: perinatal sex steroids; development of reproductive organs; epididymo-testicular inflammatory change; autoimmune disease; regulatory T lymphocyte

Results on neonatal estrogen-induced epididymo-testicular lesions and their experimental analyses have been described and discussed relating to possible mechanisms.

Effects of neonatal sex steroid treatment or thymectomy on the development and functions of reproductive organs

Experimental studies mainly using female laboratory rodents clarified that perinatal exposure to sex steroids has marked effects on differentiation of the reproductive organs and the brain, and that the male type of brain involved in male type of behavior and acyclicity of gonadotropic hormones is induced by androgen

in this critical period irrespective of the genetic type^{1,2,3}. Curiously, estrogen was also capable to cause persistent vaginal cornification in adult female mice⁴.

In males it was shown that spermatogenesis is markedly suppressed by long-term perinatal exposure to estrogen^{5,6}. Arai (1970)⁷ showed that the suppressing effect of estrogen from birth to 30 days of age on the growth of reproductive organs was inhibited by concomitant administration of testosterone⁷.

On the other hand, Nishizuka and Sakakura (1969)⁸ found that neonatal thymectomy of mice resulted in the developmental arrest of ovary and caused sterility in females. Such

Table 1. Histological changes in the testis and epididymis of neonatally estrogenized mice⁹⁾

A. Patterns of histologic changes

Histologic changes	Pattern				
	A	B	C	D	E
Cell infiltration					
Epididymis					
interstitial	- ~ ±	+	+	+	+
intratubular	-	-	+		
Testis					
interstitial	-	-	- ~ +	+	+
intratubular	-	-	- ~ +		
Necrosis					
Epididymis	-	-	- ~ + (p)	+	(t)
Testis	-	-	- ~ + (p)	+	(t)
Fibrosis	-	-	-	-	+

p, partial; t, total.

B. Frequency of each pattern of histologic changes

Age (days)	Total no. of mice	Pattern of histologic changes				
		A	B	C	D	E
30	15	15 (100%)	—	—	—	—
45	16	1 (6.3%)	15 (93.7%)	—	—	—
60	31	5 (16.1%)	15 (48.4%)	4 (12.9%)	7 (22.6%)	—
120	23	3 (13.0%)	1 (4.35%)	2 (8.7%)	1 (4.35%)	16 (69.6%)

ovarian dysgenesis was observed when mice were thymectomized at 3 days of age, but not at 7 days or later; it was prevented by thymus grafting.

Changes of epididymis and testis in neonatally estrogenized mice: effects of testosterone or hemiorchectomy and possible mechanisms

While we were investigating the relationship between the pineal and hypothalamus using neonatally sex hormone-treated mice, we found by chance in 1969 that neonatal single injection of estrogen induced marked inflammatory changes in the epididymis and testis and reported the results in 1973. Since the mechanism of such pathological changes is still enigmatic and interesting, we describe those results briefly in English here^{9,10)}.

Experiment 1⁹⁾. The study was performed

using mice of Japanese dd-strain which were originally provided from Professor Makino S, Hokkaido University Faculty of Science and most commonly used in Japan. A total of 167 mice were used. In male dd-mice given a single injection of estradiol benzoate (EB) (10 µg per animal) on the first day after birth, epididymo-testicular lesions appeared after they had reached adolescence, and the lesions progressed with advancing age. The development of the lesions was systematically examined at 2-4, 30, 45, 60 and 120 days of age (Table 1).

In the neonatally estrogen-treated group, at 30 days of age, no sperm was seen in the seminiferous tubules in more than half cases, and at 45 days of age, sperms were seen in the epididymal duct in all the cases. After 45 days of age, various patterns of inflammatory changes were often found in the epididymis and testis. It was considered that such changes occurred first

Table 2. Weight values of body, testis, seminal vesicles, thymus and spleen in normal and neonatally estrogenized mice (mean \pm SD) (modified from references^{9,10})

Age (days)	Group of mice	Number (g)	Body (mg)	Testis (mg)	Seminal vesicles (mg)	Thymus (mg)	Spleen (mg)
Experiment-1, 2							
30	N	20	20.0 \pm 3.0	56.2 \pm 7.9	30.0 \pm 12.8	59.2 \pm 10.5	97.2 \pm 24.7
	E-10 μ g-1d	15	16.5 \pm 2.6*	35.0 \pm 10.1**	7.5 \pm 3.1**	61.9 \pm 15.7	88.7 \pm 26.7
45	N	17	25.0 \pm 1.1	87.4 \pm 8.3	131.7 \pm 24.9	34.6 \pm 7.4	78.0 \pm 20.9
	E-10 μ g-1d	16	25.3 \pm 1.3	84.0 \pm 19.2	62.9 \pm 27.1**	52.1 \pm 12.0**	110.4 \pm 27.1**
60	N	20	28.1 \pm 2.4	102.8 \pm 10.7	197.0 \pm 34.1	25.9 \pm 6.8	82.7 \pm 17.4
	E-10 μ g-1d	31	26.6 \pm 3.1	—	78.5 \pm 45.7**	22.7 \pm 10.8	148.6 \pm 91.8
120	N	13	29.7 \pm 3.4	105.7 \pm 10.9	212.4 \pm 41.7	19.7 \pm 2.9	91.4 \pm 25.1
	E-10 μ g-1d	23	31.0 \pm 2.6	—	78.5 \pm 59.7** ^a	23.0 \pm 10.9	121.4 \pm 58.9
Experiment-2							
60	E-1 μ g-1d	11	26.0 \pm 4.7	110.4 \pm 12.1	143.6 \pm 44.3*	16.9 \pm 6.1*	79.2 \pm 14.8
	E-10 μ g-1,2,3d	10	26.8 \pm 1.2	—	42.9 \pm 21.7**	24.7 \pm 8.1	220.2 \pm 149.6**
120	E-1 μ g-1d	9	31.4 \pm 1.8	113.4 \pm 6.5	205.7 \pm 29.3	14.3 \pm 3.1*	88.0 \pm 17.6
	E-10 μ g-1,2,3d	10	29.5 \pm 1.5	—	94.9 \pm 66.4** ^b	20.9 \pm 6.3	122.7 \pm 58.8
	E-10 μ g-1d, T-2,3d	14	30.1 \pm 2.2	107.1 \pm 7.3	189.5 \pm 26.1 ^a	12.9 \pm 2.5**	80.3 \pm 12.6
	E-10 μ g-1,2,3d, T-4,5d	10	30.0 \pm 1.3	99.4 \pm 8.3	171.8 \pm 39.7 ^b	14.3 \pm 4.0*	110.1 \pm 62.4

N, normal, untreated; E, estrogen-treated; T, testosterone (250 μ g) -treated.

Significantly different compared with normal control: * P<0.01, ** P<0.001.

Significantly different: ^a P<0.001, ^b P<0.01.

in the epididymis and then in the testis. The epididymis was often infiltrated by lymphocytes or by lymphocytes, macrophages and neutrophils in the interstitial tissue, and its epithelial cells had occasional cytoplasmic vacuoles.

At 60 days, marked changes occurred not only in the epididymis but also in the testis. In about 35% of the cases, the epididymis or both the epididymis and testis were macroscopically swollen and hardened with yellowish appearance, and they were often fused with the surrounding tissue. Microscopically, in about half of the cases, the epididymis underwent interstitial cell infiltration. In 13% of the cases, marked neutrophil leucocyte infiltration occurred not only in the interstitial tissue of the epididymis but also within the epididymal tubules and in the testis, and occasional necrotic foci were also found in these organs. In 23%, both the epididymis and testis appeared necrotic almost totally. At 120 days, the incidence of the cases with above-mentioned remarkable macroscopic

lesions increased further to about 75%.

Body and seminal vesicle weights showed lower values, and the relative weight of thymus at 30 days of age (P<0.001) and both absolute and relative weights of thymus at 45 days of age showed higher values, in neonatally estrogenized male mice than in controls (Table 2).

From the reported results on experimental auto-allergic epididymo-testicular lesions¹¹⁻¹³), a possibility was proposed that an autoimmune mechanism might participate in the pathological mechanisms of these changes.

1) Experiment 2¹⁰). The epididymo-testicular lesions in neonatally estrogenized mice were studied in relation to the dosage of estrogen, age of injection and influences of testosterone administration and hemiorchiectomy following injection, using 268 dd-mice. Mice were given a single injection of 1 or 20 μ g of EB at 1 day of age. An injection of 1 μ g of EB produced no change in both organs at 60 and 120 days. All mice given

- 20 μg of EB died shortly after injection.
- 2) Injections of EB (10 $\mu\text{g}/\text{day}$) at 1, 2 and 3 days of age caused epididymo-testicular lesions such as seen in mice given a single injection of 10 μg at 1 day. Particularly necrosis and/or fibrosis were seen in about 50 % at 60 days and in 60 % at 120 days.
 - 3) Injections of EB (10 $\mu\text{g}/\text{day}$) at 1, 3, 5, 7 and 9 days produced inhibition of spermatogenesis, and their epididymis underwent interstitial leucocyte infiltration, but marked inflammatory lesions did not appear at 45 and 60 days. At 120 days, however, marked lesions appeared such as seen in mice given injections of EB one or three times, the necrosis and/or fibrosis were seen in 60 %.
 - 4) Administration of EB (10 $\mu\text{g}/\text{day}$) at 3 days or EB (20 $\mu\text{g}/\text{day}$) at 3 days, 3 and 4 days or 3, 4 and 5 days produced no changes in the epididymis and testis at 120 or 240 days except for a few cases with slight interstitial leucocyte infiltration in the epididymis.
 - 5) Following injections of EB (10 $\mu\text{g}/\text{day}$) at 1 day, or 1, 2 and 3 days of age, testosterone (250 $\mu\text{g}/\text{day}$) was injected at 2 and 3 days or 4 and 5 days of age, respectively. In these mice, epididymo-testicular lesions were not seen at 120 days.
 - 6) Hemi-orchectomy was performed at 30 days of age in mice which had been injected EB (10 $\mu\text{g}/\text{day}$) at 1 day, or 1, 2 and 3 days. No lesions appeared on the operated side at 120-210 days, but about 55% of the cases showed the above-mentioned lesions on the contralateral side.

From these results, it was evident that epididymo-testicular lesions induced by neonatal estrogen exposure were triggered by the appearance of sperms in the epididymal duct, and that such lesions were blocked by neonatal exposure to testosterone following estrogen. Although the appearance of abnormal sperms due to neonatal estrogen exposure has been

reported¹⁴, it remains to be determined whether abnormal sperms are the cause of these lesions. In addition, it has been thought that sperms are separated from immune mechanisms by the barrier system in the epithelium of seminiferous tubules, i.e. the blood-testis barrier¹³, or epididymal duct. Is there evidence of any developmental defects of the epididymal duct epithelium, i.e. insufficient immunological barrier, and/or defects of immune regulatory mechanisms? What is the target of the blocking effect of testosterone?

Following our reports, the appearance of spermgranuloma in the adulthood was also shown by Rustia and Shubik (1976)¹⁵ in the male offspring of pregnant Syrian golden hamsters in which diethylstilbestrol (DES) was injected during the late gestational period. On the other hand, Nishizuka and coworkers found various autoimmune diseases including orchitis induced by neonatal thymectomy¹⁶⁻¹⁹.

Aromatase, estrogens as carcinogen, estrogen receptors, and alpha-fetoprotein

MacLusky and Naftolin (1981)²⁰ proposed that the aromatase which converts androgen to estrogen exists in the brain and that estrogen has masculinizing capacity in the male brain of rodents, although the situation appears to be somewhat different in primates. From many experimental results on the effects of neonatal estrogen on the development of male reproductive organs^{14,21-24}, it became apparent that estrogen has pathological, i.e. teratogenic and tumorigenic, activities^{21,22,24}, as suggested by Takasugi (1963)⁴. These results may suggest that the blocking effect of testosterone in our experiment 2 is due to peripheral action mechanisms. On the other hand, Kalland and Holmdahl (1988)²⁵ stated in their review: "The study of the biological consequences of perinatal DES exposure has mostly concentrated on implications for tumor development. We regard

it highly probable, however, that disturbances in immune regulation will lead to autoimmune manifestations and enhance susceptibility to infections.... Tolerance is the central issue of immune deviations such as allergy and autoimmune disease. ...exogenous factors can have strong imprinting effects on the immature immune system..." Forsberg (1996)²⁶⁾ intensively investigated the thymus in neonatally DES-treated female mice. Their results on body and thymus weights were similar to ours. Neonatal DES treatment resulted in an ovary-independent thymus enlargement and a reduced bone marrow cellularity at 8 weeks after the treatment, whereas neonatal treatment with a LH-releasing hormone antagonist reduced thymus weight at 8 weeks. In the same year, the Gustafsson's group reported important findings that estrogen receptors (*ER α* and *ER β*) exist in the prostate gland and ovary^{27,28)}.

It seems interesting to note here that the onco-fetal protein, α -fetoprotein (AFP), is also able to bind estrogen and was used as a serum marker of cancer and more recently employed in the detection of congenital defects²⁹⁾. AFP exists in developing murine brains throughout fetal and postnatal development for up to 20-25 days following birth. Recently it was reported that AFP null females are infertile because of a dysfunction of the hypothalamo-pituitary system involving positive feedback control by estrogen which leads to anovulation³⁰⁾. Interestingly, the development of experimental autoimmune thyroiditis was reported to be suppressed in transgenic mice producing human AFP, and AFP was suggested to modulate T cell development and/or T-cell dependent immune responses³¹⁾.

Effects of neonatal estrogen on reproductive organs

From many experimental data on the prostate gland³²⁻³⁸⁾, Prins and coworkers proposed that neonatal estrogen causes prostatic growth and

differentiation defects which result in shifting the prostate from an androgen-dominated gland to that regulated by estrogens and retinoids and possibly predisposed to the neoplastic state³⁸⁾.

On the other hand, recently Atanassova et al. reported using male rats firstly (2001)³⁹⁾ that during postnatal development of the epididymis and vas deferens, *ER α* (but not *ER β*) showed age-, cell- and region-specific immunoeexpression, of which pattern was disrupted by neonatal DES treatment; and secondly (2005)⁴⁰⁾ that neonatal DES treatment induced stromal-epithelial abnormalities of the vas deferens and cauda epididymis in adulthood following delayed basal cell development, which was blocked by neonatal cotreatment of testosterone. Rivas et al. reported using male rats firstly (2002)⁴¹⁾ that reproductive tract developmental abnormalities were induced by lowering androgen production or action in combination with a low dose of DES, showing the evidence for the importance of the androgen-estrogen balance; and secondly (2003)⁴²⁾ that the neonatal coadministration of testosterone with DES prevented DES induction of most reproductive tract abnormalities in male rats, coincident with the restoration of normal/supranormal testosterone levels and the normal immunoeexpression of androgen receptors and *ER α* in tissues studied. Therefore, it is likely that neonatally estrogenized mice show insufficient development of the epithelia of epididymal duct and seminiferous tubules, which suggests defects of the immunological barrier to sperm.

Autoimmune diseases, regulatory T lymphocytes and effects of sex steroids on immune mechanisms

It has long been known that many autoimmune diseases are much more common in women than in men, and numerous experimental studies have been done about the gender difference of immune mechanisms⁴³⁻⁴⁷⁾. For example, estrogens have been shown to exacerbate

systemic lupus erythematosus in murine models of the disease^{48,49}. Quantitative differences in relative numbers of functional T cells have been related to gender⁵⁰⁻⁵³, and higher CD4:CD8 ratios due to relatively lower numbers of circulating CD8 T cells have generally been seen in females and hypogonadal males⁵⁴. According to Olsen et al. (1998)⁵⁵, acceleration of thymocyte apoptosis by androgens may mediate processes of thymocyte selection, with the potential to impart gender-specific characteristics on the peripheral T cell repertoire. Estrogen has been shown to interfere with tolerance induction of naïve autoreactive B cells, of which the presence in the periphery is associated with up-regulation of the antiapoptotic Bcl-2 protein⁵⁶. On the other hand, ER- α appears to be necessary for development of full-size thymus and spleen⁵⁷ and normal proportions of bone marrow B cells⁵⁸, whereas expression of ER- β is required for estradiol-mediated, possibly pregnancy-induced, thymic atrophy. In addition, the suppressive effect of androgens on developing B cells has been reported to be mediated by TGF- β in bone marrow stromal cells⁵⁹.

Sakaguchi et al. (1982, 1995) found that the neonatal thymectomy-induced autoimmune oophoritis is prevented by a single intraperitoneal injection of thymocytes from normal adult female mice⁶⁰ and that the CD25 molecule (the interleukin (IL)-2 receptor α -chain) is a reliable marker of regulatory T cells⁶¹. Davidson and Diamond (2001)⁴⁵ presented following views on autoimmune diseases. A low level of autoreactivity is physiologic and crucial to normal immune function. Since there is no fundamental difference between the structure of self antigens and that of foreign antigens, lymphocytes are thought to have evolved not to distinguish self from foreign, but to respond to antigen only in certain microenvironments, generally in the presence of inflammatory cytokines. Several kinds of regulatory cells are

important in controlling autoreactivity: CD1-restricted T cells, T cells with γ/δ receptors, CD4⁺CD25⁺ T cells, and T cells that produce cytokines that suppress pathogenic autoreactive cells. Some of these regulatory cells must mature in the thymus; others require activation by autoantigens in the periphery.

According to Sakaguchi et al. (2001)⁶², there is accumulating evidence that T cell-mediated dominant control of self-reactive T-cells contributes to the maintenance of immunologic self-tolerance and its alteration can cause autoimmune disease. CD25⁺ cells in the CD4⁺ population in normal naïve animals bear the ability to prevent autoimmune disease *in vivo* and, upon antigenic stimulation, suppress the activation/proliferation of other T cells *in vitro*. The CD25⁺CD4⁺ regulatory T cells are naturally anergic and suppressive, and appear to be produced by the normal thymus as a functionally distinct subpopulation of T cells. They play critical roles also in controlling tumor immunity and transplantation tolerance. CD25⁺ T cells constitute 5~10% of peripheral CD4⁺ T cells and less than 1% of peripheral CD8⁺ T cells in normal naïve mice and healthy humans. Furthermore, Aluvihare et al. (2004)⁶³ reported in relation to pregnancy that maternal regulatory T cells suppress an aggressive allogeneic response directed against the fetus in addition to their function in suppressing autoimmune responses and their absence leads to a failure of gestation due to immunological rejection of the fetus.

It has recently been shown that regulatory T cells richly contain Foxp3 molecules and that the mutation causing IPEX (Immune dysregulation, Polyendocrinopathy, Enteropathy, X-linked syndrome) is the mutation of FOXP3-related gene⁶⁴⁻⁶⁶. IPEX is an immune disease caused by the mutation of gene on the X chromosome, and therefore IPEX occurs usually in males in which cells do not have two X chromosomes.

In any case, is the generation of CD25⁺ CD4⁺ regulatory T cells in adolescence more potently suppressed by neonatal estrogen treatment compared with other types of immune cells? If the generation is suppressed, does the neonatal androgen treatment reverse such long-term effect of estrogen on the regulatory T cell production?

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References

- 1) Barraclough CA. Modifications in reproductive function after exposure to hormones during the prenatal and early postnatal period. In: Martini L, Ganong WF, eds. *Neuroendocrinology*. New York: Academic Press, 1967;2:61-99.
- 2) Dörner G. *Hormones and Brain Differentiation*. Amsterdam: Elsevier, 1976.
- 3) Sakuma Y. Mechanisms for brain sex differentiation. In: Hirota K, Nakane A, Suda T, et al., eds. *Emerging Theories of Host Defense*. Hirosaki: Hirosaki Univ Press, 2007; in press.
- 4) Takasugi N. Vaginal cornification in persistent-estrous mice. *Endocrinology* 1963;72:607-619.
- 5) Mori T. Effects of early postnatal injections of estrogen on endocrine organs and sex accessories in male C3H/MS mice. *J Fac Sci, Univ Tokyo, Sec IV* 1967;11:243-54.
- 6) Takasugi N. Testicular damages in neonatally estrogenized adult mice. *Endocrinol Jpn* 1970;17: 277-81.
- 7) Arai Y. Nature of metaplasia in rat coagulating glands induced by neonatal treatment with estrogen. *Endocrinology* 1970;86:918-20.
- 8) Nishizuka Y, Sakakura T. Thymus and reproduction: Sex-linked dysgenesis of the gonad after neonatal thymectomy in mice. *Science* 1969; 166:753-5.
- 9) Kachi T, Kachi H. Morphological changes of the epididymis and testis in neonatally estrogenized mice. *Acta Anat Nippon* 1973;48:202-11.
- 10) Kachi H. Experimental studies of morphological changes in the epididymis and testis of neonatally estrogenized mice. (In Japanese) Thesis, Hokkaido J Med Sci 1974;289-301.
- 11) Waksman BH. A histologic study of the auto-allergic testis lesion in the guinea pig. *J Exp Med* 1959;109:311-24.
- 12) Brown PC, Glynn LE. The early lesion of experimental allergic orchitis in guinea-pigs: an immunological correlation. *J Path* 1969;98:277-82.
- 13) Johnson MH. An immunological barrier in the guinea pig testis. *J Path* 1970;101:129-39.
- 14) Arai Y, Mori T, Suzuki Y, Bern HA. Long-term effects of perinatal exposure to sex steroids and diethylstilbesterol on the reproductive system of male mammals. *Int Rev Cytol* 1983;84:235-68.
- 15) Rustia M, Shubik P. Transplacental effects of diethylstilbestrol on the genital tract of hamster offspring. *Cancer Lett* 1976;1:139-46.
- 16) Kojima A, Tanaka-Kojima Y, Sakakura T, Nishizuka Y. Spontaneous development of autoimmune thyroiditis in neonatally thymectomized mice. *Lab Invest* 1976;34:550-7.
- 17) Kojima A, Taguchi O, Sakakura T, Nishizuka Y. Experimental production of possible autoimmune gastritis followed by macrocytic anemia in athymic nude mice. *Lab Invest* 1980;42:387-95.
- 18) Taguchi O, Nishizuka Y. Experimental autoimmune orchitis after neonatal thymectomy in the mouse. 1981; *Clin Exp Immunol* 1981;46:425-34.
- 19) Taguchi O, Kojima A, Nishizuka Y. Experimental autoimmune prostatitis after neonatal thymectomy in the mouse. *Clin Exp Immunol* 1985;60:123-9.
- 20) MacLusky NJ, Naftolin F. Sexual differentiation of the central nervous system. *Science* 1981;211: 1294-303.
- 21) Herbst AL, Bern HA, eds. *Developmental Effects of Diethylstilbestrol (DES) in Pregnancy*. New York: Thieme-Stratton, 1981.
- 22) Mori T, Nagasawa H, eds. *Toxicity of Hormones in Perinatal Life*. Boca Raton: CRC Press, 1988.

- 23) Tena-Sempere M, Pinilla L, Gonzalez LC, Anguilar E. Reproductive disruption by exposure to exogenous estrogenic compounds during sex differentiation: lessons from the neonatally estrogenized male rat. *Curr Top Steroid Res* 2000;3:23-37.
- 24) Bern HA. Mammalian endocrinology - the developmental effects of estrogens. *Endocr News* 2006;Oct:36.
- 25) Kalland T, Holmdahl R. Estrogens and immunity: long-term consequences of neonatal imprinting of the immune system by diethylstilbestrol. In: Mori T, Nagasawa H, eds. *Toxicity of Hormones in Perinatal Life*. Boca Raton: CRC Press, 1988;111-25.
- 26) Forsberg JG. The different responses of the female mouse thymus to estrogen after treatment of neonatal, prepubertal, and adult animals. *Acta Anat* 1996;157:275-90.
- 27) Kuyper GGJM, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson J-Å. Cloning of a novel estrogen receptor expressed in rat prostate and ovary. *PNAS* 1996;93:5925-30.
- 28) Gustafsson J-Å. Estrogen receptor β —a new dimension in estrogen mechanism of action. *J Endocrinol* 1999;163:379-83.
- 29) Mizejewski GJ. Biological roles of alpha-fetoprotein during pregnancy and perinatal development. *Exp Biol Med* 2004;229:439-63.
- 30) Gabant P, Forrester L, Nichols J, et al. Alpha-fetoprotein, the major fetal serum protein, is not essential for embryonic development but is required for female fertility. *PNAS* 2002;99:12865-70.
- 31) Matsuura E, Kang Y, Kitakawa H, et al. Modulation of T cell function by alpha-fetoprotein: a *in vivo* study on porcine thyroid peroxidase-induced experimental autoimmune thyroiditis in transgenic mice producing human alpha-fetoprotein. *Tumour Biol* 1999;20:162-71.
- 32) Naslund MJ, Coffey DS. The differential effects of neonatal androgen, estrogen and progesterone on adult rat prostate growth. *J Urol* 1986;136:1136-40.
- 33) Prins GS. Neonatal estrogen exposure induces lobe-specific alterations in adult rat prostate androgen receptor expression. *Endocrinology* 1992;130:3703-14.
- 34) Prins GS, Birch L, Couse JF, et al. Estrogen imprinting of the developing prostate gland is mediated through stromal estrogen receptor α : studies with α ERKO and β ERKO mice. *Cancer Res* 2001;61:6089-97.
- 35) Prins GS, Chang WY, Wang Y, Van Breemen RB. Retinoic acid receptors and retinoids are up-regulated in the developing and adult rat prostate by neonatal estrogen exposure. *Endocrinology* 2002;143:3628-40.
- 36) Gilleran JP, Putz O, DeJong M, et al. The role of prolactin in the prostatic inflammatory response to neonatal estrogen. *Endocrinology* 2003;144:2046-54.
- 37) Bianco JJ, McPherson SJ, Wang H, Prins GS, Risbridger GP. Transient neonatal estrogen exposure to estrogen-deficient mice (aromatase knockout) reduces prostate weight and induces inflammation in late life. *Am J Pathol* 2006;168:1869-78.
- 38) Prins GS, Huang L, Birch L, Pu Y. The role of estrogens in normal and abnormal development of the prostate gland. *Ann NY Acad Sci* 2006;1089:1-13.
- 39) Atanassova N, McKinnell C, Williams K, et al. Age-, cell- and region-specific immunoreexpression of estrogen receptor α (but not estrogen receptor β) during postnatal development of the epididymis and vas deferens of the rat and disruption of this pattern by neonatal treatment with diethylstilbesterol. *Endocrinology* 2001;142:874-86.
- 40) Atanassova N, McKinnell C, Fisher J, Sharpe RM. Neonatal treatment of rats with diethylstilboesterol (DES) induces stromal-epithelial abnormalities of the vas deferens and cauda epididymis in adulthood following delayed basal cell development. *Reproduction* 2005;129:589-601.
- 41) Rivas A, Fisher JS, McKinnell C, Atanassova N, Sharpe RM. Induction of reproductive tract developmental abnormalities in the male rat by lowering androgen production or action in combination with a low dose of diethylstilbestrol: evidence for importance of the androgen-estrogen balance. *Endocrinology* 2002;143:4797-808.
- 42) Rivas A, McKinnell C, Fisher JS, et al.

- Neonatal coadministration of testosterone with diethylstilbestrol prevents diethylstilbestrol induction of most reproductive tract abnormalities in male rats. *J Androl* 2003;24:557-67.
- 43) Ahmed SA, Penhale WJ, Talal N. Sex hormone, immune responses, and autoimmune diseases: Mechanisms of sex hormone action. *Am J Pathol* 1985;121:531-51.
- 44) Olsen NJ, Kovacs WJ. Gonadal steroids and immunity. *Endocr Rev* 1996;17:369-84.
- 45) Davidson A, Diamond B. Autoimmune diseases. *N Engl J Med* 2001;345:340-50.
- 46) Imamov O, Shim G-J, Warmer M, Gustafsson J-Å. Estrogen receptor beta in health and disease. *Biol Reprod* 2005;73:866-71.
- 47) Warner M, Gustafsson J-Å. Estrogen receptors in diseases of the immune system. In: Hirota K, Nakane A, Suda T, et al., eds. *Emerging Theories of Host Defense*. Hirosaki: Hirosaki Univ Press, 2007; in press.
- 48) Roubinian JR, Papoian R, Talal N. Androgenic hormones modulate autoantibody responses and improve survival in murine lupus. *J Clin Invest* 1977;59:1066-76.
- 49) Roubinian JR, Talal N, Greenspan JS, Goodman JR, Siiteri PK. Effect of castration and sex hormone treatment on survival, anti-nucleic acid antibodies, and glomerulonephritis in NZB/NZW F₁ mice. *J Exp Med* 1978;147:1568-83.
- 50) Nagel JE, Chrest FJ, Adler WH. Enumeration of T lymphocyte subsets by monoclonal antibodies in young and aged humans. *J Immunol* 1981;127:2086-8.
- 51) Hallgren HM, Jackola DR, O'Leary JJ. Unusual pattern of surface marker expression on peripheral lymphocytes from aged humans suggestive of a population of less differentiated cells. *J Immunol* 1983;131:191-4.
- 52) Mylvaganam R, Ahn YS, Harrington WJ, Kim CI, Gratzner HG. Differences in T cell subsets between men and women with idiopathic thrombopenic purpura. *Blood* 1985;66:967-72.
- 53) Amadori A, Zamarchi R, De Silvestro G, et al. Genetic control of the CD4/CD8 T-cell ratio in humans. *Nature Med* 1995;1:1279-83.
- 54) Bizzarro A, Valentini G, Di Martino G, et al. Influence of testosterone therapy on clinical and immunological features of autoimmune diseases associated with Klinefelter's syndrome. *J Clin Endocrinol Metab* 1987;64:32-6.
- 55) Olsen NJ, Viselli SM, Fan J, Kovacs WJ. Androgens accelerate thymocyte apoptosis. *Endocrinology* 1998;139:748-52.
- 56) Bynoe MS, Grimaldi CM, Diamond B. Estrogen up-regulates Bcl-2 and blocks tolerance induction of naïve B cells. *PNAS* 2000;97:2703-8.
- 57) Erlandsson MC, Ohlsson C, Gustafsson J-Å, Carlsten H. Role of oestrogen receptors α and β in immune organ development and in oestrogen-mediated effects on thymus. *Immunology* 2001;103:17-25.
- 58) Olsen NJ, Gu Xiuqing, Kovacs WJ. Bone marrow stromal cells mediate androgenic suppression of B lymphocyte development. *J Clin Invest* 2001;108:1697-704.
- 59) Thurmond TS, Murante FG, Staples JE, et al. Role of estrogen receptor alpha in hematopoietic stem cell development and B lymphocyte maturation in the male mouse. *Endocrinology* 2000;141:2309-18.
- 60) Sakaguchi S, Takahashi T, Nishizuka Y. Study on cellular events in post-thymectomy autoimmune oophoritis in mice. II. Requirement of Lyt-1 cells in normal female mice for the prevention of oophoritis. *J Exp Med* 1982;156:1577-86.
- 61) Sakaguchi S, Sakaguchi N, Asano M, Ito M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor α -chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* 1995;155:1151-64.
- 62) Sakaguchi S, Sakaguchi N, Shimizu J, et al. Immunologic tolerance maintained by CD25⁺CD4⁺ regulatory T cells: their common role in controlling autoimmunity, tumor immunity, and transplantation tolerance. *Immunol Rev* 2001;182:18-32.
- 63) Aluvihare VR, Kallikourdis M, Betz AG. Regulatory T cells mediate maternal tolerance to the fetus. *Nat*

Immunol 2004;5:266-71.

64) Bennett C, Christie J, Ramsdell F, et al. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. Nat Genet 2001;27:20-1.

65) Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. Science 2003;299:1057-61.

66) Fehervari Z, Sakaguchi S. Peacekeepers of the immune system. Sci Am 2006;295:56-63.