Genetic Variation in Susceptibility to Endocrine Disruption by Estrogen in Mice

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Large (more than 16-fold) differences in susceptibility to disruption of juvenile male reproductive development by 17β-estradiol (E_2) were detected between strains of mice. Effects of strain, E_2 dose, and the interaction of strain and E_2 dose on testes weight and spermatogenesis were all highly significant ($P < 0.0001$). Spermatid maturation was eliminated by low doses of E_2 in strains such as C57BL/6J and C17/Jls. In contrast, mice of the widely used CD-1 line, which has been selected for large litter size, showed little or no inhibition of spermatid maturation even in response to 16 times as much E_2. Product safety bioassays conducted with animals selected for fecundity may greatly underestimate disruption of male reproductive development by estradiol and environmental estrogenic compounds.

Estrogens profoundly affect sexual differentiation, reproductive function, and behavior in diverse vertebrate species. Estrogenic activity is associated with several xenobiotics or environmental estrogens that can cause cancer, impair reproductive development, and lead to irreversible abnormalities in early development (*1*, *2*). For example, current xenobiotic exposure can have deleterious effects on reproductive development in wildlife (*1*, *2*). Decreased human sperm counts and increased incidence of human pathologies such as hypospadias, cryptorchidism, and prostate, testicular, and breast cancers are also concerning, but the relation of these trends to environmental factors is not established (*2*, *3*). Environmental estrogens bind to estrogen receptors and mimic the actions of E_2, including inhibition of hypothalamic gonadotropin-releasing hormone release, which decreases follicle-stimulating hormone (FSH) and luteinizing hormone (LH) secretion, thereby leading to decreased testicular function. Estradiol and several estrogenic xenobiotics also act to increase germ cell apoptosis and decrease sperm counts (*4*). In contrast, FSH, LH, and testosterone are survival factors that generally inhibit apoptosis and support germ cell maturation.

In several mammalian species, traits such as testis weight, ovulation rate, and litter size show genetic variation and can be altered by selective breeding (*5*, *6*). For economic efficiency, most commercial "outbred" strains of laboratory animals were selected over the long term for large litter size and vigor. Selection for large litters increased ovulation rate, in part by altering follicular populations and ovarian responsiveness to gonadotropins and decreasing negative feedback on gonadotropins (*7*). Even unselected strains of mice differ 6- to 20-fold in ovarian response to gonadotropins (*7*, *8*). In response to selection for large litter size, correlated changes in males include increased testes weight and decreased sensitivity to the negative feedback of estrogen on testicular and vesicular gland weights (*9*–*11*). We hypothesize that genetic selection for increased litter size alters the sensitivity to estrogen of many commonly used outbred stocks of mice and rats. Our concern is that the use of laboratory animals selected for large litter size in product safety testing might underestimate the role of estrogenic agents in disrupting juvenile reproductive development in other genotypes.

Although the mechanisms of estrogen action have been determined with biochemical, molecular biology, and gene knockout approaches on a single genetic background, relatively little attention has been devoted to identifying the genes controlling differences in hormonal sensitivity between individuals and populations. Thus, we studied the effects of E_2 on male reproductive traits in several strains of mice including line CD-1 from Charles River, strain C57BL/6J (B6) from the Jackson Laboratory, and strains C17/Jls, S15/Jls, E/Jls, and CN-/Jls, developed from a common base population (*12*). Strain C17/Jls was developed by random selection followed by inbreeding. Strain S15/Jls was developed by selection for large litter size followed by inbreeding (*13*). After exposure to increasing doses of E_2 in silastic implants starting at 22 to 23 days of age, susceptibility to endocrine disruption by estrogen (SEDE) was measured on day 43 (*14*).

E_2 treatment during juvenile development resulted in the suppression of testis weight in all strains of mice. Testis weight was affected by strain, dose of E_2, and the strain by dose interaction ($P < 0.0001$). Testis weight in control males not treated with E_2 also differed between strains ($P < 0.0001$), with heavier gonads found in males of large litter size–selected outbred line CD-1 and inbred strain S15/Jls ($P < 0.01$). Genotype accounted for more variation in testes weight than dose of E_2. B6 mice were extremely sensitive; the lowest E_2 dose produced a 60% suppression of testis weight ($P < 0.0001$). C17/Jls and S15/Jls males also showed greater susceptibility than CD-1 males to the negative effects of E_2 on testicular weight. Even with the highest E_2 dose, CD-1 mice showed only a 30% inhibition of testes weight.

We also analyzed the effects of E_2 dose per gram of body weight (E_2 dose/g) (Fig. 1). The effects of strain and E_2 dose/g on testis weight and testis weight per gram of body weight (TW/gBW) were highly significant ($P < 0.0001$). Furthermore, the linear regression of E_2 dose/g on testes weight differed among strains ($P < 0.0001$). These data demonstrate that CD-1 mice are far more resistant to endocrine disruption by estrogen than other strains. This study confirms previous reports of genetic variation in testicular sensitivity to estrogen and diethylstilbestrol in mice and rats (*10*, *11*, *15*).

Testicular histology also revealed genetic differences in susceptibility to endocrine disruption of spermatogenesis and testicular development (Fig. 2, A to F). Unlike B6 and C17/Jls mice, wherein low to moderate doses of E_2 obliterated spermatogenesis, CD-1 mice showed very little inhibition of spermatogenesis in response to increasing doses of estradiol.

Strain variation in SEDE was also examined by "blind" histological evaluation of spermatogenesis (*16*). The effects of strain, dose of E_2, and the strain by dose interaction

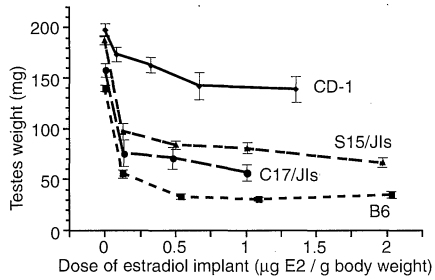

Fig. 1. Effect of E_2 implants on testes weight differs among strains of mice even after correction for differences in body weight. Mean ± SEM for an average of 16 individuals per strain × E_2 dose treatment group (*14*).

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on the percentage of seminiferous tubules with elongated spermatids were all highly significant ($P < 0.0001$) (Fig. 3). Gamete maturation to the elongated spermatid stage of development was completely eliminated in B6 males treated with a low E_2 dose (10 μg) and in randomly selected C17/Jls males treated with a moderate E_2 dose (20 μg) ($P < 0.0001$). In marked contrast, an abundance of normally maturing elongated spermatids was found in the testes of all CD-1 mice treated with up to 20 μg of E_2 and in the vast majority of CD-1 mice treated with 40-μg E_2 implants (Fig. 2C). Analysis of spermatogenic index (*17*) indicated that CD-1 mice were also much more resistant to E_2 than C17/Jls or B6 mice ($P < 0.0001$).

The 50% inhibitory dose for E_2 in CD-1 mice could only be estimated by extrapolation to doses above those used. Logistic curve-fit analysis estimated that relative to that of B6 and C17/Jls mice, CD-1 mice were 46 times more resistant to the inhibition of TW/gBW, 126 times more resistant to the inhibition of percentage of tubules with elongated spermatids, and 467 times more resistant to the inhibition of spermatogenic index. The resistance of CD-1 males to the suppressive effects of E_2 on gonadal function is more than 16 times that of other strains, and in actuality, CD-1 appears to be about 100 times more resistant.

We also compared the SEDE of a separate group of CD-1 males with mice of four contemporary strains (A/J, C8/Jls, E/Jls, and CN-/Jls) that were not selected for large litters. As previously described, SEDE was measured after exposure to increasing doses of E_2 starting at 3 weeks of age. Testes weights were measured at day 43 on an average of 10.7 mice per strain × treatment. The effects of strain, dose, and strain × dose interaction on testis weight and TW/gBW were all highly significant ($P < 0.0002$) (*18*). Once again, CD-1 males were much more resistant to suppression of testicular weight by estrogen than the average of other strains ($P < 0.0001$) (*19*).

The demonstration of major genetic differences in sensitivity to the disruption of juvenile male reproductive development and spermatogenesis by estrogen has widespread implications. Prolificacy has a large amount of genetic variance, as evidenced by successful selection for this trait in a wide range of mammalian species (*6*). Because genes controlling prolificacy are associated with genetic differences in SEDE and resistance to estrogens, genetic or demographic variation in SEDE is probable in diverse populations and species. Monitoring endocrine disruption will therefore require consideration of both susceptibility genotype and environmental exposure. This is particularly important because marked variation in the endocrine regulation of reproduction has resulted from natural and artificial selection for reproductive traits; for example, pituitary, gonadal, and uterine function vary considerably between lines of laboratory, domestic, and farm animals. In mice, litter size, hormone-induced ovulation rate, and aromatase activity and uterotrophic responses to estrogens differ between strains (*7, 8, 20*). Furthermore, quantitative trait loci controlling these traits have been mapped in this species (*21*). These data as well as individual differences in sensitivity to contraceptive steroids in humans (*22*) emphasize the need for considering hormone response genotype when optimizing doses of estrogenic and other steroidal agents for contraception, hormone replacement therapy, and the prevention and treatment of breast and prostate cancer.

Because several genes are expressed in a developmental and sex-specific manner, the strain differences in SEDE observed in juvenile to young adult males may not be concordant with those of fetal, neonatal, or adult female exposures. Addressing this issue is essential, as is asking how genetic differences in SEDE operate. Do they regulate estrogen catabolism or the mechanisms by which estrogens regulate gonadotropin secretion, gametogenesis, or steroidogenesis? Identification of the genes controlling SEDE is especially important in light of the profound resistance of CD-1 line mice to E_2 suppression of testicular function. CD-1 is among the strains most commonly used for toxicology and pharmacology research as well as product safety assays. Our results show that estimates obtained from CD-1 may underestimate those of other hormone response genotypes. Use of an animal model with a highly resistant genotype to assess deleterious effects

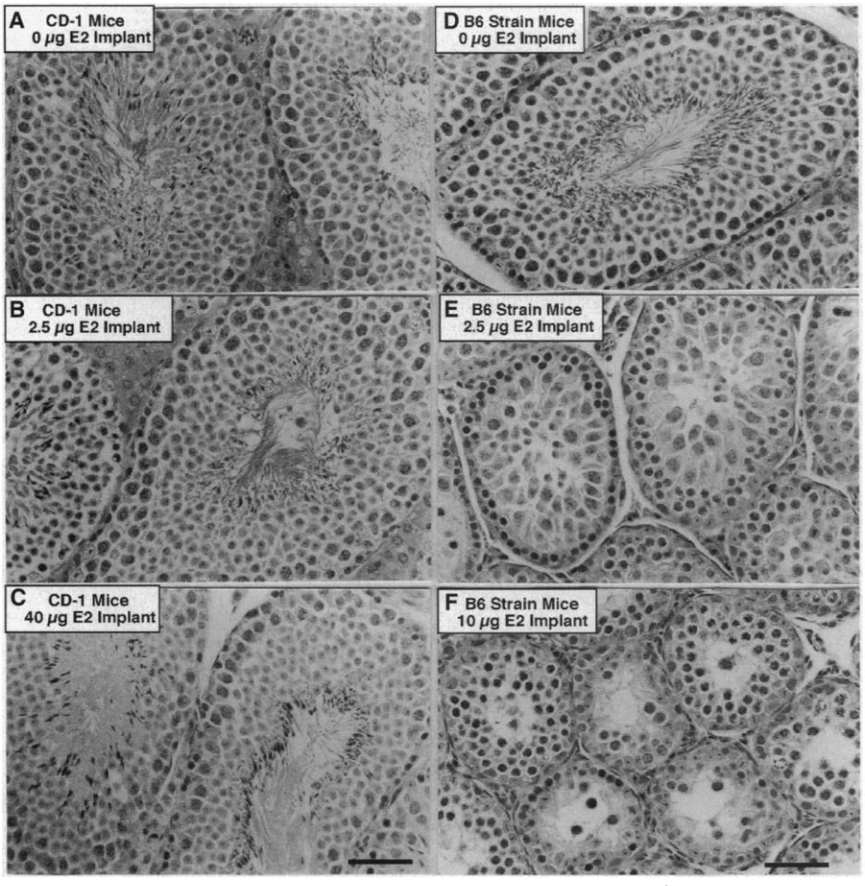

Fig. 2. Effects of E_2 implants on testicular histology in CD-1 (**A** to **C**) and C57BL/6J (B6) (**D** to **F**) strain mice. All micrographs are at the same magnification. Scale bar, 50 μm.

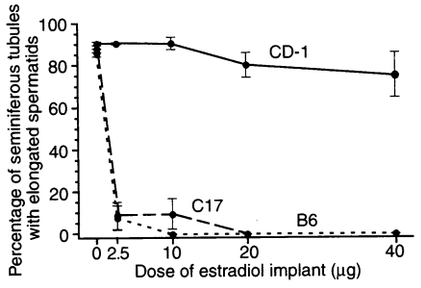

Fig. 3. Effect of E_2 implants on percentage of seminiferous tubules with elongated spermatids differs among strains of mice (*16*). Mean ± SEM for an average of six individuals per strain × E_2 dose treatment group.

of estrogenic agents on reproduction may be misleading and could mask our appreciation of how global exposure to estrogenic xenobiotics threatens wildlife, domestic animals, and our own species.

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14. Mice were maintained at 20° to 23°C on a 14 hours light/10 hours dark photoperiod and received Agway 2000 rodent chow (18% protein, 4% fiber, and 9% fat) ad libitum. At 22 to 23 days of age, mice were anesthetized with Avertin (0.012 ml per gram of body weight) and implanted subcutaneously with silastic implants (5 to 10 mm long, 1.5 mm inner diameter, 3.5 mm outer diameter) containing 0, 2.5, 10, 20, or 40 μg of ethanol-dissolved E_2 in Silgard184. At 43 days of age, mice were killed, testis weight was measured, and testes were fixed in phosphate-buffered formalin [3.7% formaldehyde and 75 mM $NaPO_4$ (pH 7.3)], embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin and eosin (H&E). Experiments were conduced in accordance with Association for Assessment and Accreditation of Laboratory Animal Care–approved protocols. Statistical analyses were conducted with one- and two-way analysis of variance with Tukey-Kramer multiple range tests. Initial E_2 release rates averaged 40.25 and 219.5 ng/day from 2.5- and 10-μg E_2 implants, respectively (*23*).
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16. Histological analysis was conducted on phosphate-buffered, formalin-fixed, paraffin-embedded, H&E- and periodic acid–Schiff–stained testicular sections. H&E-stained histological sections from an average of six mice per strain × E_2 dose treatment group were coded and evaluated "blindly" to determine spermatogenic index and the percentage of seminiferous tubules showing sperm maturation to the elongated spermatid stage of development. Germ cells of all CD-1 mice treated with up to 20-μg E_2 implants and seven out of eight mice treated with 40-μg E_2 implants progressed through spermatogenesis as shown by the abundance of normally maturing elongated spermatids and the presence of mature spermatids undergoing spermiation (Fig. 2C). All histological indicators of testicular development declined in B6 mice in response to as little as 2.5-μg E_2 implants (Fig. 2E) and were maximally disrupted in response to 10-μg E_2 implants. Treatment of B6 mice with 10-μg E_2 resulted in vacuolized seminiferous epithelium with disorganized germ cells in the majority of tubules (Fig. 2F). Several B6 mice treated with 10-μg E_2 showed even more extreme testicular atrophy (*18*).
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Rapid Infection of Oral Mucosal-Associated Lymphoid Tissue with Simian Immunodeficiency Virus

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The early events during infection with an immunodeficiency virus were followed by application of pathogenic simian immunodeficiency virus atraumatically to the tonsils of macaques. Analyses by virologic assays and in situ hybridization revealed that the infection started locally in the tonsils, a mucosal-associated lymphoid organ, and quickly spread to other lymphoid tissues. At day 3, there were few infected cells, but then the number increased rapidly, reaching a high plateau between days 4 and 7. The infection was not detected in the dendritic cell–rich squamous epithelium to which the virus was applied; instead, it was primarily in $CD4^+$ tonsillar T cells, close to the specialized antigen-transporting epithelium of the tonsillar crypts. Transport of the virus and immune-activating stimuli across this epithelium would allow mucosal lymphoid tissue to function in the atraumatic transmission of immunodeficiency viruses.

Virologic and immunologic events during the initial period of human immunodeficiency virus–type 1 (HIV-1) infection have important consequences for vaccine design and the eventual clinical course (*1, 2*). The level of virus or set-point that develops after acute infection correlates with the rapidity with which the disease develops and acquired immunodeficiency syndrome (AIDS) appears (*3*). To observe early events during the transmission of an immunodeficiency virus, and to establish the role of mucosal-associated lymphoid tissue (MALT) at body surfaces, we applied simian immunodeficiency virus (SIV) directly to the surface of the tonsils in macaques. We then followed the kinetics of virus multiplication and spread within and from a single lymphoid organ.

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This experimental design also provided information on the relative roles of two types of tissue in the early replication of virus. One tissue is the stratified squamous epithelium that overlies the tonsils. It is rich in dendritic cells (DCs) and comparable to the lining of the vagina and anus, tissues that are implicated in genital transmission of HIV-1. The other tissue is the lymphoid component of the tonsil and is comparable to MALT found in the rectum. A critical feature of MALT is a specialized epithelial covering that contacts the underlying lymphoid tissue and contains membranous or microfold "M" cells. Antigens, including virions (*4, 5*), are transported through M cells without the need for trauma or inflammation (*6–9*). Beneath the epithelium lie numerous DCs (*10, 11*), which are important for capturing antigens and initiating T cell–mediated immunity (*12*). There are many observations in tissue culture indicating that DCs contribute to the capture of HIV-1 and SIV and subsequent transmission to T cells (*13–17*).