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12 August 1999; accepted 28 October 1999

Postnatal Sex Reversal of the Ovaries in Mice Lacking Estrogen Receptors α and β

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Mice lacking estrogen receptors α and β were generated to clarify the roles of each receptor in the physiology of estrogen target tissues. Both sexes of αβ estrogen receptor knockout (αβERKO) mutants exhibit normal reproductive tract development but are infertile. Ovaries of adult αβERKO females exhibit follicle transdifferentiation to structures resembling seminiferous tubules of the testis, including Sertoli-like cells and expression of Müllerian inhibiting substance, sulfated glycoprotein-2, and *Sox9*. Therefore, loss of both receptors leads to an ovarian phenotype that is distinct from that of the individual ERKO mutants, which indicates that both receptors are required for the maintenance of germ and somatic cells in the postnatal ovary.

Reports of estrogen synthesis in the fetal ovaries of several species suggest the involvement of the estrogen signaling system in ovarian development (*1*). Insights into the physiological roles of estrogen have been gained from the study of mice lacking the capability to synthesize either estradiol (ArKO mice) (*2*) or one of the two cognate estrogen receptors (ERs) ERα (αERKO) and ERβ (βERKO) (*3*). However, conclusions drawn from these mutant mice are confounded by possible compensatory mechanisms provided by (i) the opposite ER in each respective ERKO mutant or (ii) maternal estrogens during gestation or estradiol-independent ER actions in the ArKO mutant, or both. Therefore, to further elucidate the role of estrogen signaling in reproductive tract development and function, mice homozygous for a targeted disruption of both ER genes (*Estra* and *Estrb*), termed αβERKO mice, were generated (*4*). Adult (2.5 to 7 months) αβERKO mice of both sexes survive to adulthood and exhibit no marked abnormalities as compared to control littermates, thereby challenging earlier speculations that the ER is essential to survival (*5*).

αβERKO males are infertile but possess a grossly normal reproductive tract, in agreement with past evidence that estradiol is unnecessary for the development of male gonads and reproductive structures. The testes of adult (2.5 to 7 months) αβERKO males exhibited various stages of spermatogenesis, yet the numbers and motility of epididymal sperm were reduced by approximately 80 and 5%, respectively (Fig. 1). This phenotype is similar to that of the αERKO male and is therefore characteristic of the loss of ERα; it does not occur in βERKO males, which exhibit normal fertility (*3*) and sperm counts (Fig. 1E). The αβERKO testicular phenotype also does not resemble that reported in ArKO mice, which exhibit arrested spermatogenesis but no αERKO-like tubule dysmorphogenesis (*2*). This discrepancy between male mice lacking estradiol and those lacking both ERs suggests the existence of undocumented aromatase- or ER-encoding genes or estradiol-independent ER actions within the male reproductive tract (or both).

In agreement with classical fetal castration studies indicating that differentiation of the female genital ducts is independent of ovarian steroids (*6*), αβERKO females exhibit proper differentiation of the Müllerian-derived structures (the uterus, cervix, and upper vagina). The functional uterine compartments are present in the uteri of αβERKO females, yet the dependency of each on estradiol for postnatal growth is definitively illustrated by their severe hypoplasia in adult (2.5 to 7 months) αβERKO females (Fig. 2, A and B). Similar uterine hypoplasia occurs in αERKO but not in βERKO females (*3*), which corresponds to reports of ER localization and is characteristic of the loss of

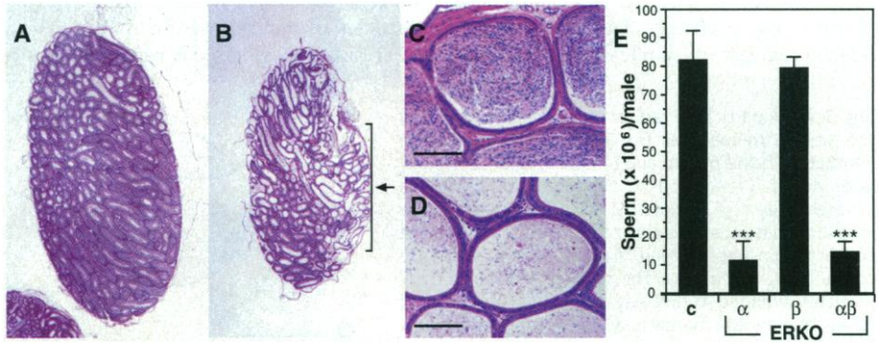

Fig. 1. Morphological and functional phenotypes of the αβERKO male reproductive tract. Testes were fixed overnight in cold Bouin's fixative, passed through several changes of cold water over 2 days, transferred to cold 50% ethanol for 24 hours, and then immersed in cold 70% ethanol until paraffin embedding. Shown are 5-μm sections stained with hematoxylin and eosin (H&E). Low-power magnification of a testis from a representative age-matched (**A**) control male and (**B**) an αβERKO adult male (2.5 to 7 months) illustrates the luminal swelling and loss of germinal epithelium of the seminiferous tubules in the αβERKO testis, which is most evident along the region indicated by the arrowed bracket. High-power (×66) magnification of the caudal epididymis of a representative (**C**) wild-type male and (**D**) of an αβERKO adult male illustrates the reduced density of the sperm population in the αβERKO male. (**E**) Epididymal sperm counts carried out as previously described (*25*) on males ≥100 days old indicate the significant reduction in sperm number in the αβERKO male that is characteristic of that observed in age-matched αERKO males. For control (c), αERKO, and βERKO, mice, $n = 4$ animals analyzed; for αβERKO mice, $n = 5$ animals analyzed. Scale bar, 100 μm. ***ANOVA, $P < 0.001$.

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ERα in the uterus. Therefore, early differentiation of the female reproductive tract can occur in the absence of functional ERα and ERβ.

The ovaries of prepubertal αβERKO females possessed adult-like follicles with defined antra and theca (Fig. 2), which is characteristic of hypergonadotropic-precocious maturation of the ovary. Serum luteinizing hormone (LH) levels in αβERKO females were higher than the elevated levels observed in αERKO females (*3, 7*), which suggests that both ERs are required for estradiol-mediated regulation of LH secretion in the hypothalamic-hypophyseal axis.

In contrast, ovaries of adult (2.5 to 7 months) αβERKO females exhibited morphological phenotypes that were clearly distinct from those of the prepubertal αβERKO and individual ERKO models (*3*). Appropriate structures in the adult αβERKO ovary included primordial and growing follicles, some possessing a large antrum (Fig. 2), yet no corpora lutea were observed. The most remarkable features of the adult αβERKO ovary were structures resembling the seminiferous tubules of the testis (Fig. 2, F and I through K), which were not observed in the ovaries of the prepubertal αβERKO mice or in individual ERKO mice of any age. These structures composed large portions of the ovary and possessed intact basal lamina but lacked the granulosa cell layers characteristic of a maturing follicle. In some, a recognizable but degenerating oocyte was present, whereas others showed no evidence of germ cells. Within the lumen of the tubule-like structures were degenerating granulosa cells and cells resembling Sertoli cells of the testis. Morphological features of the latter indicating a Sertoli cell phenotype included alignment with the basal lamina, a tripartite nucelolus, and numerous veil-like cytoplasmic processes extending inward toward the lumen (Fig. 2, J and K) (*8*).

Certain characteristics of the apparent sex reversal in the adult αβERKO ovary indicate redifferentiation of ovarian components rather than a developmental phenomenon, including (i) the absence of similar structures in prepubertal αβERKO ovaries; (ii) the consistent spherical shape of the "tubules," suggesting origination from a once healthy follicle; and (iii) age-related increases in the area of transdifferentiation. For further characterization, we examined the expression of known biochemical indices of Sertoli cell differentiation: Müllerian-inhibiting substance (MIS) (*9, 10*), sulfated glycoprotein-2 (SGP-2) (*10*), and *Sox9* (*11*). Significant levels of MIS mRNA were detected in the ovaries of all four genotypes (*12*); however, the relative levels of MIS mRNA were clearly elevated in the αβERKO ovaries (Fig. 3). Levels of *Sox9* mRNA were significantly increased in the αβERKO ovaries only, whereas levels in control, αERKO, and βERKO ovaries were below levels of detection (Fig. 3). Elevated *Sox9* mRNA levels were also detected in the testes of adult αβERKO and αERKO males, in contrast to levels in control and βERKO testes and previous descriptions of *Sox9* ontogeny in the adult mouse (*11*) (Fig. 3). Immunoreactivity for MIS and SGP-2 protein was localized to the Sertoli-like cells lining the basal lamina of the sex-reversed follicles in the αβERKO ovary (Fig. 3, C and D).

Morphological sex reversal of the ovary has been described in several species under different conditions, including in the fetal rodent ovary after transplantation to an adult host, after in vitro exposure to purified MIS, or in vivo via transgenic MIS overexpression (*13*). The sex reversal of the αβERKO ovary shares both morphological and biochemical similarities with the findings of these studies, including aberrant expression of the genes encoding MIS, SGP-2, and *Sox9* (*10, 11*). However, a remarkable difference is the postnatal onset of the αβERKO ovarian phenotype, in contrast to previous reports that only fetal ovaries were susceptible to the redifferentiating effects of MIS or transplantation

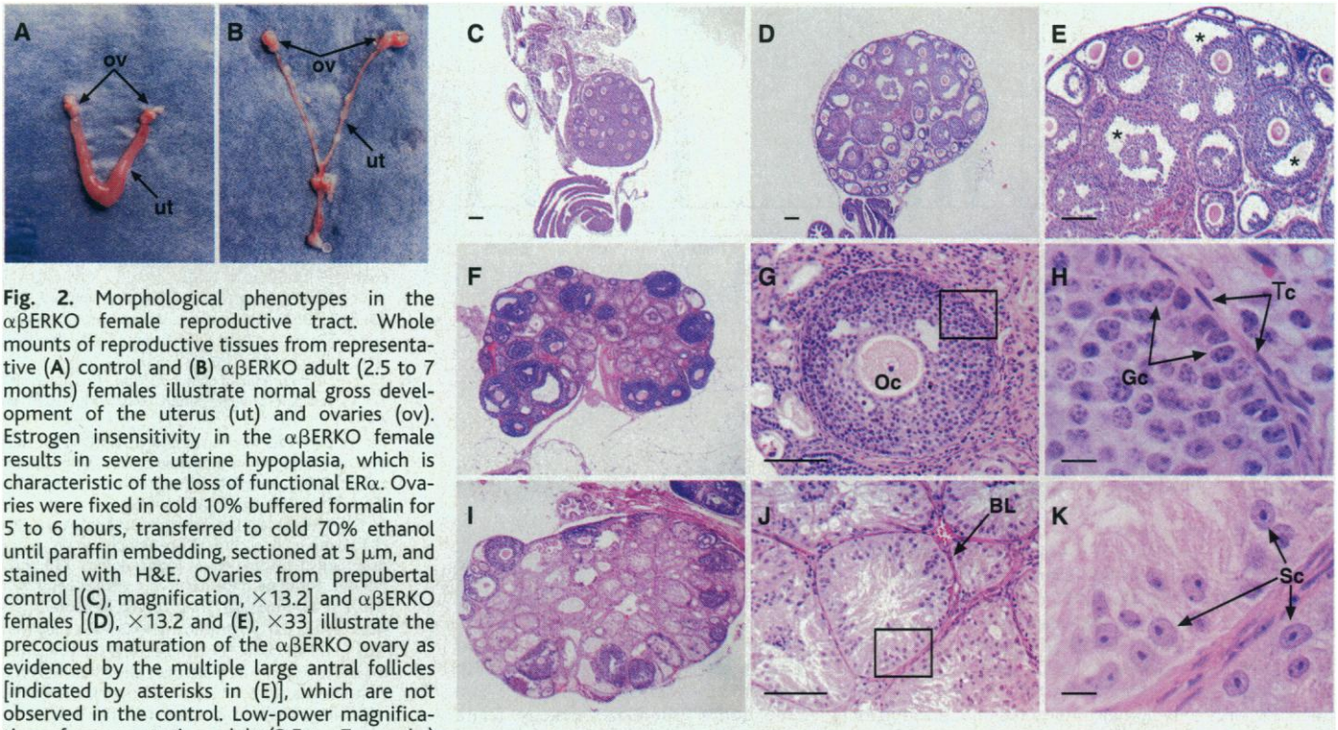

Fig. 2. Morphological phenotypes in the αβERKO female reproductive tract. Whole mounts of reproductive tissues from representative (**A**) control and (**B**) αβERKO adult (2.5 to 7 months) females illustrate normal gross development of the uterus (ut) and ovaries (ov). Estrogen insensitivity in the αβERKO female results in severe uterine hypoplasia, which is characteristic of the loss of functional ERα. Ovaries were fixed in cold 10% buffered formalin for 5 to 6 hours, transferred to cold 70% ethanol until paraffin embedding, sectioned at 5 μm, and stained with H&E. Ovaries from prepubertal control [(**C**), magnification, ×13.2] and αβERKO females [(**D**), ×13.2 and (**E**), ×33] illustrate the precocious maturation of the αβERKO ovary as evidenced by the multiple large antral follicles [indicated by asterisks in (E)], which are not observed in the control. Low-power magnification of representative adult (2.5 to 7 months) αβERKO ovaries (**F** and **I**) illustrates the diverse structures present, including relatively healthy, maturing follicles and the sex-reversed follicles that occupy large portions of the gonad. High-power magnification of a healthy follicle in an adult αβERKO ovary [(**G**), ×66 and (**H**), ×330] shows a single oocyte (Oc), several layers of granulosa cells (Gc), and an intact basal lamina and thecum (Tc). High-power magnification of an area of sex-reversed follicles [(**J**), ×66 and (**K**), ×330] shows that the oocyte has degenerated and the somatic cells have undergone redifferentiation to a Sertoli cell (Sc) phenotype. The preservation of the basal lamina (BL) of the follicle accounts for the tubular appearance of the cords of the testis. The Sertoli-like cells possess the characteristic tripartite nucleolus and veil-like cytoplasmic extensions (K). Scale bar in (C) through (E), (G), and (J), 100 μm; scale bar in (H) and (K), 10 μm).

(*14*). Therefore, the αβERKO ovarian phenotype is the first illustration of sex reversal in the adult mouse gonad, indicating that female somatic cells retain the capacity to redifferentiate to Sertoli-like cells throughout life in the mouse.

Although the intraovarian functions of ERα and ERβ are not well understood, it is known that the two are not equally expressed within the functional components of the ovary. Whereas ERα is predominant in the stromal/thecal component, ERβ is localized to granulosa cells of maturing follicles (*15*). Nonetheless, the αβERKO phenotype indicates that both ERs are required for ovarian function and oocyte survival in the adult. The mechanisms by which the loss of both ERs results in postnatal ovarian sex reversal are unclear. The degenerative state or complete absence of oocytes in the redifferentiated follicles of the αβERKO ovaries is consistent with previous descriptions of sex reversal in the mammalian ovary (*13*). Transgenic female mice possessing elevated serum LH levels also exhibit progressive follicle loss but no evidence of sex reversal (*16*), which indicates that both ERs are necessary to maintain ovarian morphology. In contrast, redifferentiation of the ovary has not been reported in ArKO mice (*2*), again suggesting the existence of unidentified aromatase- or ER-encoding genes or estradiol-independent functions of the ER (or both).

Overexpression of a MIS transgene gene results in oocyte loss and sex reversal in the developing mouse ovary (*17*). Although data concerning estrogen regulation of the MIS gene are conflicting (*18*), in vitro estradiol-ERα binding and transactivation of the human MIS gene promoter have been described (*19*). Therefore, a lack of ER may result in elevated MIS levels and oocyte death, yet normal differentiation of the Müllerian duct in the αβERKO female indicates proper repression of the MIS gene during development. *Sox9* activity in the fetal testis is critical to commitment to the male pathway, because inactivating mutations of the *SOX9* gene in human XY males lead to sex reversal of the gonads and reproductive tract (*20*). The mouse *Sox9* promoter shows no evidence for ER-mediated regulation but does indicate consensus binding sites for GATA-1 (*21*). GATA-1 is a transcription factor expressed in Sertoli cells of the fetal testis but repressed by the presence of germ cells in the adult testis (*22*), which may explain the increased *Sox9* mRNA levels in the adult αERKO and αβERKO testes, both of which exhibit substantial germ cell attrition. Furthermore, estradiol-ERα–mediated inhibition of GATA-1 transactivational activity through direct protein-protein interaction has been reported (*23*). A related GATA-binding protein, GATA-4, is estrogen-regulated in the maturing follicle and may play a role in granulosa cell maintenance (*24*). Therefore, the lack of ERα-ERβ actions resulting in ovarian sex reversal in the adult αβERKO mouse may be due to a loss of survival factors for oocyte and granulosa cells (such as GATA-4) and the enhanced activity of factors involved in testicular differentiation (such as GATA-1, *Sox9*, and MIS).

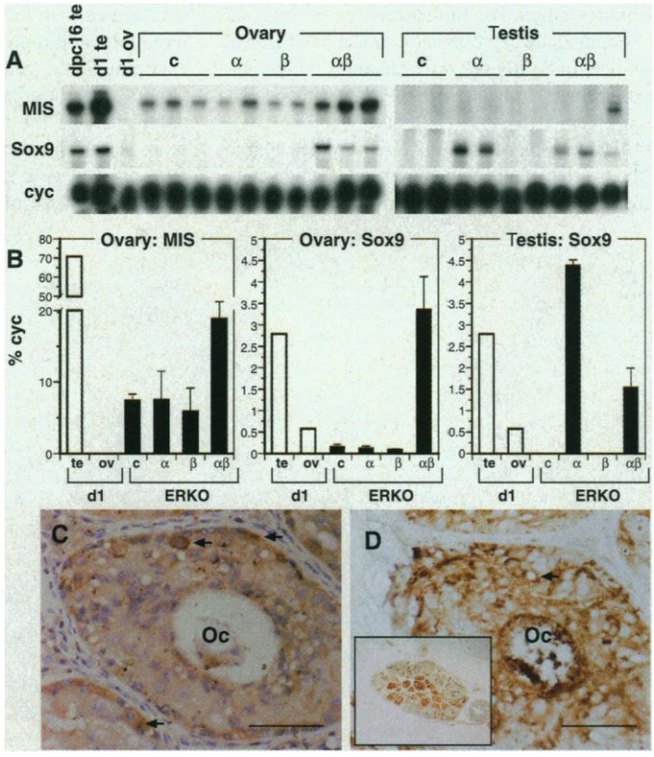

Fig. 3. Biochemical phenotypes in the gonads of αβERKO mice. (**A**) RPAs for MIS and *Sox9* transcripts in the ovaries and testes of individual adult (2.5 to 7 months) control (c), αERKO, βERKO, and αβERKO mice. RPAs for cyclophilin (cyc) mRNA were carried out for normalization and quantification purposes. Total RNA from 16 days post coitus testes (dpc 16 te), day-1 testes (d1 te), and day-1 ovaries (d1 ov) were used as controls, showing the appropriate expression pattern of the MIS and *Sox9* genes during development. (**B**) Quantitative analysis (percent cyclophilin; average ± SEM) of the RPA data illustrated above. Normal levels of MIS mRNA were detected in control (c) ovaries, which is in agreement with past reports of MIS ontogeny in the adult mouse ovary (*26*), and comparable levels were detected in the αERKO and βERKO ovaries. However, relative MIS mRNA levels are clearly elevated in the αβERKO ovaries. Increased levels of MIS mRNA were also detected in the testes of one adult αβERKO (not graphed). Levels of *Sox9* mRNA in the ovaries and testes of αβERKO mice and the testes of αERKO mice are significantly elevated relative to the control tissues. The levels of *Sox9* mRNA in adult αβERKO ovaries were similar to those in day 16 post coitus mouse testes, a tissue in which appropriate *Sox9* expression is high. (**C** and **D**) Immunohistochemistry was carried out on 5-μm sections with anti-human MIS polyclonal antibodies (SC-6886; Santa Cruz Biotechnology, Santa Cruz, California) or anti-human SGP-2 polyclonal antibodies (SC-6419; Santa Cruz Biotechnology); immunoreactivity was detected as described previously (*27*). Serial sections of an adult αβERKO ovary illustrate specific immunoreactivity (indicated by arrows) for MIS (C) and SGP-2 (D) localized to the Sertoli-like cells lining the basal lamina of a sex-reversed follicle (Oc, oocyte) (magnification, ×132; scale bar, 100 μm). The inset in (D) illustrates a low-power magnification of the whole ovary immunostained for SGP-2, illustrating localization of the cytoplasmic protein to the somatic cells of the sex-reversed follicles in the center of the ovary. All micrographs were taken with an Olympus BX-50 microscope and a PM-20 35-mm camera or DP-10 digital camera.

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by electrophoresis on a 1% agarose and 1× tris/borate/EDTA (TBE) gel. Ribonuclease protection assays (RPAs) were carried out on 5 μg of total RNA with the Maxiscript and Hybspeed (Ambion, Austin, TX) reagents as previously described [J. F. Couse, J. Lindzey, K. Grandien, *Endocrinology* **138**, 4613 (1997)]. All riboprobes were radiolabled with ^{32}P-CTP using the following templates: The antisense riboprobe for MIS corresponds to base pairs 379 through 720 of the mouse MIS mRNA (GenBank number X63240) and was kindly provided by A. Themmen of Erasmus University of Rotterdam; the antisense riboprobe for *Sox9* corresponds to the Swa I fragment of the mouse *Sox9* gene and was kindly provided by B. Capel of Duke University; and the antisense riboprobe for mouse cyclophilin mRNA was generated from the pTRI-cyclophilin template (Ambion). Final protected products were electrophoresed on a 6% acrylamide, 1× TBE, 7 M urea gel (Novex, San Diego, CA); dried; quantified with a Storm 860 Phosphorimager and accompanying software (Molecular Dynamics, Sunnyvale, CA); and then exposed to x-ray film.

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29 September 1999; accepted 15 November 1999

Isolation of West Nile Virus from Mosquitoes, Crows, and a Cooper's Hawk in Connecticut

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West Nile (WN) virus, a mosquito-transmitted virus native to Africa, Asia, and Europe, was isolated from two species of mosquitoes, *Culex pipiens* and *Aedes vexans*, and from brain tissues of 28 American crows, *Corvus brachyrhynchos*, and one Cooper's hawk, *Accipiter cooperii*, in Connecticut. A portion of the genome of virus isolates from four different hosts was sequenced and analyzed by comparative phylogenetic analysis. Our isolates from Connecticut were similar to one another and most closely related to two WN isolates from Romania (2.8 and 3.6 percent difference). If established in North America, WN virus will likely have severe effects on human health and on the health of populations of birds.

An outbreak of arboviral encephalitis associated with mosquitoes was recognized in late August 1999 to be occurring in New York City (*1*). St. Louis encephalitis virus (SLE) was identified initially as the causative agent, but a Kunjin/WN-like virus was later reported to be the likely etiologic agent (*2*). We began trapping mosquitoes for the testing of viruses on 5 September 1999 (*3*). Traps were placed first in the field in Greenwich, Connecticut, a town located about 18 miles (29 km) northeast of Bronx county, New York City. They were placed in the adjacent town of Stamford on 9 September 1999, and in the following weeks, mosquito traps were placed in 12 additional towns in Fairfield County, Connecticut.

American crows, *Corvus brachyrhynchos* (*4*), were reported dying in Fairfield County, Connecticut, in the second week of September 1999. One crow was collected from Westport, Connecticut, on 13 September 1999 and was tested for virus. Subsequently, 30 additional dead crows from 18 additional towns in Fairfield and New Haven Counties and a Cooper's hawk, *Accipiter cooperii*, from the town of East Haven in New Haven County, were tested for virus. We report isolations of WN virus in the New World from two species of mosquitoes, American crows, and a Cooper's hawk.

A total of 1361 mosquitoes was collected and tested for virus by 14 October 1999 from Greenwich and Stamford, Connecticut, and 2037 additional mosquitoes were captured and tested from the other 12 towns sampled in Fairfield County (*5*). Virus was isolated from one pool of 12 *Culex pipiens* (Fig. 1) and one pool of six *Aedes vexans* collected the evening of 14 September 1999 at the Innis Arden Country Club located in the southern parts of both Greenwich and Stamford, Connecticut. Cell lysate antigen from both isolates reacted in an enzyme-linked immunosorbent assay (ELISA) with mouse antisera to SLE but not with antisera to species in the Togaviridae or Bunyaviridae (*6*). Titers to SLE mouse antisera were 1:320.

Virus was isolated from brain tissue of the dead crow collected from Westport, Connecticut, on 13 September 1999 (*7*). This bird had histopathologic evidence of encephalitis characterized by perivascular cuffs of mononuclear cells, predominately lymphocytes, and multifocal neuronal satellitosis and neuronophagia, consistent with viral encephalitis. Cell lysate antigen was prepared and found to react in an ELISA at a titer of 1:640 with mouse immune antisera to SLE (*6*).

Virus isolations were made from 27 of 30 additional crows that died in Fairfield and New Haven Counties, Connecticut, in September through 12 October 1999 (*8*), and from the brain of a Cooper's hawk (*9*). Crows died in Connecticut along a 62-mile (100-km) corridor from Greenwich on the New York border eastward to Madison, Connecticut, in towns bordering directly on Long Island Sound or inland by about 15 miles (24 km). The gross lesions in the crows consisted of subdural hemorrhage or coelomic hemorrhage, or both, and, in about one-third of the birds, emaciation and occasional fecal staining of feathers (suggestive of seizure activity). Microscopically, these crows had evidence of multifocal viral encephalitis. Cell lysate antigen from all isolates reacted in an ELISA at titers of ≥1:320 with mouse immune antisera to SLE. None reacted with the reference antisera to the other species of viruses tested (*6*).

A portion of the genome of virus isolates from four different hosts, *Ae. vexans* (isolate 2738, GenBank accession AF206517), *Cx. pipiens* (isolate 2741, GenBank accession AF206518), the crow from Westport (isolate 86814, GenBank accession AF206519), and the Cooper's hawk (isolate 86815, GenBank accession AF206520), was sequenced and analyzed genetically by comparative phylogenetic analysis (maximum likelihood, maximum parsimony, and neighbor joining) with PAUP 4b1 (*10*). The

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References and Notes

[2] **Characterization of Mice Deficient in Aromatase (ArKO) because of Targeted Disruption of the Cyp19 Gene**

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[2] **Impairment of Spermatogenesis in Mice Lacking a Functional Aromatase (cyp 19) Gene**

Kirsten M. Robertson; Liza O'Donnell; Margaret E. E. Jones; Sarah J. Meachem; Wah Chin Boon; Carolyn R. Fisher; Kathy H. Graves; Robert I. McLachlan; Evan R. Simpson

Proceedings of the National Academy of Sciences of the United States of America, Vol. 96, No. 14. (Jul. 16, 1999), pp. 7986-7991.

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[4] **Alteration of Reproductive Function but Not Prenatal Sexual Development After Insertional Disruption of the Mouse Estrogen Receptor Gene**

Dennis B. Lubahn; Jeffrey S. Moyer; Thomas S. Golding; John F. Couse; Kenneth S. Korach; Oliver Smithies

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