Insights from the Study of Animals Lacking Functional Estrogen Receptor

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Estrogen hormones produce physiological actions within a variety of target sites in the body and during development by activating a specific receptor protein. Hormone responsiveness for the estrogen receptor protein was investigated at different stages of development with the use of gene knockout techniques because no natural genetic mutants have been described. A mutant mouse line without a functional estrogen receptor was created and is being used to assess estrogen responsiveness. Both sexes of these mutant animals are infertile and show a variety of phenotypic changes, some of which are associated with the gonads, mammary glands, reproductive tracts, and skeletal tissues.

Estrogen was first discovered in the 1920s, and scientists believe that the hormone plays a crucial role in embryonic and fetal development to influence female secondary sexual characteristics, reproductive cycle, fertility, and maintenance of pregnancy (*1*). Normal postnatal female physiology has been linked to estrogen action in several target sites in the body, most noticeably the reproductive tract, breast, and neuroendocrine tissues, in which it elicits a variety of tissue-specific responses. A dramatic lowering of estrogen levels during menopause, which is associated with osteoporosis and cardiovascular disease, has suggested it functions in bone tissue and in the cardiovascular system, but these effects are poorly understood because of the lack of appropriate physiological model systems. The lack of knowledge in these systems has stirred a debate as to whether estrogen elicits a direct tissue action or indirect effects that involve other regulators or signaling systems. Estrogens trigger a broad array of physiological responses which are tissue- and organ-specific by binding to a nuclear receptor protein within target cells (*2*); these include tissue differentiation, growth, protein synthesis, and secretion (*3*, *4*). The estrogen receptor is a ligand inducible transcription factor that modulates target gene expression after binding hormone (Fig. 1).

Past findings had indicated that estrogen steroid hormones are required for tissue effects mediated by the receptor. In addition to ligand activation, recent observations have implicated other cellular signaling systems in the mechanism of estrogen stimulation (*5*, *6*). We demonstrated that specific growth factors, such as EGF, could mimic estrogen by affecting nuclear estrogen receptor protein properties and by stimulating some biological responses (*5*, *7*). Mechanistically, this growth factor action appears to operate by coupling the estrogen receptor to multiple signaling pathways that converge in a tissue specific response (Fig. 1). Development of an animal model in which the two signaling systems were uncoupled, for example by eliminating a functional estrogen receptor system, would allow the evaluation of the role of the dual signaling systems in physiological regulation.

The precise role for estrogen in humans is not totally understood; nevertheless, the importance of estrogen in development and physiological function, in combination with uncertainties regarding the role, mechanisms, and sites of normal and pathological action for the hormone, made the use of a gene knockout to disrupt the expression of functional estrogen receptor (ER) protein an attractive experimental approach. But it was also an approach that was considered unlikely to result in viable animals (*8*). On the other hand, if lethality did occur, we could determine the stage and possible sites during development for which estrogen was critical.

Clinical evidence increased the suspicion that the gene disruption would be lethal because no conditions of estrogen insensitivity or estrogen receptor gene mutations had been reported (*1*, *8*). In contrast, conditions of resistance to other hormones, as a result of defects in other members of the hormone receptor gene family, have been reported. Androgen insensitivity caused by disruptive mutations of the androgen receptor protein results in abnormal male sexual differentiation and development (*9*). Thyroid (*10*) and glucocorticoid hormone (*11*) resistance are other examples of endocrine clinical conditions that can result from receptor gene defects. The lack of reported cases of estrogen insensitivity in humans and in experimental animals was explained by the prevailing view that disruption of the estrogen receptor gene would be lethal during development as a result of the defective implantation of the embryo

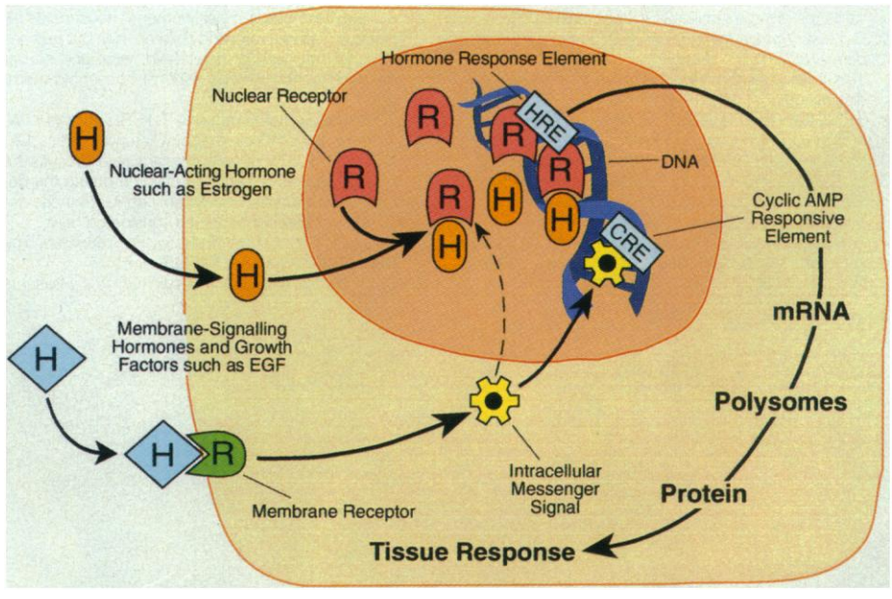

Fig. 1. Examples of cellular mechanisms for hormonal stimulation. Steroid, thyroid, and retinoid hormones diffuse into cells in which they interact with nuclear receptor proteins that function as ligand-activated transcription factors. The receptor ligand complex dimerizes and binds to specific DNA sequences (HRE) upstream of genes regulated by the hormone. Regulation results in an increase in specific gene transcription that influences responses within target cells. Protein hormones and growth factors are examples of stimulants which interact with membrane receptors eliciting a cellular response mediated by an intracellular second messenger signaling pathway.

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(*1*). Blastocysts and two-cell embryos express estrogen receptor mRNA, which supports the possibility that estrogen has a role in early development (*12*).

Gene Disruption

Development of the homozygous estrogen receptor mutant mouse was accomplished in collaboration with Oliver Smithies' laboratory, in which a sequence that encodes neomycin resistance was inserted into exon 2 of the mouse estrogen receptor gene (*8*). The neomycin insert also includes premature stop codons and polyadenylation sequences that inhibit proper transcription and translation of the ER gene, and thereby functionally inhibit expression. Successful targeting of the sequence by homologous recombination (*13*, *14*) occurred in only 2 out of 1800 embryonic stem (ES) cell clones. Several chimeric mice were produced, one of which successfully transmitted the disrupted gene in the germ line and produced heterozygous mice of both sexes. The heterozygotes containing one copy of the wild-type ER gene and one copy of the inactivated gene were detected by Southern (DNA) blot analysis and polymerase chain reaction, and were found to be fertile and exhibited no obvious phenotype in association with the disrupted ER genotype. Ligand binding assays and Western protein immunoblot analyses of uteri from heterozygous females indicate they have about half of the ER amount when compared with wild type. This suggests that in heterozygotes, the remaining wild-type allele is not expressed at a higher level in order to compensate for the loss of a wild-type allele. The findings also indicate that heterozygotes can function normally with half of the normal receptor level, which supports earlier views that uterine ER levels were in excess relative to biological activity (*15*). Crosses of the heterozygous mice resulted in the live birth of normal litter sizes of offspring containing the traditional Mendelian genotypes. A balanced sex ratio was seen in the homozygous ER mutant mice which indicates that sex determination was not affected by absence of the estrogen receptor. This data suggested that disruption of the estrogen receptor was not lethal. However, mating studies with the homozygous ER mutant mice showed that both sexes were infertile.

Phenotype Characterization

We are presently analyzing the tissues of heterozygous and homozygous animals for any alteration that may be associated with the inactivation of the estrogen receptor. This transgenic estrogen receptor knockout (ERKO) line will be further characterized in order to analyze the activity and molecular effects of various hormonally active compounds, such as diethylstilbestrol (DES), and the triphenylethylene antiestrogen compound tamoxifen to determine if other, as yet undescribed proteins such as orphan receptors are present that can mediate estrogenic activity. Antiestrogen binding proteins have been described that were distinct from the ER (*16*) and could possibly mediate the activity of antiestrogens. When treated with tamoxifen, no uterine stimulation (uterotropic response) was seen in the ERKO mice as compared to wild-type litter mates (Fig. 2), even though tamoxifen is an estrogen agonist in the mouse (*17*). This result indicates that the biological activity of this compound is associated with the estrogen receptor in uterine target tissue.

The tamoxifen result is of particular interest because the exposure to environmental chemicals has been associated with estrogenic-like effects. Once the ERKO mouse is characterized, it should be useful in investigating whether the activities of environmental chemicals associated with estrogenic effects operate through the classical estrogen receptor signaling pathway. The mice could also be used to assess the activity of various drug type preparations for either the absence or presence of possible estrogen-like activities. DES exposure during early development induces reproductive tract cancers in mice and humans (*18*, *19*). Similarly, to test for the relationship of the ER and these cancers, groups of ERKO mice will be treated with DES to determine if the same reproductive tract and other target tissue cancers develop in these mice as they do in wild-type animals.

Initial analyses of the first homozygous mutant females have shown that they contain reproductive tract structures, but lack uterine response to estrogen treatment (Fig. 2). Biochemical analyses detect 5 to 10% levels of residual ligand binding in uteri from ERKO animals. Binding was undetectable in other tissues such as the liver, spleen, brain, and kidney. No detection of the full-length or truncated forms of the ER protein by Western blot was made with the use of the H-222 antibody, which recognizes an epitope in the COOH-terminus near the ligand-binding region of the receptor. It is not possible to conclude currently whether this binding in the uterus is the result of some other non-ER estrogen-binding protein that is detectable by binding assays because of the lack of ER in the tissue. Conversely, it could arise from a small amount of altered protein product transcribed from the disrupted ER gene which is biologically inactive. Studies of the ligand structural specificity of the residual binding activity are being attempted as mutant tissue becomes available.

Estrogen stimulates early responses of water imbibition and hyperemia in the uterus (*15*). These responses may be nongenomic actions of estrogen and not involve the receptor (*20*). Estradiol treatment and experiments with hydroxy tamoxifen indicate that there is no response to treatment in the ER mutant animals (Fig. 2). Thus, the early responses of hyperemia and water imbibition require the presence of a functional estrogen receptor. The homozygous mutant females are infertile and have hemorrhagic cystic ovaries (Fig. 2), which suggest they are excessively stimulated by gonadotropins as a result of the lack of a functional negative feedback mechanism. It is not clear whether this ovarian condition

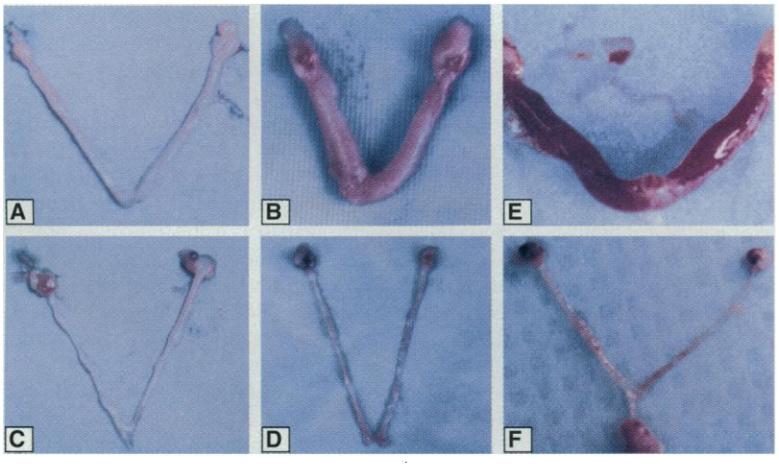

Fig. 2. Uterotropic 3-day bioassay. Wild-type littermates (**A**, **B**, and **E**) or ER mutant animals (**C**, **D**, and **F**) were treated for 3 days with vehicle (A and C); estradiol, 40 μg/kg (B and D); or hydroxy tamoxifen, 1 mg/kg (E and F). The uteri of wild-type animals show the characteristic increase in size following treatment with estradiol or hydroxy tamoxifen, whereas uteri from ERKO animals do not show any increase after treatment with either compound. Note also the hemorrhagic ovaries of the ERKO animals. Portions of figure are reproduced from (*8*).

is representative of the polycystic ovary syndrome in humans (*21*). A similar ovarian histological profile has been suggested to occur after prolonged treatment with an antiestrogen (*22*), which indicates that a common phenotypic effect is produced by loss of estrogen action in the ovary.

Ovarian histology of the ER mutants shows no functional corpora lutea, even though granulosa and theca cells are present. Follicle development appears to proceed through primary and secondary stages, but arrests prior to formation of ovulatory follicles. Preliminary attempts to overcome this arrest with exogenous gonadotropins have led to formation of a few antral or ovulatory follicles, but result principally in development of unviable (cystic atretic) follicles. Further analyses of the ovaries are being performed in order to evaluate the biochemical indexes of response. For instance, ribonuclease protection assay analysis of the ovaries indicates that they contain extremely low amounts of progesterone receptor mRNA. Because of the low progesterone receptor (PR) gene expression it is possible that the phenotypic effects seen in the ovary could be a result of compromised action of both estrogen and progestogen biological activities. ERKO mice may provide a physiological model for critically testing the role and action of estrogen in the ovary.

An unexpected finding was that sexually mature adult ERKO male progeny were also infertile, but appear to have anatomically normal male accessory sex organs. Histological analyses indicate that the testes are reduced in size, with some intact seminiferous tubules, but the majority are highly dysmorphogenic and contain few germ cells. Sperm are present in the testis and epididymis, however the sperm count is very low when compared to wild type (less than 10%). A developmental analysis of younger males is being performed to determine when the testicular phenotype appears and whether it is influenced by hormonal exposure. Male infertility as a result of the inactivation of the estrogen receptor was unexpected. In contrast, studies with a progesterone receptor knockout (PRKO) mouse indicate that females are infertile, but males are fertile (*23*). ER homozygous male mice should be a useful model system in which to evaluate the role of the estrogen receptor mechanisms in male reproductive biology. Absence of functional ER affects other organs in addition to the female and male reproductive tracts. For example, adult female mice lacking the ER have undeveloped mammary glands with only vestigial ducts present at the nipples. Currently, stimulation studies are under way with different hormones and growth factors to evaluate their action. We are now attempting to cross mice having altered oncogene expression, which have an increased incidence of mammary cancer, with ERKO heterozygotes, so we can test whether the estrogen receptor is a needed component for the development of breast cancer.

Another area of interest has been the action of estrogen in bone and skeletal tissues. In the past several years estrogen receptors were identified in bone cells (*24*) implying that estrogen may have some direct effects (*25*). Of particular relevance to those studies was the observation that the bone density of ERKO males and females was 20 to 25% lower than in wild-type mice. Findings with this ERKO transgenic mouse suggest a direct role for estrogen receptor action in bone physiology.

ERKO transgenics are being used as the background strain to reintroduce mutant estrogen receptor protein (for example, TAF-1 or TAF-2 deletion mutants) (*26*) expression by insertional transgenic technology. Previously, analyses of the expression and function of these mutant hormone receptors could only be analyzed in vitro with DNA transfection techniques. Animals can be produced that express only the mutant receptors, to determine tissue and gene regulatory specificity of the mutant receptor protein under in vivo physiological conditions.

ERKO mice can also be used for studies of potential significance to human health. Species differences have been reported for the biological activity of antiestrogens. It is now possible to create a mouse line that expresses only human estrogen receptor to study these differences. Such an animal line can be highly effective as a physiological model system for the evaluation of the activities of antihormonal therapies. Furthermore, by the reintroduction of ER using a tissue- or cell type–specific promoter, such as lactoferrin for the uterus (*27*), or follicle-stimulating hormone receptor for the ovary (*28*), it should be possible to test for rescue of the recessive phenotypes and the role of the ER in specific tissues and organs.

Clinical Findings

Although the findings in the ERKO mouse are exciting, their application and relevance to human physiology may be questionable. Because of the species differences in genetic backgrounds, some experimental knockout mice do not reflect what is expected in the human condition (*29*). The absence of reports of parallel human syndromes or gene mutations has been surprising. Therefore, it was of great interest to learn of a 28-year-old fully masculinized male patient who was seeking correction of a skeletal problem of knock-knees and was found to have unclosed epiphysis. Serum steroid and gonadotropin levels were elevated in this patient which suggested his condition was not a result of mutations of the aromatase gene as recently reported (*30*). He was later shown to be insensitive to high-dose estrogen treatment and had no expected side effects such as breast enlargement (gynecomastia). Because the animal studies had indicated an ER gene disruption was not lethal, it was possible that the patient had some defect in his estrogen receptor function. For example, this could be the result of the expression of a dominant negative form of the receptor (*31*), such as a RNA splicing variant described in some breast tumors that inactivates estrogen receptor activity. Molecular genetic analyses demonstrated this patient was homozygous for a mutation in exon 2 of the estrogen receptor gene, which resulted in the creation of a stop codon that produced a premature truncation of the estrogen receptor protein (*32*). This becomes the first description in the human population and clinical literature of a loss of function mutation in the estrogen receptor gene that produces an example of an estrogen insensitivity syndrome. A major phenotype of this patient is the nonclosure of his epiphysis and a dramatic decrease in his bone density (*32*), similar to that observed in the ERKO mice. Limited analysis of this patient's sperm levels and functionality makes his fertility unknown at the present time. Therefore, it appears that the ERKO mice may be an acceptable model for the evaluation of a variety of estrogen responses and accompanying mechanisms related to the human population. For instance, it may be plausible, because ER gene mutations appear to be carried in the human population, that some etiology of human infertility may arise from such mutations.

The ERKO knockout mouse is an experimental system that promises to have extreme utility for investigations of basic, applied, and clinical research that involves estrogen hormone studies. Creation of a physiological animal model without a functional estrogen receptor allows the further evaluation of the role of estrogen hormone action in a variety of tissues during early developmental stages. The importance of estrogen in the mediation of physiological responsiveness is not presently appreciated, and its role in pathological conditions and cancer could finally be evaluated. Application of the findings in this experimental system related to clinical questions regarding osteoporosis, cardiovascular biology, and breast, endometrial and ovarian cancers, hopefully will be forthcoming.

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