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It is known that the actions of estrogen in mammary development are mediated primarily by the estrogen receptor alpha, but it is not known which estrogen receptors, those in stroma or those in epithelium mediate mammary development. Our purpose is to probe this question by constructing transgenic mice in which wild type human estrogen receptor (hER) and mutants of the receptor that are super-active either at the classical ERE or alternative AP-1 pathway are selectively expressed in epithelium or stroma. A further purpose is to explore the importance of the AP-1 versus the classical pathway in estrogen effects on ductal development. In the first year of this project we have constructed vectors for expression of wild type and mutant human estrogen receptors in the mammary epithelium of transgenic mice, and have made a pilot study of the effects of expressing these receptors in the reproductive epithelium of transgenic mice. The mice transgenic for a human ER super-active at AP-1 show gross abnormalities and hyperproliferation of the reproductive tract.

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Introduction and abstract:

It is known that the actions of estrogen in mammary development are mediated primarily by the estrogen receptor alpha, but it is not known which estrogen receptors, those in stroma or those in epithelium mediate mammary development. Our purpose is to probe this question by constructing transgenic mice in which wild type human estrogen receptor (hER) and mutants of the receptor that are super-active either at the classical ERE or alternative AP-1 pathway are selectively expressed in epithelium or stroma. A further purpose is to explore the importance of the AP-1 versus the classical pathway in estrogen effects on ductal development. In the second year of this project we have developed lines of trangenic mice with wild type and super-active mutant human estrogen receptors driven by the MMTV promoter. We will now characterize the mammary proliferative response to estrogen in these animals.

Body:

We divided up this application into two tasks:

Task 1. To Analyze whether estrogen treatment causes mammary ductal hyperplasia in transgenic mice in which human estrogen receptor and pathway specific super active varients thereof are over-expressed in mammary stromal fibroblasts, adipocytes and epithelial cells (months 1-36).

-Construct vectors that will allow expression of human ER and super-active varients of ER in the mammary stromal fibroblasts and other mammary tissues of transgenic mice (months 1-12).

-Create mice carrying the expression vectors for human ER and test them for expression of human ER in mammary gland and other tissues (months 6-24).

-Determine whether mice that express high levels of either wild type or super-active hERs in mammary compartments develop ductal hyperplasia (months 18-36).

Task 2. To determine whether ERKO transgenics that have hER expression in specific tissues of the mammary gland display restored mammary ductal development in response to estrogen

-Establish a colony of ERKO +/- mice (months 1-12)

-Bred these mice to transgenics expressing human ER (months 12-24)

-Analyze mammary development in ERKO -/- transgene offspring (months 18-36)

In the first year we have made progress on three fronts, vector construction, pilot studies in mice, and establishment of the ERKO colony. In the second year we have made progress in the creation of transgenic mice carrying expression vectors for human ER, wild type and AP-1 super-active driven by the MMTV promoter. In addition, our pilot study has been submitted for publication. The submitted version is appended. The abstract is below.

Generation of transgenic mice with MMTV:hER and MMTV:hERK206A.

The cellular targets of estrogen in mammary ductal development:
Summary of Activity (summer 2000-summer 2001)

The transgenes containing either the wild type human estrogen receptor (hER) or the superactive mutant (hER-K206A) that were constructed and tested in vitro (see 2000 report) were given to the UCSF Transgenic Core for injection. Injection of fertilized oocytes was completed in the fall of 2000. A total of 400 injections were made for each transgene, and successfully injected oocytes were implanted into pseudopregnant female FVB/n uteri. Implantations yielded a total of 40 pups from the hER injections and 19 pups from hER-K206A injections. Pups were weaned and tested for transgene expression (hER mRNA; primers were used that recognized both the hER and hER-K206A, but did not recognize mouse ER) by genomic PCR from tail DNA. A total of 7 (4 female, 3 male) hER pups tested positive for the transgene, and 4 (2 female, 2 male) hER-K206A were positive. These "founder" mice were then bred with wild type FVB/n mice to create heterozygous lines of mice expressing the hER and hER-K206A transgenes.

After breeding all founders, one hER (#6, a male) and two hER-K206A (#1 and #4, a female and male, respectively) were found not to transmit the transgene to their offspring. Of the 6 remaining hER lines and the 2 remaining hER-K206A lines, two of each will be further analyzed based on equivalency of transgene expression levels. Both RT-PCR and Northern blotting are currently being used to analyze transgene mRNA expression. Western blot and immunohistochemistry are being used to test for protein from the transgenes. Both whole mount and thin section histology of the mammary glands is also currently being done to analyze the mammary morphology of both lines as well as their non-transgenic littermates. Thus far, gross analysis (palpation and dissection) has not shown evidence of mammary tumors. However, histological analysis shows more promise.

Manuscripts in preparation:

Price, RH and Kushner, PJ (in preparation) Transgenic mice bearing a superactive estrogen receptor in mammary epithelial cells; analysis of ductal development.

Key Research Accomplishments:

- Developed trangenic mice in which human estrogen receptor and super-active varients are under the regulation of the Mouse Mammary Tumor Virus promoter.

- Demonstrated that expression in the genital tract of mice of the human estrogen receptor super-active at AP-1 targets leads to enlargement, hyper-proliferation, and cyclin D1 overexpression.

Reportable Outcomes:

Manuscripts-One submitted study is being reviewed. The title and abstract are below.

Estrogen receptor superactive at AP-1 induces hyper-proliferation

Birgit Anderegg [1,5,8], Rosalie M. Uht[2,6,8], Adriana C. Rossi[1], Paul Webb[2], Richard H. Price[4], D. Barry Starr[3,7], Jeffrey M. Arbeit[1,9] & Peter J. Kushner[2,4,9]

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Estrogen stimulates proliferation via estrogen receptor alpha (hERα), which induces target genes either with classical estrogen response elements (EREs) [1] or AP-1 sites [2-5]. We show that a mutant hERα underactive at EREs and superactive at AP-1 induces enlargement, hyper-proliferation, and cyclin D1 overexpression [6,7] in transgenic genital tract indicating that the AP-1 pathway mediates proliferation.

Abstracts-

Nuclear Receptors 2000, "A point mutation in the ER DBD separates the AF-dependent from the AF-independent pathways of AP-1 stimulation and generates an abnormal phenotype in transgenic mice."

R. Uht, B. Anderegg, P. Webb, C. Anderson, J. Arbeit, and P. Kushner.

Talks.

Nobel Symposium- Karlskoga Sweden June 1999

Gordon Research Conference on Breast and Prostate Cancer August 1999

Merck Symposium on Estrogen Action, Philadelphia November 1999

University of Colorado Health Sciences Cancer Research Mini Symposium, March 2000

University of California San Francisco, Breast Oncology Group Seminars, June 2000

Frontiers of Estrogen Action, New Castle on Fergus, Ireland, April 2001

Patents;

One patent has been applied for."Expression of human steroid receptors in transgenic animals. UC case no. 99-382 Jeffrey M. Arbeit et al. Inventors.

US Patent Application No. 09/365,614

Conclusion:

From the first and second years of this study we can conclude that a point mutation in the DNA binding domain of the human estrogen receptor that confers super-activity selectively on AP-1 targets and is at the same time under-active at target genes with classical EREs causes enlargement, hyperproliferation, and cyclin D1 over-expression in transgenic mice genital tract. This points to the estrogen receptor-AP-1 pathway in proliferation.

We now have mice that should express the same super-active receptor in mammary gland. We are now eager to see whether they have hyper-proliferative responses to estrogen.

"So What?" If expressing the K206A mutant human ER in the mammary gland also leads to hyperproliferation, it will suggest that in hormone dependent breast cancer and premalignant states, the pathway of hormone action leads to AP-1 regulated target genes. "So What?" Well once we know the pathway, we can take steps to block hormone action, and maybe some day develop better antiestrogens to treat or prevent breast cancer. Do we still want to ask, "So what?"?

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CAR APPROVAL LETTER

Project # 99016122

May 9, 2001

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Study Title: The Cellular Targets of Estrogen in Mammary Ductal Development

APPROVAL NUMBER: A6532-16122-03

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This number is a UCSF Committee on Animal Research (CAR) number which should be used for ordering animals for this study. This number may only be used by the principal investigator and those listed as participants included in the protocol and should be referenced in any correspondence regarding this study. The committee must be notified in writing of any changes to the approved protocol including changes in personnel.

Please distribute the final approved protocol to all individual participants so that they are familiar with the procedures that have been approved.

Note: All personnel are to be fully trained before independently undertaking any procedures. All first-time animal users at UCSF are required to take the LARC BRER training course; any user with less than 1 year of experience in the specific species must attend the appropriate species-specific course(s). For specific details about these training requirements, refer to the CAR website at: *www.ucsf.edu/ora/car/policy/training.htm.*

If you have any questions, please contact the Committee on Animal Research office at (415) 476-2197, Suite 315, Laurel Heights, Box 0962, or by electronic mail at carora@itsa.ucsf.edu.

SPECIES NAME	TOTAL NUMBER APPROVED					
	Purchased			Bred		
	Category A	Category B	Category C	Category A	Category B	Category C
Mice	128	0	0	400	72	0

Kimberly S. Topp, Ph.D., Vice Chair
Committee on Animal Research

An estrogen receptor that is superactive at alternative response elements causes hyper-proliferation

Birgit Anderegg [1,5,8], Rosalie M. Uht[2,6,8], Adriana C. Rossi[1], Paul Webb[2], Richard H. Price[4], D. Barry Starr[3,7], Jeffrey M. Arbeit[1,9] & Peter J. Kushner[2,4,9]

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Estrogen stimulates proliferation via estrogen receptor alpha (ERα), which activates expression of target genes with classical estrogen response elements (EREs), or with alternative response elements including AP-1 and variant CRE sites. We show that a point mutant in human ERα, K206A, is superactive at AP-1/ CRE sites and underactive at EREs. Targeted expression of ERαK206A, but not of ERα, in female mouse genital tract causes enlargement of the vagina and cervix and hyper-proliferation. Expression of cyclin D1, an alternate response gene is elevated in ERαK206A transgenics, whereas expression of lactoferrin, an ERE gene, is not. These observations implicate the ERα pathway to alternative response elements in estrogen-induced proliferation.

Introduction

Estrogen stimulates proliferation of epithelial cells in the reproductive tract and mammary gland of females, and in the prostate of males, via estrogen receptor alpha (ERα), which functions as transcription factor to regulate expression of target genes (Parker 1998). Female mice in which ERα has been knocked-out (αERKOs) lack estrogen-induced proliferation of the uterus, cervix and vagina (Lubahn et al. 1993; Korach 1994). Male αERKO mice are completely resistant to estrogen-induced prostate metaplasia and cancer (Risbridger et al. 2001). In contrast, both male and female mice lacking the second estrogen receptor, ERβ, have normal or even hyper-normal responses to estrogen (Weihua et al. 2001).

ERα works by modulating the transcription of target genes, whose products are thought to be responsible for estrogen-dependent phenotypes such as the proliferation and development of estrogen-regulated target tissues. However, the identity of many of the specific estrogen-regulated genes that regulate proliferation, and the mechanism of their regulation, is not yet clear. In general, ERα activates expression of two types of target genes. One type has classical estrogen response elements (EREs) in the promoter region which allow the receptor to bind via its centrally located DNA binding domain, and then to recruit coactivator complexes that remodel chromatin and switch on the transcriptional machinery (Parker 1998). A second type of ERα target genes has alternate response elements, which bind heterologous transcription factors but not ERα. This category includes AP-1 sites that bind a Jun/Fos complex (Gaub et al. 1990; Philips et al. 1993; Kushner et al. 2000), or variant cyclic-AMP response elements(CREs) that bind a Jun/ATF-2 complex (Sabbah et al. 1999), or Sp1 sites that bind Sp1 (Saville et al. 2000). The DNA of alternative response elements does not bind ERα, which is believed to participate via protein-protein interactions with the heterologous transcription factors that are bound to these elements or their coactivators (Webb et al. 1995; Webb et al. 1999; Kushner et al. 2000).

Presently, the significance of ERα action at alternate response elements is not understood. Some correlative evidence, however, has pointed towards the importance of the ERα-AP-1 pathway in estrogen effects on proliferation. When the partial agonist tamoxifen mimics estrogen and

stimulates cell proliferation, it also stimulates AP-1 but not necessarily ERE transactivation. (discussed in (Webb et al. 1995; Paech et al. 1997; Philips et al. 1998; Webb et al. 1999; Kushner et al. 2000)). Moreover, ERα can either stimulate or repress the growth response of different breast cell lines in cell culture, and the nature of this response correlates with the effects of ERα on AP-1 activity in the same cell types (Philips et al. 1998). Other correlative evidence comes from the study of specific target genes believed to mediate the proliferative effects of estrogen in the mammary gland. One of the key genes mediating such effects is cyclin D1, which is induced by estrogen, but has no ERE. Induction of cyclin D1 is transcriptional and appears to be mediated mainly by a variant CRE element that binds the AP-1 related factors Jun/ATF-2(Sabbah et al. 1999).

Here, we test the hypothesis that ERα action at alternative response elements is important for estrogen-dependent proliferation in vivo. We describe a mutant of human ERα that is selectively superactive at target genes with alternative response elements, but not at target genes with classical EREs. We explore the effect of the superactive receptor on proliferation by targeting expression to the epithelium of the genital tract of female mice.

Results

ERα K206A is superactive at target genes with alternative response elements.

To probe the role of the ERα/AP-1 pathway in vivo, we looked for an ERα mutation that would affect action at AP-1 sites and other alternate response elements without affecting action at classical EREs. It has previously been shown that a mutation in a conserved lysine residue at the base of the first zinc finger of the DNA binding domain converts the glucocorticoid and thyroid hormone receptors from inhibitors to activators at AP-1 without affecting their action at classical hormone response elements (Starr et al. 1996). We therefore investigated the phenotype of a similar point mutation in hERα (K206A, Fig. 1A).

We first examined the activity of the mutant ERα on reporter genes with different response elements in HeLa cells. In accordance with our previous results, hERα stimulated transcriptional activity on an AP-1 reporter (human collagenase promoter) by two to five fold (Fig.1B). However,

in parallel hERαK206A was 5 to 200 fold more active than wild type hERα. This phenotype was also observed on a range of AP-1 responsive reporter genes (not shown) and in different cell types (Fig. 1C). As noted above, the cyclin D1promoter does not have an ERE and responds to hERα mainly through a variant CRE that binds AP-1 related proteins and is located near the promoter with minor contribution of a more distant consensus AP-1 site (Altucci et al. 1996; Sabbah et al. 1999). Again, hERαK206A was superactive on the cyclin D1 promoter compared to wild type hERα (Fig. 1B). As expected, these estrogen effects required the integrity of both the CRE and AP-1 sites (in preparation). In contrast, the hERαK206A mutation seemed to reduce the efficiency of ERα action at a promoter with Sp1 sites (Saville et al. 2000; Safe 2001). Thus, ERaK206A super-activates a subset of estrogen-regulated promoters with alternate response elements.

The super-activity of K206A is selective and does not extend to target genes with classical EREs. We then examined the effect of the hERαK206A mutation at reporter genes with classical EREs. ERαK206A had modestly reduced activity at the lactoferrin and pS2 gene promoters which are regulated by near-consensus EREs (Nunez et al. 1989; Liu and Teng 1992) (Fig. 2). ERα K206A also failed to super-activate at a promoter in which a consensus classical ERE replaced the AP-1 site in collagenase, nor at several target genes with synthetic consensus EREs (data not shown). Thus, hERK206A selectively super-activates target genes that, like cyclin D1, have AP-1 sites or variant CRE sites and but not target genes with EREs.

Expression of ERαK206A causes enlargement in mouse genital tract.

To determine the effect of super-activation at AP-1 and CRE sites (hereafter AP-1/CRE sites) within an intact organism, we targeted expression of hERαK206A or wild type hERα to the squamous epithelium of the female genital tract, an estrogen responsive tissue. We used the human keratin-14 gene promoter, which allows expression in basal epithelial cells of the vagina and cervix, in basal epithelial cells of the glandular uterus, but not in the luminal epithelial cells of the

uterus ((Arbeit et al. 1996), and data not shown). Two independent transgenic mouse lines were established for each type of transgenic (K14-hERα, and K14-hERαK206A) and observed for up to 24 months of age. Thirty of 36 female mice from the two lines of K14-hERαK206A mice spontaneously developed perineal swelling beginning at 6 months of age, which on examination of internal organs was due to marked vaginal and cervical enlargement (Fig. 3, top row). The vagina in K14-hERαK206A transgenics was folded in pleats, and the vaginal and cervical squamous epithelium was thickened, papillomatous, and hyperplastic (Fig. 3 bottom row, quantitative data not shown). No abnormalities occurred in the uterine luminal epithelium, which does not express the transgene, but the uterine glands, which do express the transgene, are enlarged (not shown). K14-hERα transgenics, by contrast, had normal reproductive tracts (Fig. 3, bottom row center). Expression of human estrogen receptors in the squamous epithelium of the K14-hERαK206A transgenics was not greater than in the K14-hERα transgenics (Fig. 4, top row). Thus the abnormal vaginal-cervical phenotype of K14-hERαK206A transgenics is a feature of the mutation.

K14-hERαK206A transgenics exhibit hyper-proliferation and over-expression of cyclin D1,with normal expression of lactoferrin.

To determine whether the hERαK206A genital enlargement was associated with increased cellular proliferation, the pattern, distribution, and expression level of proliferating cell nuclear antigen (PCNA) and cyclin D1 were analyzed (Fig.4, second and third rows). The frequency and expression level of PCNA was increased in both basal and suprabasal squamous epithelial cells in the K14-hERαK206A transgenic mice, but not in wild type hERα transgenics. Cyclin D1 expression was also elevated in the K14-hERαK206A transgenics, but not in the wild type ERa transgenics. Moreover, the pattern of cyclin D1 overexpression was identical to the distribution of PCNA suggesting that these two markers of proliferation are up-regulated in the same cells. Therefore, increased cyclin D1 expression correlates with the increased cell cycle activity and hyperplasia in the cervical and vaginal squamous epithelium of K14-hERαK206A transgenic mice.

This is consistent with other studies that have shown a role for cyclin D1 up-regulation in estrogen stimulated proliferation in mammary cells in culture and in mice (Sicinski et al. 1995; Prall et al. 1998). Expression of lactoferrin, a classical ERE regulated gene, is no greater in K206A transgenics than in wild type transgenics, consistent with the selective super-activity of the mutant receptor. In summary, genital tract enlargement in female hERaK206A transgenics is associated with hyper-proliferation and overexpression of cyclin D1, an alternative target gene, without overexpression of a classical target gene.

Discussion

While it is clear that estrogens regulate proliferation through ERα, it is not clear which ERα target genes mediate these proliferative effects, nor how ERα regulates these genes. These studies show that mutation of the conserved lysine, K206, at the base of the first zinc finger in the DNA binding domain of hERα profoundly changes its ability to activate different types of target genes. The mutant, hERαK206A, activates target genes with classical EREs with a similar efficiency to wild type hERα, but selectively super-activates target genes with AP-1 or CRE sites. This pattern occurs with a variety of promoter contexts containing either EREs or AP-1/CRE sites and in several different types of transfected cells in culture.

The pattern of activation is also preserved in vivo, as the hERαK206A mutant leads to the overexpression of cyclin D1, an AP-1/CRE regulated gene, in transgenic genital tract but not overexpression of lactoferrin, an ERE regulated gene. Targeted expression of ERαK206A, but not wild type ERα, in female genital tract leads to enlargement and hyper-proliferation as noted by dramatic increases in PCNA staining and cyclin D1 overexpression. Interestingly, hyper-proliferation is restricted to tissues expressing the transgene. Thus, the uterine luminal epithelium, which is highly sensitive to estrogen provoked proliferation, but which does not allow expression of the transgene, is normal in these transgenics. The uterine glands, which do express the transgene, are enlarged. Together these observations suggest that the selective action of hERαK206A at AP-

1/CRE sites is responsible for the hyper-proliferation of the reproductive tract and, in turn, suggests that the ERα pathway to AP-1/CRE sites is important for proliferation in vivo.

While our evidence is consistent with the notion that ERαK206A causes hyperproliferation by super-activating genes with alternate response elements, the identity of these estrogen-regulated genes is not clear. We have seen that one candidate gene that contains alternate response elements, cyclin D1, is overexpressed. Because the pattern of staining for PCNA and cyclin D1 appear to be the same, hyper-proliferation may stem, in part, from overexpression of cyclin D1. Previous studies with MMTV:cyclin D1 and K5:cyclin D1 transgenic mice revealed hyper-proliferation in mammary gland and reproductive epithelia respectively, suggesting that cyclin D1 overexpression was sufficient for hyper-proliferation (Wang et al. 1994; Robles et al. 1996). However it is also possible that the hERαK206A transgene additionally causes overexpression of other D-type cyclins or other pro-proliferative genes with a similar function.

Our studies do not address the reasons that ERαK206A super-activates genes with AP-1 sites. The conserved lysine, K206, appears to be a unique site for mutations of hERα that confer super-activity at AP-1 sites while preserving activity at EREs (data not shown). Likewise, the homologous residue in the glucocorticoid receptor, K461, is the only residue among 30,000 tested mutants of the DNA binding domain that can will confer a similar profound change of target gene preference at AP-1 sites (Starr et al. 1996). We show elsewhere that the mutant super-activates AP-1/CRE target genes through its AF-1 and AF-2 functions, and suggest that the mutation may relieve inhibition mediated by the DNA binding domain when the receptor is not bound to DNA (R Uht et al. in preparation).

Finally, we speculate that ERα action at alternate response elements will prove to be important for estrogen-dependent proliferation in other settings. If further studies with K206A transgenics that allow expression in the uterus, mammary gland, ovary, and prostate confirm hyper-proliferation, it will suggest a potential role for over-stimulation of the ER-AP-1/CRE pathway in the pathogenesis of estrogen induced proliferative disorders. Since the ER-AP-1/CRE

pathway has distinguishing functional requirements (Webb et al. 1999), such studies would suggest it as a potential target for drugs that disrupt the pathway and that might be useful for cancer prevention.

Materials and Methods

Plasmids

Reporters genes driven by the collagenase promoter and by the cyclin D1 promoter were previously described (Albanese et al. 1995; Webb et al. 1995). HSV-TK:LUC contains Herpes Simplex Virus TK promoter sequences –109/+45 which contains two SP-1 sites. and was prepared by removing the consensus ERE from ERE II TK:LUC (Webb et al. 1995). hER and hERG400V expression vectors were previously described (Webb et al. 1995). The K206 mutation was introduced by site-directed mutagenesis (Quickchange kit, Stratagene). To construct transgene expression vectors hERα and hERα.K206A were isolated by EcoR1 digest, converted to blunt ends with Klenow and ligated into a K14 transgene vector (Munz et al. 1999) digested with SmaI.

Transfections

Transfection were performed by electroporation using 2μg reporter, 1μg actin-βgalactosidase expression vector as an internal control and 5μg ER expression vectors or empty vector (Webb et al. 1995). Luciferase and βgal activities were determined by standard methods 36-40hrs after plating. To calculate relative luciferase activity, luciferase activities were divided by βgal activity and the value obtained in the absence of ER expression vector and estradiol was set at one.

Histopathology and immunohistochemistry

As described previously (Arbeit et al. 1996) mice were sacrificed by perfusion of the ascending aorta with 3.75% paraformaldehyde under Avertin anesthesia. Reproductive tracts were dissected and post-fixed overnight at 4°C. After removal of the posterior vaginal wall, tissues were rinsed in

phosphate buffered saline (PBS), dehydrated through graded alcohols and xylene, embedded in paraffin, and 5um sections were stained with hematoxylin/eosin (Sigma). PCNA immunohistochemistry was carried out as described previously (Arbeit et al. 1996). Briefly, 5um tissue sections were deparaffinized, rehydrated and subjected to antigen retrieval in 10mM citrate buffer, pH 6.0 by microwave for two 5-minute high-power pulses. Sections were blocked in 3% bovine albumin (Sigma) in PBS, and subsequently-incubated with a 1:200 dilution of mouse anti-PCNA monoclonal antibody (Biogenex). Signal development was performed by using a biotinylated goat anti-mouse IgM secondary antibody (diluted 1:200; Vector), the Vector Elite immunoperoxidase reagent (Vector), and NovaRed solution (Vector) as a substrate. Sections were counterstained with Gill's #1 hematoxylin (Sigma). Human ERα IHC was carried out similarly, using a 1:200 dilution (in 0.2% bovine albumin) of anti-human ERα monoclonal antibody D75 (Greene et al. 1980) and biotinylated anti-rat IgG secondary antibody (1:200 in 0.2% bovine albumin; Vector). Immunohistochemistry for cyclin D1 (1:500; Upstate Biotechnology) and Lactoferrin (1:2000; a generous gift from C.T. Teng, NIEHS, North Carolina) was performed as described for PCNA, but additionally included blocking of endogenous peroxidase by a 20-minute incubation in 3% hydrogen peroxide in methanol following tissue dehydration. A 1:200 dilution of a biotinylated anti-rabbit IgM (Vector) was followed by 3,3'-diaminobenzidine (Sigma) as the chromogen.

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Figure legends

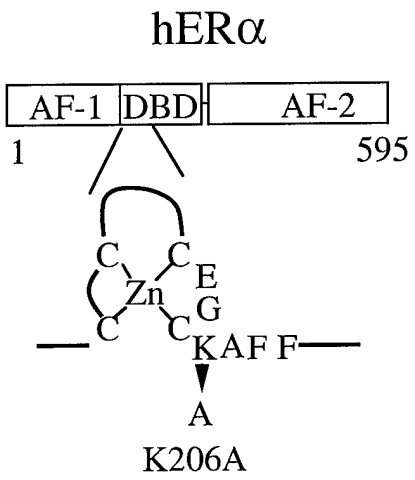
Figure1 A point mutation in the ERα DNA binding domain (DBD) results in super-activation at promoters regulated by estrogen through AP-1/CRE sites. A, Location of the hERαK206A mutation in the first zinc finger of hERα B, Ability of the hERαK206A mutant to enhance expression of a reporter genes regulated by estrogen through an AP-1 site (human collagenase promoter), CRE (cyclin D1), or Sp1 site (HSV-TK). C, hERαK206A super-activity on an AP-1 reporter in a variety of cell lines in culture: HeLa, cervical cancer; Ishikawa, endometrial cancer; MDA-MB & MCF-7, breast cancer; DU145, prostate cancer; GT1, hypothalamic; GHT1-5, pituitary lactotroph; CEF, chick embryo fibroblasts; COS, SV40 transformed kidney fibroblast.

Figure 2 The hERαK206A mutant is underactive at target genes with classical EREs. Ability of the mutant and wild type hERs to activate expression of reporter genes with the lactoferrin and pS2 promoters, which contain near consensus EREs, and at a promoter with a consensus ERE replacing the AP-1 site of collagenase (ERE-Coll),

Figure 3 Reproductive tract abnormalities in female K14-hERαK206A transgenic mice. A, Vaginal enlargement (but note normal uterus) develops in K14-hERαK206A transgenic (right) but not in age-matched (9 months) nontransgenic (left) or wild type K14-hERα transgenic (center) mice. B, K14-hERαK206A mice develop cervical and vaginal enlargement with squamous epithelial thickening not seen in nontransgenic (left) or K14-hERα transgenic (center) mice.

Figure 4 Hyper-proliferation in K14-hERαK206A transgenic genital epithelium. First row shows that human ERα (huERα), assayed with a specific monoclonal antibody, is not more strongly expressed in vaginal squamous epithelium of K14-hERαK206A transgenic mice (right) than in K14-hERα transgenic (center) or nontransgenic (FVB/n, left) control mice (200x). Second row shows that ERαK206A transgenic mice have more proliferating cells than ERα transgenic or nontransgenic mice as revealed by staining for proliferating cell nuclear antigen (PCNA) in both basal and suprabasal cell layers (400x). Third row shows that cyclin D1 immunoreactivity is increased and detected in multiple basal and suprabasal vaginal epithelial cell layers in ERαK206A transgenic mice, compared to low-level basal cell expression in ERα transgenic and nontransgenic mice (400X). Fourth row show that lactoferrin expression is not elevated in the K14-hERαK206A transgenics.

A



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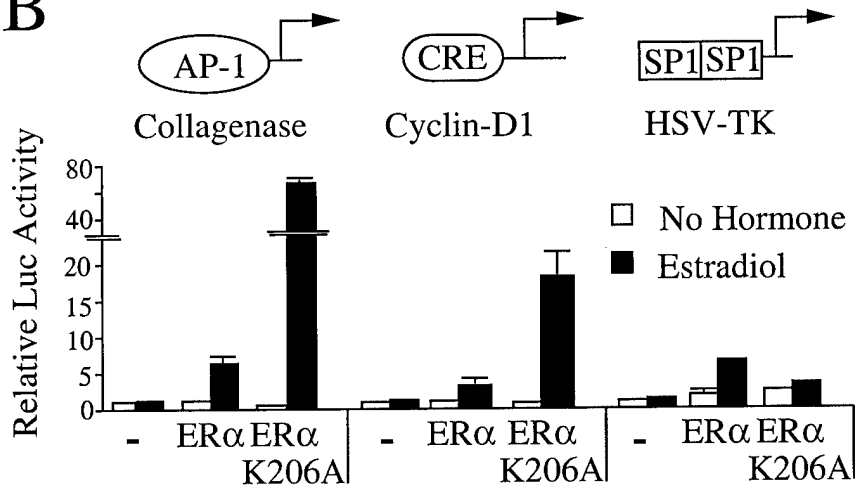


C

	Fold Induction	
Cell Line	hERα	K206A
HeLa	4.3	46.7
Ishikawa	2.1	31.1
MCF-7	2.2	214.3
MDA-MB453	2.2	224.0
MDA-MB231	2.3	81.5
DU145	4.2	22.3
GT1	1.2	88.0
GHFT1-5	1.8	101.1
CEF	1.9	5.2
COS	1.7	7.7

Fig.1

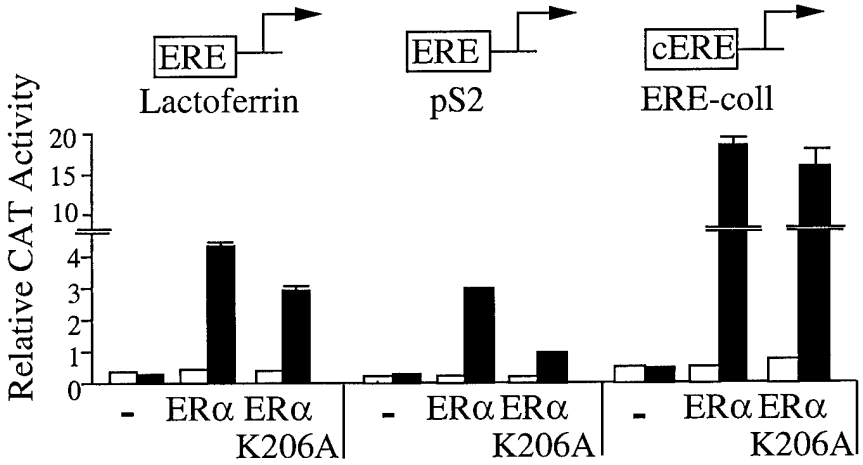

ERE
Lactoferrin
ERE
pS2
cERE
ERE-coll
Relative CAT Activity
20
15
10
4
3
2
1
0
-
ERα
ERα K206A
-
ERα
ERα K206A
-
ERα
ERα K206A

Fig. 2

Fig. 3

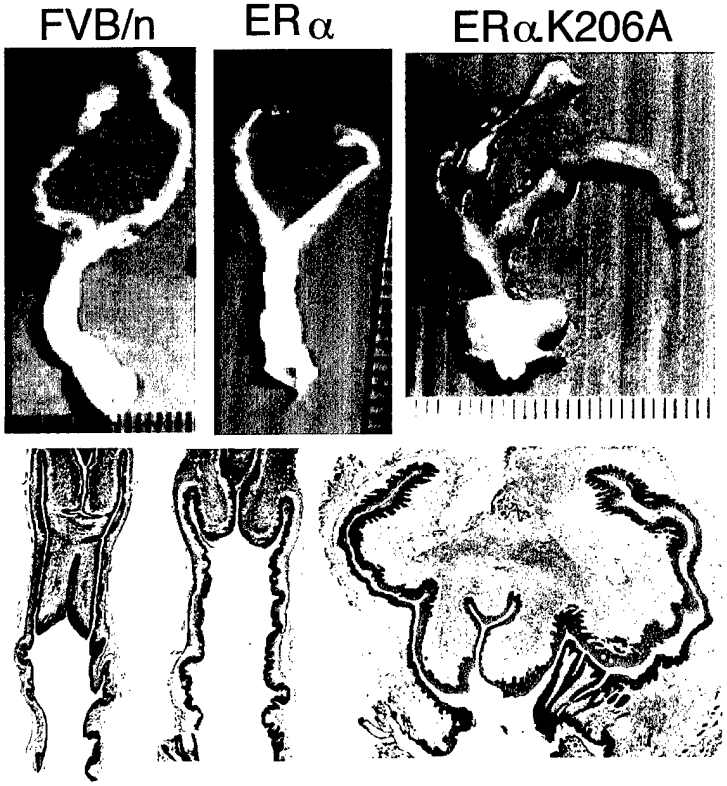
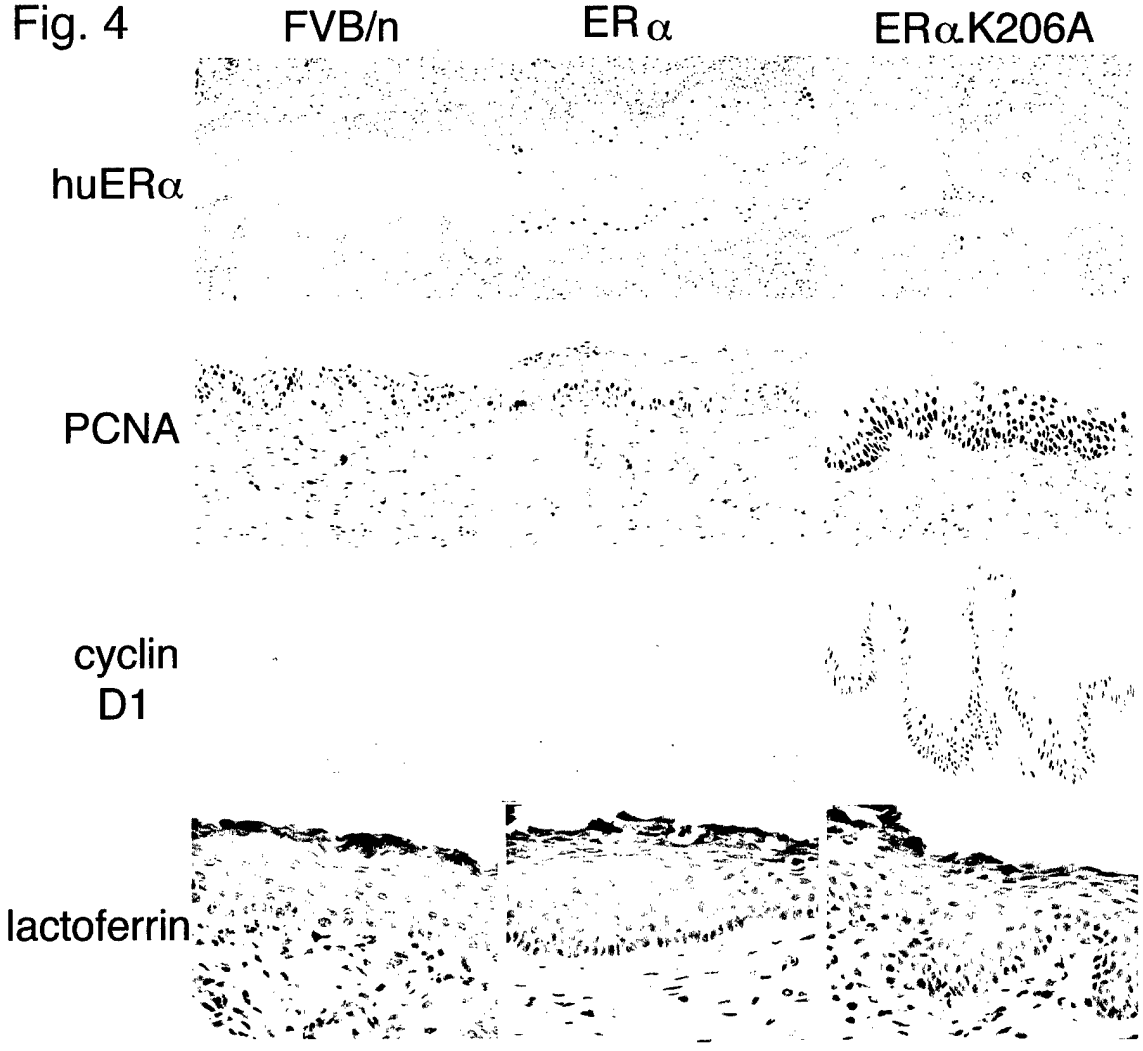

FVB/n
ERα
ERαK206A

Fig. 4


FVB/n
ERα
ERαK206A
huERα
PCNA
cyclin
D1
lactoferrin