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Estrogen signaling is not required for prostatic bud patterning or for its disruption by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin

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Abstract

Estrogens play an important role in prostatic development, health, and disease. While estrogen signaling is essential for normal postnatal prostate development, little is known about its prenatal role in control animals. We tested the hypothesis that estrogen signaling is needed for normal male prostatic bud patterning. Budding patterns were examined by scanning electron microscopy of urogenital sinus epithelium from wild-type mice, mice lacking estrogen receptor (ER) α , ER β , or both, and wild-type mice exposed to the antiestrogen ICI 182,780. Budding phenotypes did not detectably differ among any of these groups, strongly suggesting that estrogen signaling is not needed to establish the prototypical prostatic budding pattern seen in control males. This finding contributes to our understanding of the effects of low level estrogen exposure on early prostate development. *In utero* exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) can greatly alter the pattern in which prostatic buds form and reduce their number. For several reasons, including a prior observation that inhibitory effects of TCDD on prostatic budding in rats depend heavily on the sex of adjacent fetuses, we tested the hypothesis that estrogen signaling is needed for TCDD to disrupt prostatic budding. However, budding did not detectably differ among wild-type mice, or mice lacking ER α , ER β , or both, that were exposed prenatally to TCDD (5 μ g/kg on embryonic day 13.5). Nor did ICI 182,780 detectably affect the response to TCDD. These results strongly suggest that estrogen signaling is not needed for TCDD to inhibit prostatic epithelial budding.

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Conflict of interest statement

The authors declare they have no conflict of interest or competing financial interests.

Keywords

Prostatic budding patterns; Estrogen signaling; 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD); ICI 182,780; Estrogen receptor knockout mice; Prostate development

Introduction

The research described in this report addresses two related though distinctly different issues: the role of estrogen signaling in prenatal prostate development in control animals, and whether estrogen signaling is essential for the disruption of prenatal prostate development caused by the environmental toxicant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD).

Prostate development begins in fetal males when urogenital sinus (UGS) epithelium forms prostatic buds under the influence of the surrounding mesenchyme. Most buds elongate, canalize, and ultimately develop into prostatic ducts. Both prenatal and postnatal prostate development are heavily androgen dependent (Cunha et al., 1987; Marker et al., 2003), yet prostatic budding in control animals occurs in the presence of high estrogen concentrations (vom Saal, 1989; Prins and Korach, 2008; Ruhlen et al., 2008).

It has long been known that excessive estrogen exposure can severely impair prostatic budding and subsequent prostate development (Green et al., 1940, 1941; Raynaud, 1942). More recently, UGS and prostate development were found to be affected by intrauterine position (Nonneman et al., 1992; Timms et al., 1999, 2002), and were reported by some (vom Saal et al., 1997; Gupta, 2000; Timms et al., 2005; Hofkamp et al., 2008) though not all (e.g., Tyl et al., 2008) laboratories to be exquisitely sensitive to stimulatory effects of low level exposure to estrogenic chemicals. Additional research demonstrates that estrogens can affect prostate development and prostate health/disease in adulthood in multiple ways (Prins et al., 2006).

Research on the question of whether estrogens are *required* for normal prostate development was facilitated greatly when estrogen receptor (ER)-deficient mice, and mice lacking the enzyme needed to convert testosterone to 17 β -estradiol (aromatase), became available. Mice lacking ER α , ER β , both ER α and ER β , or aromatase are referred to as ER α KO, ER β KO, ER $\alpha\beta$ KO, and ArKO mice, respectively. The fact that all these mice have prostates (Eddy et al., 1996; Krege et al., 1998; Couse et al., 1999; Dupont et al., 2000; McPherson et al., 2001) provides compelling evidence that estrogens are not needed for the prostate to develop (and therefore are not needed for prostatic budding to occur). Nevertheless, prostates are not entirely normal in mice lacking full estrogen signaling. Hyperplasia, changes in lobe weights, abnormalities in proliferation, differentiation, apoptosis, ductal branching morphogenesis, and stromal-epithelial cell signaling, and other anomalies have been reported in prostates from ERKO and/or ArKO mice (McPherson et al., 2001, 2007; Weihua et al., 2001, 2002; Jarred et al., 2003; Risbridger et al., 2003; Imamov et al., 2004; Omoto et al., 2005; Omoto, 2008; Chen et al., 2009). McPherson et al. (2007) summarized research in this area by noting that "... although androgens are essential for the coordinated growth of the prostate, local estrogenic activity is equally essential for the modulation of normal prostate development."

The abnormalities described above were all seen in studies where prostates were only examined postnatally. One objective of the present study, therefore, was to determine if abnormalities in prostate development caused by impaired or absent estrogen signaling can also be found *prenatally*. The hypothesis we tested is that estrogen signaling is required for establishing the normal prostatic budding pattern in fetal males. To do so, we compared prostatic epithelial bud formation in wild-type mice with that in ER α KO, ER β KO, and ER $\alpha\beta$ KO mice, as well as in

wild-type mice exposed *in utero* to the estrogen receptor antagonist ICI 182,780 (Wakeling et al., 1991).

The second issue addressed in this report is whether estrogen signaling is necessary for the region-specific disruption of prostatic epithelial bud formation caused by *in utero* TCDD exposure. In mice, maternal TCDD treatment can prevent ventral prostatic buds from forming, displace dorsal and lateral buds towards the dorsal surface of the UGS epithelium, and reduce dorsolateral bud numbers (Lin et al., 2003, Vezina et al., 2008). Long-term consequences for the prostate include ventral lobe agenesis, altered ductal branching patterns in the dorsal, lateral, and anterior lobes, impaired expression of androgen-dependent genes, abnormal retention of androgen dependence with aging, and pathological abnormalities in senescence (Ko et al., 2002; Lin et al., 2002a,b; Fritz et al., 2005). Because these prostate abnormalities in adulthood are caused by or may originate in part due to aberrant prostatic bud formation, our laboratory has been investigating the mechanisms by which TCDD affects prostatic budding. We previously reported on the potential role of androgen signaling (Ko et al., 2004), epidermal growth factor and transforming growth factor α signaling (Abbott et al., 2003), aryl hydrocarbon receptor (AHR) signaling (Vezina et al., 2008), and WNT5A signaling (Allgeier et al., 2008) in causing these abnormalities, and have now examined the relationship in mouse UGS between TCDD and estrogen signaling.

The possible connection between effects of TCDD on prostatic epithelial bud formation and estrogen signaling is based on several observations. First, there is crosstalk between the AHR signaling pathway (through which TCDD acts) and estrogen signaling pathways (Matthews and Gustafsson, 2006; Ohtake et al., 2009). Second, TCDD can functionally affect estrogen-dependent processes, as shown both by its antiestrogenic properties (Safe et al., 1998) and its ability to increase the inhibitory potency of 17 β -estradiol (Bookstaff et al., 1990). Third, according to most reports (Greene et al., 1940, 1941; Raynaud, 1942; Vannier and Raynaud, 1980; though see Timms et al., 2005) the inhibitory effects of estrogenic chemicals on prenatal prostate development, like those of TCDD (Lin et al., 2003, Vezina et al., 2008), are greatest for the ventral lobe. Fourth, both TCDD (Lin et al., 2003, Vezina et al., 2008) and low level exposure to estrogenic chemicals (vom Saal et al., 1997; Timms et al., 2005; Hofkamp et al., 2008) can change the pattern in which prostatic buds form. Finally, TCDD is significantly more effective at inhibiting prostatic budding in fetal male rats whose intrauterine position is between two females (which generally elevates their 17 β -estradiol concentrations) than when their intrauterine position is between two males (Timms et al., 2002). Collectively, these observations led us to hypothesize that the inhibitory effects of TCDD on prostatic budding by the UGS may require estrogen signaling. Therefore, we determined whether effects of TCDD on prostatic epithelial budding would be reduced or absent in ER α KO, ER β KO and ER α β KO mice, and/or in wild-type mice exposed *in utero* to ICI 182,780.

Materials and Methods

Animals and treatments

Colonies of wild-type C57BL/6J mice, one strain of *Era* knockout mice (kER α KO; B6.129P2-*Esr1*^{tm1Ksk/J}; Lubahn et al., 1993), and *Er β* knockout mice (ER β KO; B6.129P2-*Esr2*^{tm1Unc/J}; Kregge et al., 1998) were started with breeders obtained from The Jackson Laboratory (Bar Harbor, ME). kER α KO mice are widely used in estrogen signaling research despite having a truncated form of ER α that retains some residual signaling activity (Couse et al., 1995). A second strain of *Era* knockout mice was also studied (mER α KO; *Esr1*^{tm1.1Mma}; Dupont et al., 2000; used with permission from Dr. Pierre Chambon, Institut de Génétique et de Biologie Moléculaire et Cellulaire, Strasbourg, France). All knockout strains were backcrossed onto a C57BL/6J background for at least eight generations. Heterozygous males and females were mated to generate individual allele knockout fetuses and wild-type littermate controls.

$kEr\alpha^{+/-}/Er\beta^{+/-}$ and $mEr\alpha^{+/-}/Er\beta^{+/-}$ females were mated to $kEr\alpha^{+/-}/Er\beta^{-/-}$ and $mEr\alpha^{+/-}/Er\beta^{-/-}$ males, respectively, to generate $kEr\alpha\beta$ KO and $mEr\alpha\beta$ KO offspring. Animals were housed with a 24-hour light-dark cycle (lighted 0600 h to 1800 h) at $22 \pm 1^\circ\text{C}$ in clear plastic microisolator cages with corn cob bedding. Feed (5015 Mouse Diet, PMI Nutrition International, Brentwood, MO) and water were available *ad libitum*.

Timed-pregnant females were generated by housing males and females together overnight. The day after overnight mating was designated embryonic day (E) 0.5. Most pregnant dams were given a single oral dose of corn oil (5 ml/kg) or TCDD (5 $\mu\text{g/kg}$; 98% purity; Cambridge Isotopes Laboratories, Andover, MA) on E13.5. This dose was previously shown to greatly alter prostatic budding patterns in mice (Lin et al., 2003; Vezina et al., 2008). Other pregnant dams were dosed daily from E14.5 to E17.5 with 1 mg/kg (sc) of the pure antiestrogen ICI 182,780 (Wakeling et al., 1991; Tocris Bioscience, Ellisville, MO) or vehicle (10% DMSO/90% corn oil; 5 ml/kg). These dams were also given corn oil or TCDD, as described above, on E15.5. Timed-pregnant dams were euthanized on E18.5 by asphyxiation with CO_2 prior to removal of fetuses. All animal procedures were approved by the University of Wisconsin Institutional Animal Care and Use Committee. Caution: TCDD is exceptionally toxic and must be used with comprehensive safety precautions.

Genotyping was performed using the PCR cycling conditions described by Benedict et al. (2000), except that conditions for *Er\beta* in $kEr\alpha\beta$ KO fetuses were as described by Windahl et al. (1999). Primer sequences for genotyping and expected PCR product sizes are shown in Table 1.

Scanning electron microscopy

Samples were prepared for scanning electron microscopy (SEM) as previously described (Lin et al., 2003). Briefly, UGSs were incubated with 1% trypsin (Difco, Sparks, MD) at 4°C for 90 min. Protease activity was attenuated with 5% fetal bovine serum (Hyclone, Logan, UT), then mesenchyme was removed from the epithelium using fine forceps. UGS epithelial samples were fixed in 2.5% glutaraldehyde (Ted Pella, Redding, CA), dehydrated and dried by the critical-point procedure, mounted, and coated with gold for analysis using a Hitachi S-570 scanning electron microscope (Tokyo, Japan). All images were taken at the same nominal magnification, 130 $\times$.

Results

Prostatic budding in single *Er* mutant males exposed to vehicle or TCDD

Two hypotheses were tested: that estrogen signaling is required for establishing the normal prostatic budding pattern in fetal males, and that estrogen signaling is required for the impairment of bud patterning caused by TCDD.

Timed-pregnant dams were given corn oil or TCDD (5 $\mu\text{g/kg}$, po) on E13.5, and UGSs were collected from male fetuses on E18.5. UGS epithelium was isolated, fixed, and examined by SEM. The expected prototypical pattern of ventral, dorsal, lateral, and anterior prostatic buds was observed in vehicle-exposed wild-type male UGSs (Fig. 1A, 2A). Prostatic bud patterns did not detectably differ among vehicle-exposed wild-type, $kEr\alpha$ KO, $mEr\alpha$ KO, and $Er\beta$ KO mice (Fig. 1A, C, E, G). These results strongly suggest that neither $ER\alpha$ nor $ER\beta$, alone, is absolutely required for the normal prostatic bud pattern in male mice.

TCDD exposure starting on E13.5 prevented ventral prostatic bud formation in wild-type fetuses, decreased dorsolateral bud number, and dislocated dorsal and lateral buds towards the dorsal UGS epithelial surface (Fig. 1B, 2B). Budding phenotypes were not detectably different from this in TCDD-exposed $kEr\alpha$ KO (Fig. 1D), $mEr\alpha$ KO (Fig. 1F), and $Er\beta$ KO (Fig. 1H)

males. These results strongly suggest that neither ER α nor ER β , alone, is required for the abnormal prostatic budding pattern produced by TCDD.

Prostatic budding in double *Er* mutant males exposed to vehicle or TCDD

In light of the results described above, we hypothesized that prostatic buds developed in a normal pattern in *Er α* and *Er β* single mutants, and that TCDD was fully inhibitory in these mutants, because the remaining functional *Er* gene compensated for loss of the other. We therefore examined prostatic budding patterns in *Er α /Er β* double mutants. As described above, corn oil or TCDD were administered to timed-pregnant dams on E13.5, and UGSs from male fetuses were collected on E18.5, processed, and examined. Prostatic budding patterns were very similar in vehicle-exposed wild-type, kER $\alpha\beta$ KO, and mER $\alpha\beta$ KO male mice (Fig. 2A, C, E). TCDD inhibited ventral budding and displaced dorsal and lateral buds towards the dorsal UGS epithelial budding surface in wild-type (Fig. 2B), kER $\alpha\beta$ KO (Fig. 2D) and mER $\alpha\beta$ KO (Fig. 2F) males. No differences were detected in the effects of TCDD among any of these genotypes. These results strongly suggest that development of the normal prostatic bud pattern in control male mice, and disruption of this pattern by TCDD, require neither ER α nor ER β .

Prostatic budding in wild-type males exposed to an ER inhibitor plus vehicle or TCDD

The pure steroidal antiestrogen ICI 182,780 was used to independently determine whether ER signaling is required for normal prostatic bud patterning and/or for the impairment of prostatic budding caused by TCDD. Wild-type mice were exposed *in utero* to ICI 182,780 via maternal dosing (1 mg/kg/day from E14.5 to E17.5), and to vehicle or TCDD (5 μ g/kg) via maternal dosing on E15.5. This ICI 182,780 dosing regimen is strongly antiestrogenic in mice (Wakeling and Bowler, 1992) and could not be exceeded without causing abortions (unpublished observations). Prostatic budding patterns did not detectably differ between vehicle-exposed (Fig. 1A, 2A) and ICI 182,780-exposed wild-type males (Fig. 3A). Moreover, ICI 182,780 did not protect against the abnormal prostatic budding pattern caused by TCDD (Fig. 3B).

Discussion

Estrogen signaling is not required for normal prostatic bud patterning in fetal male mice

Control male fetuses are exposed to high concentrations of both androgens and estrogens during prostatic budding (vom Saal, 1989; Prins and Korach, 2008; Ruhlen et al., 2008). While the androgens are essential for prostatic budding and subsequent prostate development, and while estrogens are also needed for normal prostate development postnatally, little is known about what role estrogens play prenatally (during bud specification, initiation, and elongation) in control animals.

It is clear that alterations in prenatal estrogen levels can profoundly affect prostate development, at least at high doses (low-dose effects remain controversial). Effects of exogenous estrogens on UGS development include changes in the pattern in which prostatic buds form (vom Saal et al., 1997; Timms et al., 2005; Hofkamp et al., 2008). The relationship between fetal exposure to exogenous estrogens and prostatic budding/prostate development has been described as an inverted U-shaped dose-response curve, where low concentrations are stimulatory and high concentrations are inhibitory (Welshons et al., 2003; Richter et al., 2005). Because of evidence that early developmental exposure to estrogens can permanently imprint the prostate and increase the risk of benign prostate hyperplasia and cancer later in life (Prins et al., 2006, 2008), the effects of perinatal estrogen exposure on prostate development and health are likely to remain a toxicological risk assessment concern well into the future.

Regardless of the ultimate outcome of the debate over the nature and extent to which low-level estrogen exposure affects UGS and prostate development, our results shed light on this issue

by addressing the role of *endogenous* estrogen signaling in fetal prostate development. The question we asked is whether estrogen signaling, like androgen signaling, is involved in determining the pattern in which prostatic buds develop in control male mice. We observed no differences between prostatic budding patterns in wild-type mice, two strains of ER α KO mice, ER β KO mice, or two strains of ER $\alpha\beta$ KO mice that were greater than the variability inherently present in replicate UGSs from the same genotype and strain. Nor did we observe any difference when wild-type mice were exposed to the pure steroidal antiestrogen ICI 182,780. Obviously, we cannot exclude the possibility that estrogens can act on the UGS through ERs other than ER α and ER β (e.g., via the transmembrane G protein-coupled receptor GPR30 (Prossnitz et al., 2008)). Similarly, we cannot exclude the possibilities that estrogens can act on the UGS via ER-independent mechanisms, that the antiestrogen we used did not completely block all estrogen signaling, or that estrogen signaling deficiency caused effects that were too subtle to be detected by careful visual inspection of the samples. In addition, our results do not rule out a myriad of other possible effects that the loss of estrogen signaling could potentially have on the UGS prenatally – all we looked at was the position and shape of the prostatic buds. Nevertheless, our results strongly suggest that ER α - and ER β -mediated estrogen signaling is not needed to establish the prototypical prostatic budding pattern seen in control mice. Therefore, it appears that the effects of low-level prenatal estrogen exposure on prostatic budding patterns and subsequent prostate development studied by other laboratories were exerted in males whose control counterparts were displaying a “no estrogen signaling” rather than an “already affected by endogenous estrogen signaling” prostatic budding pattern. This finding contrasts with conclusions based on other endpoints, where the degree of endogenous estrogen signaling was already above the minimum necessary to affect phenotype, and therefore there was no threshold for effects of exogenous estrogens (Welshons et al., 2003).

Estrogen signaling is not required for prostatic budding inhibition by TCDD

As noted in the Introduction, there were several reasons for suspecting that estrogen signaling might affect the region-specific inhibition of prostatic epithelial bud formation caused in mice by *in utero* TCDD exposure. The single most important was a prior study in which effects of TCDD on prostatic budding in fetal male rats were found to be greatly influenced by intrauterine position (Timms et al., 2002). Although TCDD had no significant effect on serum testosterone concentrations in male fetuses, budding inhibition was far greater in males adjacent to two females (2F males) than in males developing next to two males (2M males). 2F males generally have higher serum 17 β -estradiol and lower testosterone concentrations than 2M males; there are differences in UGS and prostate development between 2F and 2M males in control animals, and such differences have been attributed to these hormonal differences (Timms et al., 1999). However, it is not apparent from the rat study how estrogens affected the responses of the UGS to TCDD. The considerations described above suggest that estrogen signaling greatly enhanced the inhibitory effects of TCDD. Yet TCDD significantly reduced serum 17 β -estradiol concentrations in 2F males but not in 2M males, suggesting that the effects of TCDD on budding may involve a reduction in 17 β -estradiol concentrations. However, TCDD simply reduced 17 β -estradiol concentrations in 2F males to levels comparable to those seen in TCDD-exposed 2M males, suggesting that the defining difference in how 2F and 2M male fetuses responded to TCDD may be unrelated to 17 β -estradiol concentrations. Despite these uncertainties, the study by Timms et al. (2002) strongly suggests that endogenous estrogen signaling can profoundly affect how prostatic budding responds to TCDD.

In the present study, *in utero* TCDD exposure produced comparable budding inhibition in wild-type, kER α KO, mER α KO, ER β KO, kER $\alpha\beta$ KO, and mER $\alpha\beta$ KO mice, and in wild-type mice pretreated with the antiestrogen ICI 182,780. While these results shed little light on how TCDD and estrogens interact when estrogen signaling is fully functional, they strongly suggest that estrogen signaling is not required for TCDD to inhibit prostatic epithelial budding. Previous

reports that TCDD can increase the potency of 17 β -estradiol (Bookstaff et al., 1990) and that the ventral lobe is the most susceptible part of the prostate to developmental inhibition by estrogens (Greene et al., 1940, 1941; Raynaud, 1942; Vannier and Raynaud, 1980) therefore cannot explain the UGS region- and prostate lobe-specificity with which TCDD inhibits UGS and prostate development.

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Fig. 1. Prostatic budding in vehicle- or TCDD-exposed fetal mice lacking one form of estrogen receptor (ER). Wild-type, $ER\alpha$ knockout ($kER\alpha KO$ and $mER\alpha KO$), and $ER\beta$ knockout ($ER\beta KO$) fetuses were exposed via maternal dosing on E13.5 to vehicle (5 ml corn oil/kg) or TCDD (5 $\mu g/kg$, po). UGSs were collected on E18.5, mesenchyme was removed, and underlying epithelium was visualized by SEM. At least 3 UGSs per genotype and treatment were examined. Ventral prostatic buds are pseudocolored blue, dorsal buds green, lateral buds yellow, and anterior buds red. Abbreviations used are: bladder neck (BN), ductus deferens (DD), pelvic urethra (PU), seminal vesicle (SV).

Fig. 2.

Prostatic budding in vehicle- or TCDD-exposed fetal mice lacking both ER α and ER β . Wild-type, kER $\alpha\beta$ KO, and mER $\alpha\beta$ KO fetuses were exposed via maternal dosing on E13.5 to vehicle (5 ml corn oil/kg) or TCDD (5 μ g/kg, po). UGSs were collected on E18.5, mesenchyme was removed, and underlying epithelium was visualized by SEM. At least 3 UGSs per genotype and treatment were examined. Ventral prostatic buds are pseudocolored blue, dorsal buds green, lateral buds yellow, and anterior buds red. Abbreviations used are: bladder neck (BN), ductus deferens (DD), pelvic urethra (PU), seminal vesicle (SV).

Fig. 3.

Prostatic budding in vehicle- or TCDD-exposed fetal wild-type mice exposed to the antiestrogen ICI 182,780. Wild-type fetuses were exposed to vehicle (10% DMSO/90% corn oil, 5 ml/kg, sc) or ICI 182,780 (1 mg/kg) via daily maternal dosing from E14.5 through E17.5. Dams were also given vehicle (5 ml corn oil/kg) or TCDD (5 μg/kg, po) on E15.5. UGSs were collected on E18.5, mesenchyme was removed, and underlying epithelium was visualized by SEM. At least 3 UGSs per genotype and treatment were examined. Ventral prostatic buds are pseudocolored blue, dorsal buds green, lateral buds yellow, and anterior buds red. Abbreviations used are: bladder neck (BN), ductus deferens (DD), pelvic urethra (PU), seminal vesicle (SV).

Table 1Primer sequences and PCR product sizes for genotyping *Er* mutant mice.

Transgenic Strain	Primer Sequences (5' → 3' orientation)	Product Sizes (bp)
kER α KO	CGGTCTACGGCCAGTCGGGCACC GTAGAAGGCGGGAGGGCCGGTGTC	240 (<i>Era</i> wild-type allele)
	AGCGCATCGCCTTCTATCGC GGCGCTGGGCTCGTTCTC	570 (<i>Era</i> knockout allele)
mER α KO	ACAGGCAAATACCTTCTGCTGC GCTTCCCTGGCATTACCACTTC	590 (<i>Era</i> wild-type allele)
	TCGCTTTCCTGAAGACCTTTCATAT GCTTCCCTGGCATTACCACTTC	280 (<i>Era</i> knockout allele)
ER β KO	GTTGTGCCAGCCCTGTTACT GCAGCCTCTGTTCCACATACA	106 (<i>Erβ</i> wild-type allele)
	TCACAGGACCAGACACCGTA	160 (<i>Erβ</i> knockout allele)
kER $\alpha\beta$ KO	GGAGTAGAAACAAGCAATCCAGACAT GCAGCCTCTGTTCCACATACACTTC	650 (<i>Erβ</i> wild-type allele)
	AGAATGTTGCACTGCCCCTGCTGC	450 (<i>Erβ</i> knockout allele)
	CGGTCTACGGCCAGTCGGGCACC GTAGAAGGCGGGAGGGCCGGTGTC	240 (<i>Era</i> wild-type allele)
	AGCGCATCGCCTTCTATCGC GGCGCTGGGCTCGTTCTC	570 (<i>Era</i> knockout allele)
mER $\alpha\beta$ KO	See both BERKO and mERKO	