

TECHNOLOGY REPORT

Generation of a Mouse for Conditional Excision of Progesterone Receptor

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Summary: The progesterone receptor (PR) is required for several aspects of mammalian female reproduction. PR null mice have overlapping defects that preclude an understanding of its multiple functions in ovulation, pregnancy, mammary gland biology, and sexual behavior. We have generated a PR conditional excision (PRCE) allele in which loxP sites flank exon 1. Homozygous PRCE females are fertile and appear to be functionally normal. Global cre mediated excision of the floxed exon 1 using E1a-cre mice resulted in systemic loss of exon 1 and PR protein. Female mice homozygous for this null allele were sterile, as expected for PR knockout (PRKO) females. Conditional loss of PR will facilitate investigation of the spatial and temporal roles of PR in both normal development and disease. *genesis* 44:391–395, 2006. © 2006 Wiley-Liss, Inc.

Key words: gene targeting; mammary gland; ovary; steroid receptors; uterus

INTRODUCTION

The progesterone receptor (PR) is a member of the superfamily of nuclear factors that regulates gene transcription in response to hormonal activation via progesterone (Conneely et al., 1989). The two PR isoforms, A and B, are conserved across species and are transcribed from the same gene using either distinct promoters or alternative translation initiation sites from a single mRNA (Conneely et al., 1989; Kastner et al., 1990). PR-B contains an additional amino terminal stretch of 165 amino acids that codes for a transactivation domain that specifically activates PR-B target genes (Sartorius et al., 1994) and is otherwise identical to PR-A. Despite their structural similarities, PR-A and PR-B perform distinct cellular functions. This has been demonstrated both in cultured cells and also by the generation of isoform-specific mouse knockout models (Giangrande et al., 2000; Mulac-Jericevic et al., 2000, 2003). Steroid receptors typically function in concert with transcriptional coregulators to direct a wide range of biological functions including homeostasis, differentiation, growth, and reproduction (Conneely et al., 1989). The interaction of

transcriptional cofactors with PR contributes to the diversity of targets regulated by the effects of progesterone, and also mediates changes in local chromatin architecture as well as stability of the transcriptional complex itself (Giangrande et al., 2000; Jenster et al., 1997).

PR is expressed in the mammary gland, uterus, ovary, brain, and vascular tissue (Apostolakis et al., 1996; Lee et al., 1997; Perrot-Appianat et al., 1988; Vazquez et al., 1999). While its role in the establishment and maintenance of pregnancy has been appreciated since 1930 (Allen, 1930), the extent of its influence on these and other processes has been documented more recently. Detailed examination of PR knockout (PRKO) mice that lack both PR isoforms revealed acute physiological and behavioral differences from wild-type animals (Ismail et al., 2002; Lydon et al., 1995). These studies demonstrate unequivocally that PR is required to regulate several aspects of female reproductive biology, including ovarian, uterine, and mammary development and function, as well as behavioral aspects including lordosis (sexual receptivity response).

Systemic loss of PR results in a number of complex phenotypes that preclude understanding of PR function in individual cell types and also in serial events that involve PR activity. For example, pregnancy requires the precise coordination of hormonal, ovarian, fallopian tube, and uterine function. Lack of ovulation in the PRKO animal complicates analysis of PR function in pregnancy. Further, the endometrium of homozygous PRKO females fails to support pregnancy even when blastocysts are injected (Lydon et al., 1995). As PR is expressed by epithelial, stromal, and vascular cells in the uterus, it is unclear which of these cellular compartments are critical to reproductive processes (Ismail et al., 2002). Thus, fertility may be obstructed because

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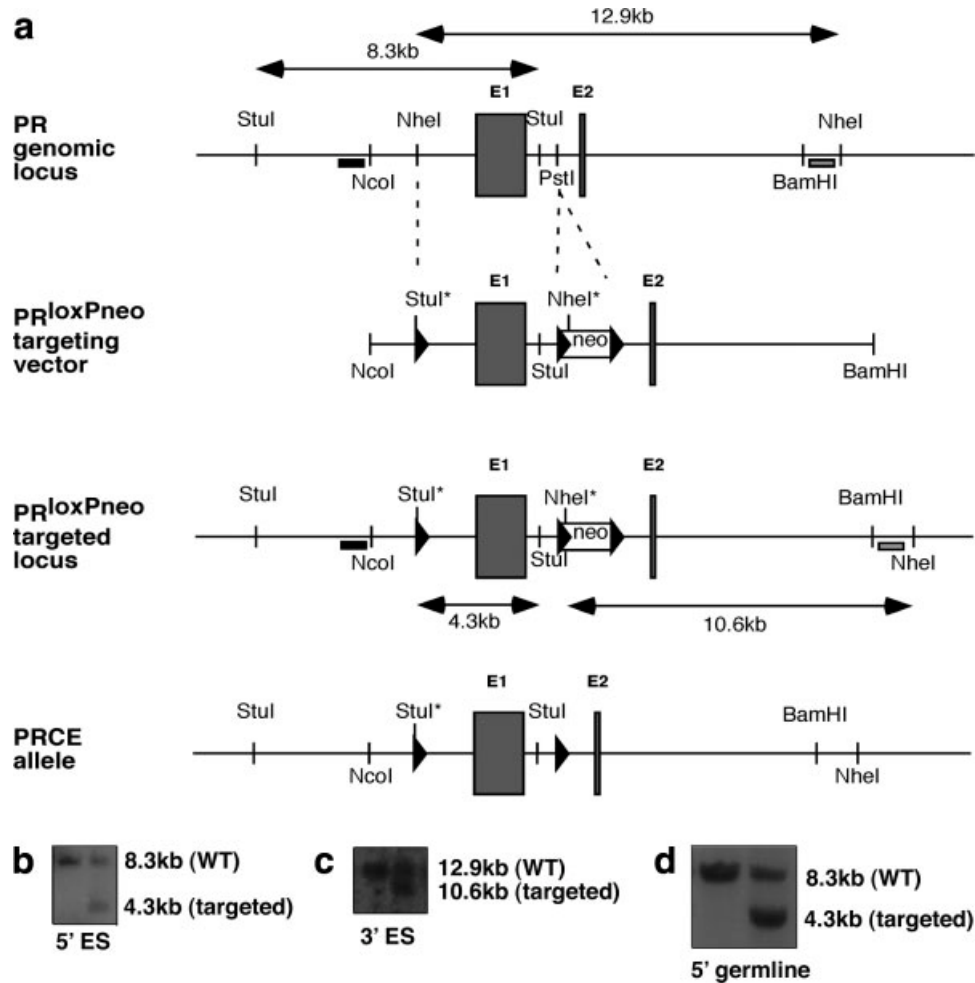


FIG. 1. Generation of $PR^{loxPneo}$ and $PRCE$ alleles. (a) A $Stul^*$ site was engineered for screening purposes, and the endogenous $NheI$ site was destroyed in cloning. A $PGKNeo$ cassette flanked by $loxP$ sites was inserted downstream of exon 1 and introduced a novel $NheI^*$ site used for screening by Southern blot analysis. 5' and 3' external probes are indicated by black and grey rectangles, respectively. Double-headed arrows indicate size of DNA fragments detected by 5' probe (4.3 kb) and 3' probe (10.5 kb) in loci that underwent homologous recombination. **b–d.** Verification of homologous recombination and germline transmission. Total DNA from single ES cell colonies (b and c) or tail DNA (d) was $Stul$ digested to assay for 5' end homologous recombination and $NheI$ for 3' homologous recombination. Homologous recombination on both 5' and 3' ends was verified for ES cell clones chosen for blastocyst injection.

epithelial cells are not receptive to interactions with the trophoblast. Alternatively, stromal cells may fail to decidualize appropriately. A conditional PRKO model will add a spatiotemporal dimension to our understanding of PR function and more clearly highlight the contribution of PR in individual cell types to normal fertility. In addition, comparisons between targeted inactivation of PR in adjacent cell populations will help distinguish between cell-autonomous and cell nonautonomous requirements for PR function. Thus, in order to clearly identify the tissue specific roles of PR, we have generated a conditional knockout mouse model that can delete PR function in a cell-type specific manner in the presence of cre-recombinase.

We targeted exon 1 of the PR genomic locus to generate a conditional allele. Deletion of exon 1 is predicted to generate a null allele as it contains the ATG for both

PR isoforms A and B. We designed the $PR^{loxPneo}$ targeting vector to contain $loxP$ sites flanking exon 1 (Fig. 1a). A single $loxP$ site was introduced into an $NheI$ site approximately 2.1 kb upstream of ATG_B that does not overlap with any known regulatory elements in the PR promoter region or any conserved sequences as determined by sequence alignment (Kraus et al., 1994). We also inserted a $PGKNeo$ cassette flanked by $loxP$ sites into a $PstI$ site downstream of exon 1. Electroporation of linearized targeting vector DNA into SvJ/129 embryonic stem (ES) cells yielded fewer G418-resistant colonies (240 total) than typically produced with smaller targeting vectors. Nonetheless, Southern analysis using both 5' and 3' external probes indicated a targeting frequency of 25% with this targeting vector (Fig. 1b). Four ES clones were chosen for injection into C57BL/6J blastocysts. Two male chimeras were obtained, both of which were

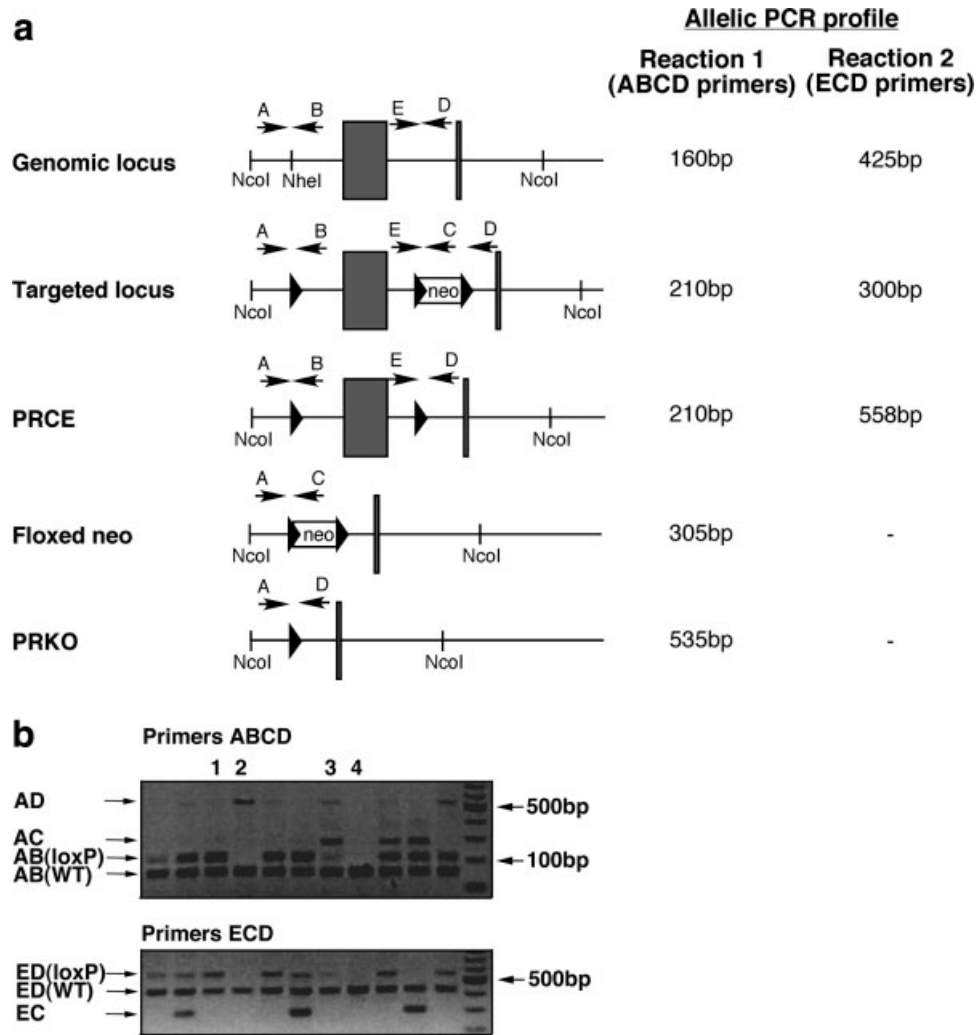


FIG. 2. Screening of EIIa-cre generated recombinant alleles. (a) Schematic of all possible alleles detected in progeny of $PR^{loxPneo}$ chimeras and EIIa-cre mates. Two diagnostic PCR reactions and associated primers are indicated to the right with predicted product sizes representing each possible allele. Conditions were optimized to exclude PCR products greater than 1 kb. (b) Results from screening of tail DNA from mosaic progeny of $PR^{loxPneo}$ chimeras and EIIa-cre mates. Each column represents one mouse and all detected alleles. Column a represents a $PRCE$ allele, b is PRKO, c is a predominantly a floxed neo and d is wild-type. All animals have one WT allele. Note varying degrees of mosaicism among different mice. PCR products were cloned and sequenced to confirm that the predicted bands were amplified.

determined to be sterile. A second round of blastocyst injections yielded germline transmission of the $PR^{loxPneo}$ allele for two of three independent clones injected, as confirmed by Southern analysis.

It is well-known that the presence of a strong PGK promoter in the positive selection cassette may induce “neighborhood effects” on local gene regulation upon genomic integration (Lewandoski, 2001). Indeed, four of five homozygous $PR^{loxPneo}$ females examined were infertile. Heterozygous $PR^{loxPneo}$ animals were crossed to EIIa-cre transgenic mice (Lakso et al., 1996) in order to remove the PGKNeo cassette and generate the $PRCE$ allele. EIIa-cre mice express cre recombinase inefficiently in the preimplantation embryo (Lakso et al., 1996). Thus, some cells within the blastocyst retain genomic loci that experience incomplete excision

before cre expression is turned off. These embryos develop into mosaic animals, whose germ cells carry a mixed population of $PR^{loxPneo}$ recombinants as shown in Figure 2. We screened the pups from $PR^{loxPneo}$ chimeras crossed to EIIa-cre mates for the loss of only the PGKNeo cassette, leaving a floxed exon 1, or PR conditional excision ($PRCE$) allele. As EIIa-cre mediated excision in this case yielded three possible recombinant events, a PCR-based strategy was devised to distinguish the resulting alleles (Fig. 2). The products of two individual PCR reactions were evaluated, each reaction containing a single forward primer, A or E, in combination with reverse primers B, C, and D. The combined data gave each of five possible alleles a distinct signature. Thirteen of 224 pups screened carried predominantly a $PRCE$ allele. These thirteen animals containing mosaic germlines

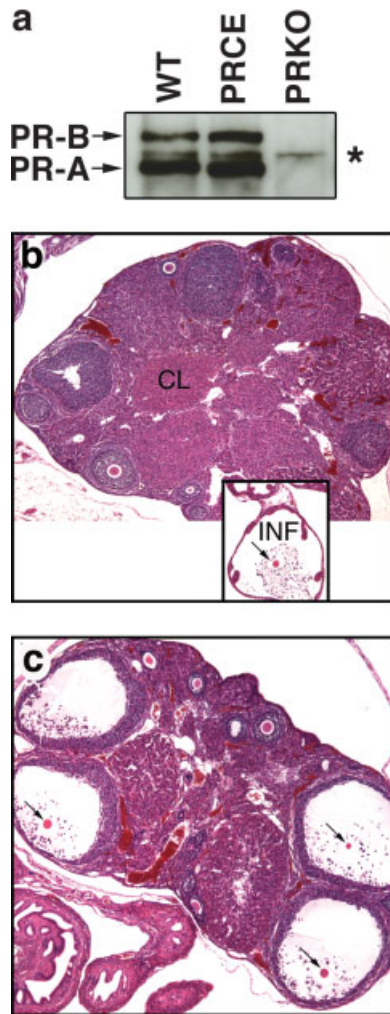


FIG. 3. Global excision of PRCE allele generates the PRKO phenotype. (a) Western blot analysis shows that PRCE homozygous females are comparable to wild-type females in expression of PR-A and PR-B, while both isoforms are lost in PRKO homozygotes (* denotes nonspecific band detected in all three lanes). Anti-enolase confirmed equal loading (data not shown). (b) Heterozygous PRKO ovary ovulated in response to hormonal stimulation as evidenced by the presence of numerous corpus lutea (CL) in the ovary and eggs in the infundibulum (inset, INF). (c) Homozygous PRKO ovaries contain large numbers of unruptured follicles containing oocytes (arrows).

were crossed to either wild-type or Rosa26 reporter (Soriano, 1999) mates and pups were screened for founders that lost the EIIa-cre allele and carried a single *PRCE* allele.

In order to verify Cre recombinase-mediated inactivation of PR function in vivo, homozygous PRKO females were evaluated for reproductive function. Characterization of EIIa-cre-generated PRKO (null) homozygous females was consistent with the published phenotype (Lydon et al., 1995). Ablation of PR-A and PR-B isoforms in homozygous PRKO females was confirmed by Western blot analysis (Fig. 3a). Homozygous female PRKO animals were sterile, while heterozygous PRKO and wild-type female littermates displayed normal fertility.

Homozygous PRKO males were as fertile as wild-type males. Infundibula were examined for the presence of eggs by either flushing or histology and homozygous PRKO ovaries were anovulatory compared to heterozygotes and wild-type littermates after exposure to superovulation hormonal regimens (Fig. 3b,c and data not shown). Homozygous *PRCE* females were, however, fully viable, fertile, and did not exhibit any obvious phenotypic abnormalities. Litters from PCRE homozygous intercrosses were of normal size (5–9 mice per litter at weaning, average 7.0). Pups born to homozygous *PRCE* females were successfully weaned, indicating that the presence of the loxP sites flanking exon 1 did not disrupt normal fertility or mammary function of the mothers.

In conclusion, we have generated a mouse model that provides for the conditional inactivation of PR. *PRCE* females are phenotypically normal, while systemic cre-mediated inactivation of this allele leads to the same phenotypic anomalies previously characterized for the PR null animal. This model will provide for spatiotemporal analysis of PR function in both normal and pathological reproductive processes.

MATERIALS AND METHODS

Generation of PR^{loxPneo} Targeting Construct

PR genomic DNA was isolated from a 129/SvJ BAC clone obtained from Genome Systems, Inc. A 13.4 kb NcoI-BamHI fragment that contains exons 1 and 2 was subcloned into pBSKSII (Stratagene, La Jolla, CA). A loxP site was inserted into the *NheI* site at the 5' end of the fragment, and a new *StuI* site was engineered adjacent to this 5' loxP site for subsequent Southern analysis. A *phosphoglycerol kinase neomycin-resistance* (PGKneo) cassette flanked by 2 loxP sites was inserted into a *PstI* site immediately downstream of exon 1.

Electroporation of ES Cells and Generation of Chimeric Mice

129/Sv ES cells were transfected with *NotI/KpnI* linearized targeting vector by electroporation and selected in G418-containing medium (UCLA ES Cell Core). Targeted clones were injected into C57BL/6J blastocysts from which chimeric mice were derived (UCLA Transgenic Facility). Briefly, 10–15 targeted ES cells were injected into the cavity of blastocysts, and embryos were transplanted into pseudopregnant foster mice to complete gestation and birth. Resulting male chimeric mice were tested for germline transmission by breeding to either C57BL/6J or Fvb wild-type females. Presence of the PR^{loxPneo} targeted allele in the F1 generation was confirmed by Southern blot analysis.

Genotype Analysis

Homologous recombination and germline transmission events were determined by Southern blot analysis using a 5' and 3' external probes as described in Figure 1. Cre-

induced recombinants were distinguished using the PCR-based assay described in Figure 2. The sequences of PCR primers used are as follows; Primer A: 5' TGT GCA CTT TTG GAG GCA AG 3', Primer B: 5' GTG GAG GCT TCT GGA CAG T 3', Primer C: 5' TAA AGC GCA TGC TCC AGA C 3', Primer D: 5' TGA TTT TGC CTT TGG CAG ATG 3', Primer E: 5' GGT CTC TGG CCT GAT TTT CC 3'. HotMaster Taq DNA polymerase (Eppendorf) was used to amplify PCR products and cycling conditions for both reactions were as follows: initial denaturation (94°C, 2 min), product amplification (27 cycles of 94°C, 20 s, 59°C, 20 s, 68°C, 40 s), with a final extension at 68°C for 5 min.

Ovarian Function

Ten-week-old virgin females were superovulated as previously described (Lydon et al., 1995). Ovaries and infundibula were collected for histological evaluation 24 h following hCG injection and fixed in 4% buffered formalin, paraffin embedded, sectioned, and stained with hematoxylin and eosin.

Western Analysis

Wild-type, homozygous PRCE, and homozygous PRKO females were given daily subcutaneous injections of estrogen (100 ng) dissolved in sesame oil for 4 days. Uterine tissue was homogenized using a mortar and pestle and rocked at 4°C in ice-cold lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 1 mM sodium orthovanadate, 10 mM β -glycerophosphate, 1 mM PMSF, 20 μ g/ml leupeptin, 20 μ g/ml aprotinin) for 1 h. The lysate was centrifuged at 10,000g for 30 min to remove cell debris and total protein was determined using a DC Protein Assay kit (Bio-Rad). Absorbance was read on a SpectraMax Plus spectrophotometer (Molecular Devices), and 3 mg protein from each sample was precleared using Protein-A agarose beads (Roche). Cleared lysates were incubated with anti-PR (13 μ g, Santa Cruz, #sc-538) at 4°C overnight and subsequently with Protein-A agarose for 2 h. Precipitated protein was washed three times with lysis buffer, then released from beads with Laemmli sample buffer containing β -mercaptoethanol and resolved using SDS-PAGE (10%). Detection of PR was done using the same antibody that was used for the pulldown (1:200).

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