Generation of a Mouse Model with a Reversible Hypomorphic Cytochrome P450 Reductase Gene: Utility for Tissue-Specific Rescue of the Reductase Expression, and Insights from a Resultant Mouse Model with Global Suppression of P450 Reductase Expression in Extrahepatic Tissues

Yuan Wei, Xin Zhou, Cheng Fang, Lei Li, Kerri Kluetzman, Weizhu Yang, Qing-Yu Zhang, and Xinxin Ding

Wadsworth Center, New York State Department of Health, and School of Public Health, State University of New York, Albany, New York

Received February 18, 2010; accepted April 5, 2010

ABSTRACT

A mouse model termed *Cpr-low* (CL) was recently generated, in which the expression of the cytochrome P450 reductase (*Cpr*) gene was globally down-regulated. The decreased CPR expression was accompanied by phenotypical changes, including reduced embryonic survival, decreases in circulating cholesterol, increases in hepatic P450 expression, and female infertility (accompanied by elevated serum testosterone and progesterone levels). In the present study, a complementary mouse model [named reversible-CL (r-CL)] was generated, in which the reduced CPR expression can be reversed in an organ-specific fashion. The neo cassette, which was inserted into the last *Cpr* intron in r-CL mice, can be deleted by Cre recombinase, thus returning the structure of the *Cpr* gene (and hence CPR expression) to normal in Cre-expressing cells. All previously identified phenotypes of

the CL mice were preserved in the r-CL mice. As a first application of the r-CL model, we have generated an extrahepatic-CL (xh-CL) mouse for testing of the functions of CPR-dependent enzymes in all extrahepatic tissues. The xh-CL mice, generated by mating of r-CL mice with albumin-Cre mice, had normal CPR expression in hepatocytes but down-regulated CPR expression elsewhere. They were indistinguishable from wild-type mice in body and liver weights, circulating cholesterol levels, and hepatic microsomal P450 expression and activities; however, they still showed elevated serum testosterone and progesterone levels and sterility in females. Embryonic lethality was prevented in males, but apparently not in females, indicating a critical role for fetal hepatic CPR-dependent enzymes in embryonic development, at least in males.

The NADPH-cytochrome P450 reductase (CPR) is the obligate redox partner for all microsomal cytochrome P450 monooxygenases (Black and Coon, 1987); the latter enzymes can metabolize numerous endogenous and exogenous compounds (Porter and Coon, 1991). Studies on the *in vivo* func-

tions of CPR and CPR-dependent enzymes have been greatly facilitated in recent years by the development of a panel of engineered mouse models harboring null or hypomorphic *Cpr* alleles. A critical role of CPR in embryonic development was demonstrated via germ-line *Cpr* deletion (Shen et al., 2002; Otto et al., 2003). The specific functions of hepatic CPR/P450 in cholesterol synthesis and homeostasis and in xenobiotic clearance were demonstrated through mouse models having a hepatocyte-specific *Cpr* deletion (Gu et al., 2003, Henderson et al., 2003). Furthermore, the roles of extrahepatic organ CPR/P450 in drug metabolism and xenobiotic toxicity were studied by using mouse models with conditional *Cpr* knock-

This work was supported in part by the National Institutes of Health National Cancer Institute [Grant CA092596]; the National Institutes of Health National Institute of Environmental Health Sciences [Grant ES007462] (to X.D.); and the National Institutes of Health National Institute of General Medical Sciences [Grant GM082978] (to Q.-Y.Z.).

Article, publication date, and citation information can be found at <http://jpet.aspetjournals.org>.
doi:10.1124/jpet.110.167411.

ABBREVIATIONS: CL, *Cpr-low*; r-CL, reversible-CL; xh-CL, extrahepatic-CL; CPR, cytochrome P450 reductase; POR, P450 oxidoreductase; LCN, liver-specific *Cpr*-null; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; OM, olfactory mucosa; LC/MS, liquid chromatography/mass spectrometry; kbp, kilobase pair; WT, wild type; ES, embryonic stem; PCR, polymerase chain reaction; Alb, albumin; G418, (2*R*,3*S*,4*R*,5*R*,6*S*)-5-amino-6-[(1*R*,2*S*,3*S*,4*R*,6*S*)-4,6-diamino-3-[(2*R*,3*R*,4*R*,5*R*)-3,5-dihydroxy-5-methyl-4-methylaminooxan-2-yl]oxy-2-hydroxycyclohexyl]oxy-2-(1-hydroxyethyl)oxane-3,4-diol; *En*, embryonic day *n*.

out in the lung (Weng et al., 2007), heart (Fang et al., 2008), or small intestine (Zhang et al., 2009). Conditional *Cpr*-knockout was also used to demonstrate the essential role of CPR during limb and skeletal development (Schmidt et al., 2009).

Numerous mutations have been reported for the human CPR gene (*POR*, for P450 oxidoreductase), and many mutant *POR* alleles have been found to be associated with congenital deficiencies in steroidogenesis/homeostasis and/or with Antley Bixler syndrome, characterized by skeletal malformation and reproductive defects (e.g., Flück et al., 2004; Fukami et al., 2005). The impact of *POR* mutations is probably systemic, affecting all cells in the body. Thus, although the conditional *Cpr*-null mice are valuable for explorations of in vivo functions of CPR/P450 in a tissue-specific manner, mouse models in which CPR expression or function is globally affected by gene targeting are more realistic for the identification of potential pathological manifestations that could potentially be found in human individuals who carry *POR* mutations. In that context, a *Cpr*-low (CL) mouse model, in which CPR expression was globally down-regulated (>70% decreases in CPR levels in all tissue tested), was generated previously by our laboratory (Wu et al., 2005). The relevance of the CL model to human *POR* deficiency is supported by the occurrence of substantial interindividual variations in levels of CPR expression in human tissues (e.g., Wortham et al., 2007). It is noteworthy that at least some of the phenotypes already identified in the CL mouse, such as female infertility and altered steroid homeostasis, are known to occur in human patients (Arlt et al., 2004; Flück et al., 2004; Fukami et al., 2005, 2006; Huang et al., 2005). Other known phenotypes of the CL mouse, such as lower cholesterol levels and reduced embryonic survival, and phenotypes yet to be identified, are also anticipated to be manifest in human patients. Therefore, the CL mouse is valuable for studies on the potential functional impact (and the underlying mechanisms) of *POR* mutations that affect CPR expression levels in human patients.

A powerful strategy for validating the role of CPR in any disease phenotypes observed in the CL mouse is to demonstrate that the given phenotype can be eliminated through reintroduction of a functional *Cpr* gene, either in the germ line or in a tissue-specific manner. For the CL mouse, low CPR expression is believed to be caused by the insertion of a neo gene in the last *Cpr* intron (Wu et al., 2005). Thus, an effective way to “rescue” CPR expression would be to remove the neo cassette through Cre-mediated recombination (Nagy, 2000). However, the *Cpr^{low}* allele contains three loxP sites (one in intron 2, two in intron 15); Cre-mediated recombination would lead to the removal of both *Cpr* (exons 3–15) and neo (Gu et al., 2007). Thus, to remove neo while keeping the *Cpr* gene intact the loxP site in intron-2 would need to be removed; the resulting mouse model is designated reversible-CL (r-CL).

In this study, we have generated the r-CL model, and then confirmed that it is essentially identical to the CL model with respect to CPR expression and biological phenotypes. Furthermore, specific rescue of hepatocyte CPR expression was achieved through intercrosses between r-CL and the albumin-Cre (Alb-Cre) transgenic (Postic et al., 1999) mice, yielding Alb-Cre(+/-)/*Cpr^{r-CL}*, an extrahepatic CL (xh-CL) mouse. We then characterized the xh-CL model to determine

whether hepatic CPR/P450 plays critical roles in the various phenotypes seen previously in the CL mouse, including mild growth retardation, reduced embryonic survival, female infertility and steroid hormone dysregulation, hepatic P450 induction, and decreased in vivo metabolism of xenobiotic compounds.

Materials and Methods

Targeting Vector. The targeting construct for the r-CL model was prepared through modification of the targeting construct used previously for generation of the *Cpr*-lox and *Cpr*-low mice (Wu et al., 2003, 2005). A 6.5-kbp BamHI fragment of the original construct, containing the furthest upstream homology arm (a part of *Cpr* intron 2), and the 5'-loxP and surrounding vector sequences, was omitted, while leaving the other parts unchanged. Thus, the new construct contained the neo marker (flanked by two loxP sites, or “floxed”) inserted in *Cpr* intron 15, the homology arms that flank the neo cassette, and the *tk* gene needed for negative selection (Fig. 1). The floxed neo marker would subsequently be inserted into intron 15 of the *Cpr* gene in the mouse genome, as the result of a successful homologous recombination event.

Electroporation and Selection of Embryonic Stem Cells. Procedures for electroporation, selection of embryonic stem (ES) cells, and blastocyst injection were performed at the Transgenic and Knockout Core Facility of the Wadsworth Center, essentially as described previously (Nagy et al., 2003). The Bruce4 (C57BL/6J-derived) ES cells (Köntgen et al., 1993), kindly provided by Dr. Colin Stewart (Institute of Medical Biology, Singapore, Singapore), were used for electroporation. After electroporation, cells were cultured on tissue culture plates containing mitomycin C-treated primary embryonic fibroblast feeder layers prepared from a transgenic mouse line that expresses the neo (Stewart et al., 1992). After 24 h, the medium was replaced with selection medium containing 250 µg/ml G418 [(2*R*,3*S*,4*R*,5*R*,6*S*)-5-amino-6-[(1*R*,2*S*,3*S*,4*R*,6*S*)-4,6-diamino-3-[(2*R*,3*R*,4*R*,5*R*)-3,5-dihydroxy-5-methyl-4-methylaminooxan-2-yl]oxy-2-hydroxycyclohexyl]oxy-2-(1-hydroxyethyl)oxane-3,4-diol]. Individual G418-resistant ES cell clones were screened by using polymerase chain reaction (PCR) and Southern blot analysis, as described previously for the *Cpr*-lox model (Wu et al., 2003).

Blastocyst Injection and Animal Breeding for the r-CL Mice. All procedures involving animals were approved by the Institutional Animal Care and Use Committee of the Wadsworth Center. ES cells from five positive clones were karyotyped, and the clone with the best karyotyping result was selected for expansion. ES cells from that clone were trypsinized, centrifuged, resuspended in ES cell growth medium, and subsequently injected into the blastocysts from albino B6(Cg)-Tyrc-2J/J female mice (The Jackson Laboratory, Bar Harbor, ME). The blastocysts were transferred into the uterus of a pseudopregnant B6CBAlF1/J mouse to generate offspring. The male chimera pups were identified by their black eyes and coat color. Adult chimeras were bred with WT B6 female mice to obtain germline-transmission F₁ mice that were heterozygous for the mutant allele. The F₂ homozygotes were obtained by intercrossing between the F₁ heterozygotes. The structure of the modified *Cpr* gene in the r-CL mice was confirmed by PCR, DNA sequencing of PCR products, and Southern blot analysis (Fig. 1). Note that, unless specified otherwise, all mentions of CL, r-CL, or xh-CL mice refer to homozygotes.

Generation of the xh-CL Mice. The xh-CL mice were generated according to a two-step cross-breeding scheme. In the first step, hemizygous female Alb-Cre mice (on a B6 background; The Jackson Laboratory) were crossed with male *Cpr^{r-CL/r-CL}* (r-CL) mice, yielding Alb-Cre(+/-)/*Cpr^{r-CL/+}* pups. In the second step, female Alb-Cre(+/-)/*Cpr^{r-CL/+}* mice were crossed again with male r-CL mice, generating pups with four distinct genotypes, including Alb-Cre(+/-)/*Cpr^{r-CL/r-CL}* (xh-CL). The number of pups in each

Fig. 1. Generation of the r-CL mouse. **A**, targeting scheme for the generation of the *Cpr*^{r-CL} allele. The *Cpr* gene sequence is indicated by a solid line. Selected exons, shown as black boxes, are numbered. The *Neo* and *tk* genes are both in the reverse orientation, relative to the *Cpr* gene. Lox P sites and their orientations are shown by arrowheads. The positions of the following genomic regions are shown: characteristic EcoRI (E) restriction fragments in the WT (*Cpr*⁺) and the targeted (*Cpr*^{r-CL}) alleles; sequence complementary to a 1.9-kbp DNA probe (Int P) for Southern blot analysis; and sequences complementary to PCR primers (F1/R1) used for detection of the *Cpr*^{r-CL} allele. **B**, PCR detection of the *Cpr*^{r-CL} allele. Genomic DNA samples from a heterozygous (*Cpr*^{r-CL/+}; HE) mouse and a WT [*Cpr*(+/+)] mouse were analyzed. A product of the expected size (2.1 kbp) was detected in heterozygous (HE), but not in WT. MK, a 1-kb DNA marker. **C**, Southern blot analysis of the *Cpr* alleles. Genomic DNA samples (10 µg) from a homozygous (*Cpr*^{r-CL/r-CL}; HO), a heterozygous (HE), and a WT mouse were digested with EcoRI. The 6.5-kbp characteristic band for the *Cpr*^{r-CL} allele was detected in HO and HE, but not in WT, whereas the 13-kbp characteristic band for the *Cpr*⁺ allele was detected in WT and HE, but not in HO. **D**, suppression of CPR expression in all tissues examined of the r-CL mice, in both genders, compared with WT mice. Immunoblot analysis was performed by using an anti-CPR antibody for microsomes from various tissues of WT and r-CL (*Cpr*^{r-CL/r-CL}) mice. Equal amounts of microsomal proteins were analyzed between strains: 5 µg for OM and liver, 10 µg for brain, and 20 µg for lung and kidney. Microsomes were prepared from pooled tissues from three 2-month-old mice.

of the four genotypes was recorded, and the information was used for detection of potential in utero lethality of a given genotype.

Determination of Serum Levels of Total Cholesterol, Testosterone, and Progesterone. Total cholesterol was determined with a cholesterol assay kit (Cayman Chemical, Ann Arbor, MI); for each assay, 5 µl of serum, prepared from tail blood, was used. For determination of testosterone and progesterone, blood samples collected by cardiac puncture were used for preparation of serum; the latter was stored at -80°C until use. For each mouse, a total of 180 µl of serum was used for detection of testosterone and progesterone. Details of the liquid chromatography/mass spectrometry (LC/MS)

methods used for determination of serum testosterone and progesterone have been described elsewhere (Zhou et al., 2009, 2010).

Other Methods. Microsomes were prepared from liver and other tissues as described previously (Ding and Coon, 1990; Gu et al., 1997). Protein concentration was determined by the bicinchoninic acid method (Pierce Chemical, Rockford, IL), with bovine serum albumin as the standard. Microsomal CPR and P450 protein expression was determined by immunoblot analysis (Gu et al., 2003); the intensity of the detected bands was determined with a densitometer.

For determination of CPR expression in fetal liver, fetuses of a given embryonic age were obtained through timed pregnancy; the day when vaginal plugs first appeared in the dams was assigned as embryonic day 1 (E1). The gender of a fetus was identified by genotyping analysis for the *Sry* (sex-determining region Y) gene, located on the Y chromosome (Wallis et al., 2008), with the following PCR primers: 5'-ggccatgtcaagcgcccat-3' and 5'-tgccatgtgggtctctgtccca-3' and an annealing temperature of 60°C; the PCR product was 325 base pairs in size.

Immunohistochemical analysis of liver CPR protein was carried out as described earlier (Gu et al., 2007). Determination of microsomal testosterone hydroxylase activity was performed by LC/MS as described recently (Zhou et al., 2009). Pentobarbital clearance was assessed by a sleeping test (Tsuji et al., 1996) performed essentially as described previously (Wu et al., 2005).

Animal treatments with 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), for pharmacokinetic studies and analysis of in vivo DNA adduct formation, were performed as described recently (Weng et al., 2007). Serum levels of NNK and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) and tissue levels of O⁶-methyl-guanine and guanine in the liver and lung of NNK-treated mice were determined by using high-performance liquid chromatography and LC/MS essentially as described previously (Weng et al., 2007), except that an ABI 4000 Q-trap LC/MS system (Applied Biosystems, Foster City, CA) was used for O⁶-methyl-guanine analysis. Pharmacokinetic parameters for NNK and NNAL clearance were calculated with WinNonlin software, version 5.0.1 (Pharsight, Mountain View, CA). Statistical analysis was carried out with SigmaStat software (SPSS Inc. Chicago, IL). Statistical significance of differences between two groups was examined by using Student's *t* test. Significance of differences in genotype distribution was analyzed with the χ^2 test.

Results

Generation of the r-CL Mouse. The structures of the WT *Cpr* allele, the targeting construct, and the targeted *Cpr*^{r-CL} allele are shown in Fig. 1. The *Cpr*^{r-CL} allele contains a *neo* gene, flanked by two loxP sites, inserted at intron 15 of the *Cpr* gene (Fig. 1A), as was the case in the original *Cpr*^{low} allele (Wu et al., 2005). However, unlike the *Cpr*^{low} allele, which contained an additional loxP site (together with a ~100-base pair flanking sequence from the pBS246 vector) in intron 2, the *Cpr*^{r-CL} allele has an intact intron 2 with no insertion of any exogenous sequence.

Although the source DNA for the homology arms in the targeting construct was from the 129/Sv mouse strain, we were successful in obtaining homologous recombinants after electroporation of the targeting construct to ES cells derived from the B6 strain. ES cells from a positive clone were used for subsequent blastocyst injection and generation of chimeric males. When bred with WT B6 females, the chimeras exhibited germ-line transmission. F₁ heterozygous r-CL mice were intercrossed for the production of r-CL, heterozygous r-CL, and WT littermates for subsequent studies.

The structure of the *Cpr*^{r-CL} allele was confirmed by both PCR and Southern blot analysis. As shown in Fig. 1A, the *Cpr*^{r-CL} allele, but not the *Cpr*(+) allele or the targeting

construct, can be detected by the allele-specific PCR primer pair: F1 and R1. R1 is external to the 3' homology arm of the targeting construct; hence, only the allele derived from homologous recombination can be detected (Fig. 1B). For Southern blot analysis (Fig. 1C), an internal DNA probe (Int P) (see Fig. 1A) was used, which detected the 13-kbp EcoRI fragment of the *Cpr*(+) allele in genomic DNA from WT and heterozygous r-CL mice and the 6.5-kbp EcoRI fragment of the *Cpr^{r-CL}* allele in genomic DNA from heterozygous and homozygous r-CL mice. The detection of only a single band in homozygous r-CL mice, and the fact that the two bands detected in heterozygous r-CL mice (representing WT and *Cpr^{r-CL}* alleles, respectively) are at approximately equal intensity, indicated that nonspecific integration of the targeting construct did not occur.

Global Suppression of CPR Expression in the r-CL Mice. CPR expression in various organs of adult r-CL mice, including liver, kidney, brain, lung, and olfactory mucosa (OM), was examined through immunoblot analysis. It is apparent from the results shown in Fig. 1D that microsomal CPR protein levels were substantially decreased in all organs examined, in both males and females. Subsequent semiquantitative immunoblot analyses of CPR expression in 2-month-old male mice (data not shown) indicated that the levels of CPR protein in the r-CL mouse were approximately 25% (liver), 5% (brain), 19% (kidney), 19% (lung), and 11% (OM) of the corresponding levels determined for the WT B6 mice. These results are comparable with the 5 to 26% residual CPR expression found in the CL mice (Wu et al., 2005). Thus, it is clear that the unique sequence insertion (including the loxP site) in *Cpr* intron 2 of the CL mice did not contribute to the down-regulation of CPR expression.

The r-CL mouse was found to be essentially identical to the CL mice in various additional phenotypes characterized. The results of those studies are described below, together with data from the xh-CL mouse.

Generation of the xh-CL Mouse Model. The xh-CL mouse was generated by cross-breeding between r-CL and Alb-Cre (Postic et al., 1999) mouse strains. In the xh-CL mouse, the Cre recombinase was specifically expressed in hepatocytes, as a result of the tissue specificity of the albumin promoter, leading to hepatocyte-specific deletion of the floxed neo cassette from the *Cpr^{r-CL}* allele (Fig. 2A). As expected, CPR expression levels were normal in liver microsomes, but were still down-regulated in microsomes from various extrahepatic tissues of the xh-CL mice, compared with WT littermates (illustrated in Fig. 2B). In additional semiquantitative immunoblot analysis (data not shown), the levels of liver microsomal CPR protein were found to be essentially identical in the xh-CL and WT mice, either male or female, whereas the levels in the other tissues tested, including brain, kidney, lung, OM, testis, and ovary, of the xh-CL mice were only 4 to 24% of the corresponding levels found in the WT mice. The liver-specific normalization of CPR expression in the xh-CL mice supports our hypothesis that neo insertion at intron 15 was responsible for the suppression of CPR expression in the CL and r-CL mice.

A centrilobular pattern of CPR protein expression was found, via immunohistochemical analysis, in the livers of both WT and xh-CL mice (Fig. 3A), with the signal intensities being similar between the two groups. Centrilobular distribution of CPR-positive cells was also found for the livers of

Fig. 2. Generation of the xh-CL mouse. A, scheme for tissue-specific conversion of a *Cpr^{r-CL}* allele to a *Cpr*(+) allele. In the xh-CL (*Alb-Cre/Cpr^{r-CL/r-CL}*) mouse, which was generated by crossing between the r-CL mouse and the Alb-Cre transgenic mouse, the floxed *neo* gene is deleted from the *Cpr^{r-CL}* allele in a hepatocyte-specific manner. The resulting recombinant *Cpr* allele is in essence identical to the WT *Cpr* allele, thus restoring normal CPR expression in essentially all hepatocytes. B, tissue-specific restoration of normal CPR expression in the livers of male and female xh-CL mice. Immunoblot analysis was performed with an anti-CPR antibody on microsomes from various tissues of WT and xh-CL mice (2 months old). Equal amounts of microsomal proteins were analyzed between strains: 10 μ g for OM and 20 μ g for brain, lung, kidney, ovary, and testis. Microsomes were prepared from pooled tissues from three mice.

the r-CL mice; however, the overall signal intensity was much lower in the r-CL liver than in the WT liver, as reported previously for the CL mouse (Wu et al., 2005; Gu et al., 2007).

The time course for the developmental expression of liver CPR protein in WT, r-CL, and xh-CL mice was also determined through immunoblot analysis of microsomal proteins (Fig. 3B). In the WT mice, CPR was already abundantly expressed at postnatal day 1; a densitometric analysis of the immunoblot results indicated that adult levels of CPR expression were reached by postnatal day 7 (data not shown). It is noteworthy that the CPR level was comparable between the xh-CL liver and the WT liver at all four ages assayed (1, 7, 30, and 60 days of age), a result suggesting that Cre-mediated removal of the *neo* gene in hepatocytes was already complete during fetal development. In contrast, the CPR level was consistently lower in the r-CL liver at all ages tested. In additional studies, hepatic CPR protein expression was analyzed for both male and female fetuses at the age of E18; no significant difference in hepatic CPR levels was observed between the xh-CL liver and the WT liver for either male or female fetuses (Fig. 3C).

Fig. 3. Regional distribution and time course of the restoration of hepatic CPR expression in the xh-CL mice. **A**, immunohistochemical analysis of hepatic CPR expression in WT B6, r-CL, and xh-CL mice. Paraffin sections (5 μ m) of livers from 2-month-old male mice were analyzed by using a rabbit anti-rat CPR antibody. Alexa Fluor 594-conjugated tyramide was used as the peroxidase substrate. Fluorescent signals were detected with a tetramethyl rhodamine isothiocyanate filter. No signal was detected for any of the three mouse strains when the primary antibody was replaced by a normal rabbit serum (Negative control). CV, central vein. Scale bar, 100 μ m. **B**, time course of the restoration of hepatic CPR expression. Immunoblot analysis was performed with an anti-CPR antibody for hepatic microsomal preparations from WT, r-CL, and xh-CL mice at 1, 7, 30, and 60 days of age. Livers, pooled from three mice in each group, were obtained from males. Equal amounts (5 μ g) of microsomal proteins were analyzed in each lane. **C**, hepatic CPR protein expression in WT and xh-CL fetuses. Microsomes were prepared from pooled livers of three to four male or female fetuses at E18. Equal amounts (10 μ g) of microsomal proteins were analyzed in each lane.

The levels of hepatic microsomal expression of several P450 isoforms were determined for adult WT, r-CL, and xh-CL mice. As reported earlier for the CL mouse (Wu et al., 2005; Weng et al., 2007), the expression of most CYP1A, CYP2A, CYP2B, and CYP3A proteins was up-regulated in the r-CL livers compared with the WT livers; however, the expression of these proteins returned to the WT levels in the xh-CL livers (Fig. 4). Thus, the up-regulation of hepatic P450 expression in the CL mice (Wu et al., 2005; Weng et al., 2007) and r-CL mice was caused by lower CPR expression in the liver as opposed to lower CPR expression in extrahepatic tissues.

Liver microsomal testosterone hydroxylase activities were compared between xh-CL and B6 mice. Microsomes were prepared from 2-month-old male mice. The rates of formation of 6 β -OH-testosterone and 16 α -OH-testosterone were determined at a testosterone concentration of 10 μ M. There was no significant difference ($P > 0.05$) in the rates between B6 mice (0.25 ± 0.10 and 0.25 ± 0.02 , for 6 β -OH-testosterone and 16 α -OH-testosterone, respectively; means $\pm$ S.D.; $n = 4$)

Fig. 4. Normal hepatic microsomal CYP1A, CYP2A, CYP2B, and CYP3A expression in the xh-CL mice. Immunoblot analysis was performed with polyclonal antibodies to rat CYP1A1/2, mouse CYP2A5, rat CYP2B1, and rat CYP3A2. Hepatic microsomal proteins (5 μ g per lane) were prepared from 2-month-old male WT, r-CL, and xh-CL mice. Each lane represents an individual mouse.

and xh-CL mice (0.22 ± 0.08 and 0.25 ± 0.02 , for 6 β -OH-testosterone and 16 α -OH-testosterone, respectively; $n = 4$). These data, which are in contrast to the 50 to 60% reductions in the rates of testosterone metabolism found previously for CL liver microsomes compared with WT liver microsomes (Wu et al., 2005) further confirm the recovery of P450 function in the livers of the xh-CL mice.

General Characterization of the r-CL and xh-CL Mice. The r-CL and xh-CL mice both were normal in general appearance. Similar to what was found previously for the CL mice, the r-CL mice showed significantly lower (by $\sim 10\%$) body weight in males and females ($p < 0.05$) and lower organ (heart, lung, and kidney) weight (by ~ 5 – 15%) in males compared with WT B6 mice (data not shown). However, we did not find a significant difference between xh-CL and WT B6 mice in body weights for either gender at 4 or 8 weeks of age or in weights of various organs tested (including liver, heart, lung, and kidney) in either males or females at 3 months of age (data not shown). Thus, it seems that the previously reported lower body weights in adult male and female CL mice, and the lower heart, lung, and kidney organ weights in adult CL males (Wu et al., 2005), were at least partly caused by the loss of hepatic CPR/P450 function during mouse development.

Similar to the female CL mice (Wu et al., 2005), female r-CL mice were infertile; as shown in Table 1, none of the nine female r-CL mice used in a breeding test yielded any litters. In contrast, male r-CL mice were fertile, as were male CL mice (Wu et al., 2005), as demonstrated by the ability of 11 of 12 “male r-CL $\times$ female heterozygous xh-CL” breeding pairs to yield normal-sized litters (~ 7 /litter). Female xh-CL mice were also infertile; none of the nine female xh-CL mice tested (in breeding pairs with male r-CL mice) yielded

TABLE 1

Reproductive ability in female r-CL and xh-CL mice

Mice were used for breeding tests at 2 to 3 months of age. The number of litters and the total number of pups produced in 2 months after establishment of the breeding pairs were recorded.

Mating Group	No. of Breeding Pairs	No. of Fertile Females	No. of Litters Born	No. of Pups Born
Male r-CL $\times$ female r-CL	9	0	0	0
Male r-CL $\times$ heterozygous female xh-CL	12	11	15	94
Male r-CL $\times$ female xh-CL	9	0	0	0

any litters. In additional breeding tests (not shown), no litters were produced when either the xh-CL females or the r-CL females that were described in Table 1 were later paired with 2-month-old WT B6 males. The fact that xh-CL females are also infertile, despite having normal hepatic CPR expression, indicates that the female infertility associated with the CL and r-CL mice did not result from lower hepatic CPR expression.

Impact of Reduced Extrahepatic CPR Expression on Circulating Testosterone and Progesterone Levels in Females. The female infertility associated with the CL mice is believed to be at least partly caused by the higher levels of testosterone and progesterone found in these mice (Wu et al., 2005). The availability of the xh-CL mouse provided a unique opportunity to test whether the hormonal disturbance in the CL mice was caused by reductions in the ability of hepatic microsomal P450 enzymes to metabolize steroid hormones. As predicted from the similar pattern of female infertility in the r-CL mice and xh-CL mice, circulating testosterone and progesterone levels in female xh-CL mice were found to be substantially higher (by 5- and 10-fold, respectively) compared with the levels in WT B6 females (Table 2). This result

TABLE 2

Levels of circulating testosterone and progesterone in female B6 and xh-CL mice

Blood samples were collected, via cardiac puncture, from 2-month-old female mice, for preparation of serum samples. Testosterone and progesterone were determined by LC/MS, as described in *Materials and Methods*. The values are presented as mean $\pm$ S.D. ($n = 9$).

Strain	Testosterone	Progesterone
	pg/ml	ng/ml
B6	53 $\pm$ 16	2.3 $\pm$ 2.5
xh-CL	256 $\pm$ 109 ^a	24.4 $\pm$ 12.9 ^a

^a $P < 0.01$, compared with B6 (Student's t test).

TABLE 3

Levels of circulating cholesterol in B6, r-CL, and xh-CL mice

The values are shown as mean $\pm$ S.D. ($n = 4$ per group). Serum samples were obtained from 2- to 3-month-old male or female mice. Cholesterol was determined as described in *Materials and Methods*.

Strain	Cholesterol Level	
	Male	Female
	mM	
B6	2.6 $\pm$ 0.2	2.2 $\pm$ 0.3
r-CL	2.0 $\pm$ 0.2 ^a	1.5 $\pm$ 0.2 ^a
xh-CL	2.4 $\pm$ 0.2 ^b	1.9 $\pm$ 0.2 ^b

^a Significantly lower than for the B6 group; $P < 0.01$ (Student's t test).

^b Not significantly different from the B6 group, $P > 0.05$; significantly higher than for the r-CL group, $P < 0.05$.

TABLE 4

Increased survival rate of xh-CL embryos

Genotype distribution was analyzed in a total of 392 pups derived from matings between female *Alb-Cre*(+/-)/*Cpr*^{r-CL/+} and male *Cpr*^{r-CL/r-CL} mice. Numbers in parentheses indicate percentage of the total number of pups.

Gender	Number of Pups in Each Genotype ^a			
	<i>Alb-Cre</i> (-/-)/ <i>Cpr</i> ^{r-CL/r-CL} (r-CL)	<i>Alb-Cre</i> (-/-)/ <i>Cpr</i> ^{r-CL/+} (heterozygous r-CL)	<i>Alb-Cre</i> (+/-)/ <i>Cpr</i> ^{r-CL/r-CL} (xh-CL)	<i>Alb-Cre</i> (+/-)/ <i>Cpr</i> ^{r-CL/+} (heterozygous xh-CL)
Female + male	75 ^b (19.1%)	115 (29.3%)	94 (24.0%)	108 (27.6%)
Female	43	60	39	44
Male	32 ^c	55	55 ^d	64

^a The number of pups in each genotype was significantly different from the 25% value, expected according to Mendelian distribution, for male pups alone ($P < 0.05$), or male and female combined ($P < 0.05$), although not for female pups alone ($P > 0.05$).

^b Significantly lower than the number of corresponding heterozygous pups ($P < 0.01$; χ^2 test).

^c Significantly lower than the number of corresponding heterozygous pups ($P < 0.05$; χ^2 test).

^d Significantly higher than the number of corresponding r-CL pups ($P < 0.05$; χ^2 test).

indicates that the reductions previously found in hepatic microsomal P450 activities toward steroid hormone metabolism in the CL females (Wu et al., 2005) did not play a significant role in the observed elevation of circulating levels of testosterone and progesterone.

Impact of Reduced Hepatic CPR Expression on Circulating Cholesterol Levels. Plasma total cholesterol levels were determined in the r-CL, xh-CL, and B6 WT mice (Table 3). As had been found previously for the CL mice (Wu et al., 2005), circulating cholesterol levels in the r-CL mice were moderately lower than in the WT (by 23% in males and 32% in females). However, circulating cholesterol levels in the xh-CL mice were significantly higher than the levels in the r-CL mice and were not significantly different from the levels in the B6 WT mice. Thus, the lower levels of circulating cholesterol in the CL and r-CL mice are caused primarily by a reduced rate of cholesterol biosynthesis in the liver, a conclusion consistent with the established role of hepatic CPR-dependent enzymes in cholesterol biosynthesis.

Impact of Reduced Fetal Hepatic CPR Expression on Embryonic Survival. Because of the female infertility, breeding pairs for the xh-CL mice were set up between male xh-CL homozygotes and female xh-CL heterozygotes. Based on Mendelian distribution, the resulting pups would have one of four possible genotypes (r-CL, heterozygous r-CL, xh-CL, and heterozygous xh-CL; as shown in Table 4) at equal frequency. An analysis of genotype distribution, among all pups obtained to date, indicated deviation from Mendelian distribution; the number of pups in each genotype was significantly different from the 25% value expected for male pups alone ($p < 0.05$) or male and female combined ($p < 0.05$), although not for female pups alone ($p > 0.05$). Further comparisons of the number of pups, either between heterozygotes and homozygotes or between r-CL and xh-CL groups clearly indicated a survival disadvantage of the r-CL embryos, given that only 19.1% of pups (male and female combined) had the r-CL genotype, compared with 29.3% of pups (male and female combined) with the heterozygous r-CL genotype (the two values were significantly different; $p < 0.01$). The same conclusion is reached when male pups alone are analyzed. Thus, the r-CL homozygotes show reduced embryonic survival; this finding is consistent with our previous report for the comparisons between CL pups and heterozygous CL pups derived from intercrosses between homozygous CL males and heterozygous CL females (Gu et al., 2007). However, the homozygous and heterozygous xh-CL embryos seemed to have equivalent survival rates. Furthermore, the genotype frequency for the male xh-CL pups was signifi-

cantly higher than the genotype frequency for the male r-CL pups. The genotype frequency for all (male and female combined) xh-CL pups (24.0%) was also higher than the frequency for all r-CL pups (19.1%), although the difference did not reach statistical significance. This result, combined with the knowledge that hepatic CPR expression was normal during fetal development in the xh-CL mice, suggests that normalized CPR expression in the embryonic liver can prevent the reductions in embryonic survival seen in the CL mice (Wu et al., 2005; Gu et al., 2007) and r-CL mice, at least in males.

Utility of the xh-CL Mouse Model for Studies on the Role of Liver and Extrahepatic Tissue P450 Enzymes in Xenobiotic Metabolism in Vivo. The impact of hepatocyte-specific rescue of CPR expression on systemic drug clearance was examined by using pentobarbital, a barbiturate, as an example. Rates of pentobarbital clearance were gauged by the length of pentobarbital-induced sleeping time after a single intraperitoneal dosing at 60 mg/kg. As shown in Table 5, pentobarbital sleep time was not significantly different between the xh-CL mice and WT B6 mice. In contrast, the sleeping time was substantially (>4-fold) longer for the r-CL mice, as had been found previously for the CL mice (Wu et al., 2005).

We also compared the abilities of the xh-CL and WT B6 mice to activate NNK, a tobacco-specific carcinogen, by measuring the abundance of O⁶-methyl-guanine adduct, formed in the livers and lungs of NNK-treated mice. As shown in Table 6, the levels of the DNA adduct in the livers were not different between the two mouse strains at 24 h after a single intraperitoneal injection of NNK at 100 mg/kg, a result further confirming the rescue of hepatic P450 activity in the xh-CL mice. However, the levels of the DNA adduct detected in the lungs were ~60% lower in the xh-CL mice than in the WT B6 mice, a finding consistent with the role of extrahepatic tissue (lung) P450 enzymes in NNK-induced O⁶-methyl-guanine DNA adduct formation

(Weng et al., 2007). In control experiments (not shown), we observed no difference in NNK/NNAL plasma levels or pharmacokinetic parameters between WT B6 and xh-CL mice.

Discussion

The *neo* gene is frequently used as a selection marker in gene-targeting experiments (Soriano et al., 1991). The insertion of *neo* may affect expression of the target gene: in the CL and r-CL mouse models the *neo* gene is in the reverse orientation to *Cpr* transcription, a situation that is likely to cause greater compromise in the expression of the target gene than if *neo* were inserted in the same orientation as the target gene (Jacks et al., 1994; Nagy, 2000). We have now confirmed through studies on the xh-CL mouse model that the presence of *neo* led to the observed global suppression in CPR expression in the CL and r-CL mouse models. Furthermore, we have taken advantage of this feature of the CL mice and designed a novel strategy to conditionally reactivate CPR expression for functional rescue studies.

The developmental time course for the rescue of hepatic CPR expression in the xh-CL mice suggested that the Alb-Cre-mediated *neo* deletion was highly efficient in this mouse model. For comparison, in the liver-specific *Cpr*-null (LCN) mouse, which has an *Alb-Cre*(+/-)/*Cpr*^{lox/lox} genotype, Alb-Cre-mediated deletion of the *Cpr* gene (exons 3–15) was not complete until the mice were 2 months old (Gu et al., 2003). The apparently higher efficiency of Cre-mediated recombination in the xh-CL mouse than in the LCN mouse may be explained by size differences of the floxed DNA fragments, differential chromosomal accessibility, or a potentially earlier expression of the Alb-Cre transgene in xh-CL than in LCN mice. In any event, the embryonic rescue of hepatic CPR expression in the xh-CL mouse was consistent with previous reports that the Alb-Cre transgene was expressed, and functional, in fetal mouse hepatocytes (Kellendonk et al., 2000; Weisend et al., 2009).

The CL and r-CL mouse strains both show reduced embryonic survival rates despite differences in genetic background. The original CL mice were on a mixed B6:129/Sv background (Wu et al., 2005), whereas the r-CL (and the xh-CL) mice were on a B6 background. The higher embryonic survival rates seen in the xh-CL male mice compared with the r-CL male mice suggest that normalization of hepatic CPR expression during embryonic development can prevent the potentially lethal metabolic disruptions that result from the global suppression of CPR expression in the CL and r-CL mice, at least in males. In that regard, previous work has suggested that dysregulation of the homeostasis of endogenous regulatory molecules, such as retinoic acids, played an important role in the embryonic lethality associated with germ-line deletion of the *Cpr* gene (Shen et al., 2002; Otto et al., 2003; Ribes et al., 2007).

The potential influence of maternal *Cpr* genotype on embryonic survival was not evaluated in the present study. In fact, that issue would be difficult to study, given the infertility of female r-CL mice. In this context, the heterozygous xh-CL dams [*Alb-Cre*(+/-)/*Cpr*^{r-CL/+}] are expected to be *Cpr*(+/+) in the liver, because of Cre-mediated recombination of the *Cpr*^{r-CL} allele. Thus, maternal hepatic CPR expression was normal in our present studies on

TABLE 5

Pentobarbital clearance in B6, r-CL, and xh-CL mice

Male mice (2 to 3 months old) were treated with pentobarbital (10 mg/ml in phosphate-buffered saline) at a dose of 60 mg/kg i.p.. The lengths of time between drug administration and the loss and the subsequent recovery of righting reflex were recorded. Values presented are means ± S.D. The numbers of animals examined in each group are shown in parentheses.

Strain	Onset of Sleep	Sleep Duration
		min
B6 (n = 6)	4.1 ± 0.7	68 ± 8
r-CL (n = 5)	4.4 ± 0.8	278 ± 47 ^a
xh-CL (n = 6)	4.2 ± 0.5	66 ± 12

^a Significantly greater than the B6 group or the xh-CL group; *P* < 0.01 (Student's *t* test).

TABLE 6

NNK-induced O⁶-mG DNA adduct formation in the B6 and xh-CL mice

Mice (2-month-old, females) were given an i.p. injection of NNK at 100 mg/kg, and tissues were obtained at 24 h after dosing. O⁶-mG levels were determined as described in *Materials and Methods*. The values shown represent means ± S.D. (n = 4).

Strain	Tissue O ⁶ -mG Levels	
	Lung	Liver
	pmol/μmol guanine	
B6	12.1 ± 1.2	194 ± 44
xh-CL	4.9 ± 0.5 ^a	196 ± 16

^a Significantly lower than in the B6 group; *P* < 0.01 (Student's *t* test).

embryonic survival rates, and that factor could potentially influence embryonic survival. For purposes of comparison, the dams used in our previous studies on embryonic survival rates were either *Cpr*(+/-) (for CL-LCN; Gu et al., 2007) or *Cpr*^{low/+} (for CL; Wu et al., 2005) in the liver. Nevertheless, the observed improvements in embryonic survival rates of the xh-CL mouse were clearly caused by fetal, rather than maternal, rescue of hepatic CPR function, given the fact that pups of all four genotypes are derived from the same dams, such that the embryos are exposed to the same maternal/placental environment.

The precise mechanisms underlying the female infertility seen in the CL mice (Wu et al., 2005) have yet to be identified. Both r-CL and xh-CL females displayed infertility in the present study, whereas the liver-Cpr-null females were previously found to have normal fertility (Gu et al., 2003). These contrasting findings clearly indicate that hepatic CPR-dependent enzymes have little direct effect on female fertility. The female infertility in the CL and xh-CL mouse strains was accompanied by elevated levels of circulating testosterone and progesterone, presumably the result of reduced activities of CPR-dependent steroidogenic P450 enzymes (such as CYP19) in reproductive/endocrine organs. It remains to be determined whether the detailed patterns of steroidogenic disturbances in the CL and xh-CL females are similar to those found in polycystic ovarian syndrome patients (Raj and Talbert, 1984). In female WT mice, circulating testosterone levels are very low; therefore, CYP19 (the aromatase), which has a low K_m for the conversion of testosterone to estradiol (Stresser et al., 2000), plays a critical role in systemic testosterone clearance. The latter point is supported by studies on *Cyp19*-knockout female mice (Fisher et al., 1998). In future studies, the reproductive functions of CPR-dependent P450 enzymes in a specific reproductive/endocrine organ (e.g., the ovary) or cell type could be determined through rescue of CPR expression specifically in that organ via crosses between the r-CL mouse and a suitable Cre-expressing mouse. In this context, the global suppression of CPR expression and the possibility to rescue CPR function through additional crosses make the r-CL mouse a powerful animal model for the exploration of additional disease phenotypes and pathogenic mechanisms of human POR genetic deficiencies.

The xh-CL mouse can serve as a useful alternative to conditional *Cpr*-knockout models that have tissue-specific *Cpr* deletion in an extrahepatic organ. The usefulness of a tissue-specific *Cpr* knockout approach depends largely on the suitability of available tissue-specific Cre-transgenic mouse strains. Although the number of Cre-transgenic mouse lines has steadily grown (Nagy et al., 2009), it is often still difficult to find a suitable mouse model with the desired pattern of Cre expression. Furthermore, for those tissues/organs composed of multiple cell types, it is difficult to achieve tissue-specific *Cpr* deletion in all cells of the organ, a situation that can potentially lead to underestimation of the contributions, by a given extrahepatic organ, to in vivo xenobiotic metabolism and toxicity. In contrast, the xh-CL mouse, with its substantial reductions of CPR expression in all cells of all extrahepatic organs, can be used for an initial determination of whether CPR-dependent enzymes in any extrahepatic organ are capable of influencing systemic clearance and/or target tissue activation of xenobiotic compounds.

It is noteworthy that liver-Cpr-null models (Gu et al., 2003;

Henderson et al., 2003) have been used for deducing whether extrahepatic organs play any substantial role in xenobiotic metabolism in vivo (e.g., Gu et al., 2005). However, the conclusions reached in those studies were based on indirect evidence, muddled by possible contributions of hepatic CPR-independent biotransformation enzymes. Furthermore, those conclusions were potentially confounded by reduced hepatic clearance of the xenobiotic compound (e.g., Gu et al., 2007). Such reductions would lead to greater systemic bioavailability of the xenobiotic compound, and, consequently, possible overestimation of the role of extrahepatic tissue metabolic activation (and correlative underestimation of any role of liver-derived reactive intermediates) in target tissue toxicity. In contrast, results obtained from the xh-CL model will provide direct evidence for any role of extrahepatic CPR-dependent enzymes in target tissue toxicity, unconfounded by reductions in hepatic xenobiotic metabolism or other factors associated with hepatic *Cpr* deletion, such as lower circulating cholesterol levels and greater liver weights. In further contrast to the liver-Cpr-null model, in which hepatic *Cpr* deletion is not yet complete by 3 weeks of age (Gu et al., 2003; Wu et al., 2005), the xh-CL model is characterized by an "extrahepatic Cpr-low" status that is already established around birth; thus, the xh-CL model is suitable for study of the role of CPR-dependent enzymes in xenobiotic metabolism and toxicity in neonatal animals.

In summary, we have generated and characterized two novel gene-modified mouse models, r-CL and xh-CL. The r-CL model enables the rescue of CPR expression and function in a tissue-specific fashion in a mouse with globally reduced CPR expression. The r-CL model will be valuable for mechanistic studies on the roles of CPR-dependent enzymes of a given organ or cell type in various known or still-to-be identified disease phenotypes associated with human POR genetic deficiencies. The xh-CL mouse, generated through the use of the r-CL model, is not only a valuable screening tool for testing the functions of extrahepatic CPR-dependent enzymes in xenobiotic metabolism and toxicity, but it is also useful for study of the potential roles of hepatic CPR-dependent enzymes in the various biological phenotypes observed in the r-CL mouse model. Of particular interest, our studies on the r-CL and xh-CL mice have revealed that fetal hepatic CPR-dependent enzymes play a critical role in embryonic development, albeit apparently only in male fetuses.

Acknowledgments

We thank the Transgenic and Knockout Mouse Core, Histopathology Core, and the Molecular Genetics Core facilities of the Wadsworth Center for providing use of their services; Dr. Adriana Verschoor for reading the manuscript; and Dr. Xiuling Zhang for assisting with statistical analysis.

References

- Arlt W, Walker EA, Draper N, Ivison HE, Ride JP, Hammer F, Chalder SM, Borucka-Mankiewicz M, Haufla BP, Malunowicz EM, et al. (2004) Congenital adrenal hyperplasia caused by mutant P450 oxidoreductase and human androgen synthesis: analytical study. *Lancet* **363**:2128–2135.
- Black SD and Coon MJ (1987) P-450 cytochromes: structure and function. *Adv Enzymol Relat Areas Mol Biol* **60**:35–87.
- Ding XX and Coon MJ (1990) Induction of cytochrome P-450 isozyme 3a (P-450IIE1) in rabbit olfactory mucosa by ethanol and acetone. *Drug Metab Dispos* **18**:742–745.
- Fang C, Gu J, Xie F, Behr M, Yang W, Abel ED, and Ding X (2008) Deletion of the NADPH-cytochrome P450 reductase gene in cardiomyocytes does not protect mice against doxorubicin-mediated acute cardiac toxicity. *Drug Metab Dispos* **36**:1722–1728.
- Fisher CR, Graves KH, Parlow AF, and Simpson ER (1998) Characterization of mice

- deficient in aromatase (ArKO) because of targeted disruption of the *cyp19* gene. *Proc Natl Acad Sci USA* **95**:6965–6970.
- Flück CE, Tajima T, Pandey AV, Arlt W, Okuhara K, Verge CF, Jabs EW, Mendonça BB, Fujieda K, and Miller WL (2004) Mutant P450 oxidoreductase causes disordered steroidogenesis with and without Antley-Bixler syndrome. *Nat Genet* **36**:228–230.
- Fukami M, Hasegawa T, Horikawa R, Ohashi T, Nishimura G, Homma K, and Ogata T (2006) Cytochrome P450 oxidoreductase deficiency in three patients initially regarded as having 21-hydroxylase deficiency and/or aromatase deficiency: diagnostic value of urine steroid hormone analysis. *Pediatr Res* **59**:276–280.
- Fukami M, Horikawa R, Nagai T, Tanaka T, Naiki Y, Sato N, Okuyama T, Nakai H, Soneda S, Tachibana K, et al. (2005) Cytochrome P450 oxidoreductase gene mutations and Antley-Bixler syndrome with abnormal genitalia and/or impaired steroidogenesis: molecular and clinical studies in 10 patients. *J Clin Endocrinol Metab* **90**:414–426.
- Gu J, Chen CS, Wei Y, Fang C, Xie F, Kannan K, Yang W, Waxman DJ, and Ding X (2007) A mouse model with liver-specific deletion and global suppression of the NADPH-cytochrome P450 reductase gene: characterization and utility for in vivo studies of cyclophosphamide disposition. *J Pharmacol Exp Ther* **321**:9–17.
- Gu J, Cui H, Behr M, Zhang L, Zhang QY, Yang W, Hinson JA, and Ding X (2005) In vivo mechanisms of tissue-selective drug toxicity: effects of liver-specific knockout of the NADPH-cytochrome P450 reductase gene on acetaminophen toxicity in kidney, lung, and nasal mucosa. *Mol Pharmacol* **67**:623–630.
- Gu J, Weng Y, Zhang QY, Cui H, Behr M, Wu L, Yang W, Zhang L, and Ding X (2003) Liver-specific deletion of the NADPH-cytochrome P450 reductase gene: impact on plasma cholesterol homeostasis and the function and regulation of microsomal cytochrome P450 and heme oxygenase. *J Biol Chem* **278**:25895–25901.
- Gu J, Walker VE, Lipinskas TW, Walker DM, and Ding X (1997) Intraperitoneal administration of coumarin causes tissue-selective depletion of cytochromes P450 and cytotoxicity in the olfactory mucosa. *Toxicol Appl Pharmacol* **146**:134–143.
- Henderson CJ, Otto DM, Carrie D, Magnuson MA, McLaren AW, Rosewell I, and Wolf CR (2003) Inactivation of the hepatic cytochrome P450 system by conditional deletion of hepatic cytochrome P450 reductase. *J Biol Chem* **278**:13480–13486.
- Huang N, Pandey AV, Agrawal V, Reardon W, Lapunzina PD, Mowat D, Jabs EW, Van Vliet G, Sack J, Flück CE, et al. (2005) Diversity and function of mutations in P450 oxidoreductase in patients with Antley-Bixler syndrome and disordered steroidogenesis. *Am J Hum Genet* **76**:729–749.
- Jacks T, Shih TS, Schmitt EM, Bronson RT, Bernards A, and Weinberg RA (1994) Tumor predisposition in mice heterozygous for a targeted mutation in *Nf1*. *Nat Genet* **7**:353–361.
- Kellendonk C, Opherk C, Anlag K, Schütz G, and Tronche F (2000) Hepatocyte-specific expression of Cre recombinase. *Genesis* **26**:151–153.
- Köntgen F, Süss G, Stewart C, Steinmetz M, and Bluethmann H (1993) Targeted disruption of the MHC class II Aa gene in C57BL/6 mice. *Int Immunol* **5**:957–964.
- Nagy A, Gertsenstein M, Vintersten K, and Behringer R (2003) *Manipulating the Mouse Embryo: A Laboratory Manual*, 3rd ed, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Nagy A (2000) Cre recombinase: the universal reagent for genome tailoring. *Genesis* **26**:99–109.
- Nagy A, Mar L, and Watts G (2009) Creation and use of a cre recombinase transgenic database. *Methods Mol Biol* **530**:365–378.
- Otto DM, Henderson CJ, Carrie D, Davey M, Gundersen TE, Blomhoff R, Adams RH, Tickle C, and Wolf CR (2003) Identification of novel roles of the cytochrome P450 system in early embryogenesis: effects on vasculogenesis and retinoic acid homeostasis. *Mol Cell Biol* **23**:6103–6116.
- Porter TD and Coon MJ (1991) Cytochrome P-450. Multiplicity of isoforms, substrates, and catalytic and regulatory mechanisms. *J Biol Chem* **266**:13469–13472.
- Postic C, Shiota M, Niswender KD, Jetton TL, Chen Y, Moates JM, Shelton KD, Lindner J, Cherrington AD, and Magnuson MA (1999) Dual roles for glucokinase in glucose homeostasis as determined by liver and pancreatic β cell-specific gene knockouts using Cre recombinase. *J Biol Chem* **274**:305–315.
- Raj SG and Talbert LM (1984) Polycystic ovarian disease. *Obstet Gynecol Annu* **13**:261–273.
- Ribes V, Otto DM, Dickmann L, Schmidt K, Schuhbauer B, Henderson C, Blomhoff R, Wolf CR, Tickle C, and Dollé P (2007) Rescue of cytochrome P450 oxidoreductase (Por) mouse mutants reveals functions in vasculogenesis, brain and limb patterning linked to retinoic acid homeostasis. *Dev Biol* **303**:66–81.
- Schmidt K, Hughes C, Chudek JA, Goodyear SR, Aspdren RM, Talbot R, Gundersen TE, Blomhoff R, Henderson C, Wolf CR, et al. (2009) Cholesterol metabolism: the main pathway acting downstream of cytochrome P450 oxidoreductase in skeletal development of the limb. *Mol Cell Biol* **29**:2716–2729.
- Shen AL, O'Leary KA, and Kasper CB (2002) Association of multiple developmental defects and embryonic lethality with loss of microsomal NADPH-cytochrome P450 oxidoreductase. *J Biol Chem* **277**:6536–6541.
- Soriano P, Montgomery C, Geske R, and Bradley A (1991) Targeted disruption of the *c-src* protooncogene leads to osteopetrosis in mice. *Cell* **64**:693–702.
- Stewart CL, Kaspar P, Brunet LJ, Bhatt H, Gadi I, Köntgen F, and Abbondanzo SJ (1992) Blastocyst implantation depends on maternal expression of leukaemia inhibitory factor. *Nature* **359**:76–79.
- Stresser DM, Turner SD, McNamara J, Stocker P, Miller VP, Crespi CL, and Patten CJ (2000) A high-throughput screen to identify inhibitors of aromatase (CYP19). *Anal Biochem* **284**:427–430.
- Tsuji R, Isobe N, and Kawasaki H (1996) Mechanism of prolongation of pentobarbital-induced sleeping time by empenethrin in mice. *Toxicology* **108**:185–190.
- Wallis MC, Waters PD, and Graves JA (2008) Sex determination in mammals: before and after the evolution of SRY. *Cell Mol Life Sci* **65**:3182–3195.
- Weisend CM, Kundert JA, Suvorova ES, Prigge JR, and Schmidt EE (2009) Cre activity in fetal albCre mouse hepatocytes: utility for developmental studies. *Genesis* **47**:789–792.
- Weng Y, Fang C, Turesky RJ, Behr M, Kaminsky LS, and Ding X (2007) Determination of the role of target tissue metabolism in lung carcinogenesis using conditional cytochrome P450 reductase-null mice. *Cancer Res* **67**:7825–7832.
- Wortham M, Czerwinski M, He L, Parkinson A, and Wan YJ (2007) Expression of constitutive androstane receptor, hepatic nuclear factor 4 α , and P450 oxidoreductase genes determines interindividual variability in basal expression and activity of a broad scope of xenobiotic metabolism genes in the human liver. *Drug Metab Dispos* **35**:1700–1710.
- Wu L, Gu J, Cui H, Zhang QY, Behr M, Fang C, Weng Y, Kluetzman K, Swiatek PJ, Yang W, et al. (2005) Transgenic mice with a hypomorphic NADPH-cytochrome P450 reductase gene: effects on development, reproduction, and microsomal cytochrome P450. *J Pharmacol Exp Ther* **312**:35–43.
- Wu L, Gu J, Weng Y, Kluetzman K, Swiatek P, Behr M, Zhang QY, Zhuo X, Xie Q, and Ding X (2003) Conditional knockout of the mouse NADPH-cytochrome P450 reductase gene. *Genesis* **36**:177–181.
- Zhang QY, Fang C, Zhang J, Dunbar D, Kaminsky L, and Ding X (2009) An intestinal epithelium-specific cytochrome P450 (P450) reductase-knockout mouse model: direct evidence for a role of intestinal P450s in first-pass clearance of oral nifedipine. *Drug Metab Dispos* **37**:651–657.
- Zhou X, Zhang X, Weng Y, Fang C, Kaminsky L, and Ding X (2009) High abundance of testosterone and salivary androgen-binding protein in the lateral nasal gland of male mice. *J Steroid Biochem Mol Biol* **117**:81–86.
- Zhou X, Zhuo X, Xie F, Kluetzman K, Shu YZ, Humphreys WG, and Ding X (2010) Role of CYP2A5 in the clearance of nicotine and cotinine: insights from studies on a Cyp2a5-null mouse model. *J Pharmacol Exp Ther* **332**:578–587.

Address correspondence to: Dr. Xinxin Ding, Wadsworth Center, New York State Department of Health, Empire State Plaza, Box 509, Albany, NY 12201-0509. E-mail: xding@wadsworth.org