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# Rescue of cytochrome P450 oxidoreductase (*Por*) mouse mutants reveals functions in vasculogenesis, brain and limb patterning linked to retinoic acid homeostasis

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**Abstract**

Cytochrome P450 oxidoreductase (POR) acts as an electron donor for all cytochrome P450 enzymes. Knockout mouse $Por^{-/-}$ mutants, which are early embryonic (E9.5) lethal, have been found to have overall elevated retinoic acid (RA) levels, leading to the idea that POR early developmental function is mainly linked to the activity of the CYP26 RA-metabolizing enzymes (Otto et al., Mol. Cell. Biol. 23, 6103–6116). By crossing *Por* mutants with a RA-reporter *lacZ* transgene, we show that $Por^{-/-}$ embryos exhibit both elevated and ectopic RA signaling activity e.g. in cephalic and caudal tissues. Two strategies were used to functionally demonstrate that decreasing retinoid levels can reverse $Por^{-/-}$ phenotypic defects, (i) by culturing $Por^{-/-}$ embryos in defined serum-free medium, and (ii) by generating compound mutants defective in RA synthesis due to haploinsufficiency of the *retinaldehyde dehydrogenase 2* (*Raldh2*) gene. Both approaches clearly improved the $Por^{-/-}$ early phenotype, the latter allowing mutants to be recovered up until E13.5. Abnormal brain patterning, with posteriorization of hindbrain cell fates and defective mid- and forebrain development and vascular defects were rescued in E9.5 $Por^{-/-}$ embryos. E13.5 $Por^{-/-}$; $Raldh2^{+/-}$ embryos exhibited abdominal/caudal and limb defects that strikingly phenocopy those of $Cyp26a1^{-/-}$ and $Cyp26b1^{-/-}$ mutants, respectively. $Por^{-/-}$; $Raldh2^{+/-}$ limb buds were truncated and proximalized and the anterior–posterior patterning system was not established. Thus, POR function is indispensable for the proper regulation of RA levels and tissue distribution not only during early embryonic development but also in later morphogenesis and molecular patterning of the brain, abdominal/caudal region and limbs.


*Keywords:* POR; Retinoids; P450 cytochromes; CYP enzymes; Limb development; Brain patterning; Vasculogenesis

## Introduction

Cytochrome P450 oxidoreductase (POR; previously named CPR and CYPOR) is an NADPH-dependent protein essential for transferring electrons to microsomal cytochrome P450 enzymes (CYP or P450; Strobel et al., 1995). This large superfamily of enzymes catalyzes oxidative metabolism of many exogenous and endogenous compounds including environmental chemicals, drugs and dietary derived-compounds, such as retinoids, as well as steroids, fatty acids and prostaglandins (Hasler, 1999). Functional inactivation of the *Por* gene in mice leads to severe developmental defects and death at embryonic day (E) 9.5 (Otto et al., 2003; Shen et al., 2002). High-pressure liquid chromatography (HPLC) analysis of $Por^{-/-}$ embryos

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showed a significantly elevated retinoic acid/retinol ratio, which could be partially rescued by feeding dams with a vitamin A-deficient (VAD) diet (Otto et al., 2003). Widespread defects are observed in *Por*$^{-/-}$ embryos, but it remains to be established whether these defects are merely a consequence of abnormal retinoid signaling, or whether other cytochrome P450-mediated metabolic pathways might be involved.

Retinoic acid (RA), an active metabolite of vitamin A, is an important signaling molecule during vertebrate embryonic development and plays a pivotal role in pattern formation, organogenesis, cell proliferation, differentiation and apoptosis (Blomhoff, 1994; Chambon, 1996; Niederreither and Dollé, in press; Ross et al., 2000; Sporn and Roberts, 1994). The three known members of the CYP26 subfamily have been shown to act on RA and oxidize it into more polar compounds that are prone to further metabolism and inactivation (Fujii et al., 1997; MacLean et al., 2001; Tahayato et al., 2003; White et al., 1997). The importance of RA metabolism by CYP26 P450s during embryonic development has been demonstrated by the *Cyp26a1* and *Cyp26b1* null mouse models. Both *Cyp26a1*$^{-/-}$ and *Cyp26b1*$^{-/-}$ genotypes are lethal. *Cyp26a1*$^{-/-}$ embryos die at mid-late gestation and have multiple organ defects, including caudal truncation with spina bifida (MacLean et al., 2001; Sakai et al., 2001), while *Cyp26b1*$^{-/-}$ pups die soon after birth due to respiratory defects (Yashiro et al., 2004). *Cyp26b1*$^{-/-}$ mutants also have severe limb defects (Yashiro et al., 2004). It has been shown in both *Cyp26a1*$^{-/-}$ and *Cyp26b1*$^{-/-}$ mice that RA signaling is increased in areas where these genes are normally expressed, suggesting that the corresponding enzymes contribute to RA degradation (MacLean et al., 2001; Sakai et al., 2001; Yashiro et al., 2004). To further demonstrate this point, Niederreither et al. (2002a) showed that the *Cyp26a1*$^{-/-}$ phenotype can be rescued genetically by conditions which decrease RA levels. Introduction of haploinsufficiency of the *retinaldehyde dehydrogenase 2* (*Raldh2*) gene, encoding for the main RA-synthesizing enzyme during early embryogenesis (Niederreither et al., 1999), rescued the lethal phenotype and development of posterior structures in *Cyp26a1*$^{-/-}$ mutants (Niederreither et al., 2002a).

*Por*$^{-/-}$ mutants are more severely affected (Otto et al., 2003), and die much earlier during development, than either *Cyp26a1*$^{-/-}$ or *Cyp26b1*$^{-/-}$ null mice. This may not be surprising, considering that lack of POR would impair the activity of all three CYP26 enzymes, and that these enzymes may have partially redundant roles in the early embryo (H. Hamada, personal communication). POR activity is necessary for the function of all P450s and an additional possibility is that other CYP subfamilies play important roles in embryonic development and that lack of POR impairs their activities too. Indeed, several P450s, including *Cyp1a1*, *Cyp2r1* and *Cyp2s1*, have been reported to be expressed during early mouse embryogenesis (Choudhary et al., 2003, 2005), although there are no available data about their tissue distribution or possible function. *Cyp1a1* knockout mouse mutants, however, are viable and do not have any obvious defects (Ghanayem et al., 2000).

In this work, we have used various approaches to dissect out in detail the link between the *Por*$^{-/-}$ embryonic phenotype and abnormal RA signaling. To analyze RA signaling at the cellular level, we crossed *Por* mutants with transgenic mice harboring a RA-sensitive (*RARE-hsp68-lacZ*) reporter gene (Rossant et al., 1991). We show that RA-reporter activity is both abnormally high in regions of normal transgene expression, and ectopically extends in cephalic and caudal regions of the *Por*$^{-/-}$ embryos. We also used two strategies to experimentally lower the retinoid levels in *Por*$^{-/-}$ mutants, either by culturing whole embryos in serum-free defined medium, or genetically by haploinsufficiency of the *Raldh2* gene. Both approaches led to a rescue of the *Por*$^{-/-}$ mutants, the latter strategy allowing mutants to be recovered up to E13.5 for further phenotypic analyses. We thus found that *Por*$^{-/-}$ fetuses have an abdominal/caudal truncation phenotype strikingly similar to that of *Cyp26a1*$^{-/-}$ mice, as well as limb defects whose molecular analysis revealed a 'proximalization' of cell fates, consistent with the abnormal limb phenotype of *Cyp26b1*$^{-/-}$ mutants. Analysis of unrescued and rescued *Por*$^{-/-}$ embryos revealed abnormal molecular patterning of various brain regions, consistent with known effects of excess RA administration to wild-type embryos (Avantaggiato et al., 1996; Simeone et al., 1995 and refs therein). Altogether, our data concur in showing that a major developmental function of POR is to allow proper homeostasis of RA both in early embryogenesis and through the activity of P450 metabolizing enzymes.

## Materials and methods

### *Mice and embryo culture*

Generation of mice with targeted disruptions of the *Por* (Otto et al., 2003) and *Raldh2* (Niederreither et al., 1999) genes has been described. For embryo culture, pregnant females were sacrificed at E9.5 and embryos explanted from uterus in DMEM (Invitrogen). Embryos with intact yolk sacs were collected and immersed in warmed (37°C) culture medium in 50 ml screw top tubes (5 ml medium/embryo; 1–2 embryos/tube). The culture medium consisted of Knockout DMEM, 10% KSR (Knockout Serum Replacement), 1× N2 supplement, plus penicillin and streptomycin (all from Invitrogen) and 2% of cell culture grade albumin (Sigma) (Moore-Scott et al., 2003). Tubes were gassed with 40% $O_2$, 5% $CO_2$ and 55% $N_2$ and placed into a roller-tube mouse incubator at 37°C (Tanaka et al., 2000). After 24 h, cultured embryos were dissected out of the yolk sac in DMEM and fixed in 4% paraformaldehyde.

### *Retinoic acid analysis and scanning electron microscopy*

RA analysis was performed on frozen embryos and medium by using automated online solid-phase extraction high-pressure liquid chromatography-electrochemical detection (Otto et al., 2003) and verified by using single wave UV detection.

For scanning electron microscopy, embryos were fixed in Peter's fixative (1.25% glutaraldehyde, 1% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.2) at 4°C. Embryos were post-fixed in 1% osmium in cacodylate buffer, dehydrated in graded ethanol, placed in acetone, critically point-dried and sputter-coated with gold/palladium. Specimens were viewed on a Philips XL30 emission scanning electron microscope.

### *In situ hybridization, X-gal assays and immunohistochemistry*

In situ hybridizations (ISH) with digoxigenin-labeled riboprobes on whole-mount embryos or on cryosections (10 μm) were performed as described (Chotteau-Lelièvre et al., 2006). An Intavis InSituPro robot was used in some cases; details at http://www.eumorphia.org/EMPReSS/servlet/EMPReSS.