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A Hypomorphic Allele of *Aryl Hydrocarbon Receptor-Associated Protein-9* Produces a Phenocopy of the *Ahr*-Null Mouse

Bernice C. Lin, Linh P. Nguyen, Jacqueline A. Walisser, and Christopher A. Bradfield

McArdle Laboratory for Cancer Research, Department of Oncology, School of Medicine and Public Health, University of Wisconsin Madison, Madison, Wisconsin

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ABSTRACT

The aryl hydrocarbon receptor-associated protein-9 (ARA9) is a chaperone of the aryl hydrocarbon receptor (AHR). The AHR has been shown to play a late developmental role in the normal closure of a fetal hepatovascular shunt known as the ductus venosus (DV). Given that *Ara9*-null mice display early embryonic lethality, we generated a hypomorphic *Ara9* allele (designated *Ara9*^{fxneo}) that displays reduced ARA9 protein expression. In an effort to demonstrate the role of ARA9 protein in AHR-mediated DV closure, we used combinations of *Ara9* wild-type [*Ara9*(+/+)], null [*Ara9*(−/−)], and hypomorphic [Ara9(*fxneo/fxneo*)] alleles to produce mice with a graded expression of the ARA9 protein. Liver perfusion studies demonstrated that although none of the *Ara9*(+/+) mice displayed a patent DV, the shunt was observed in 10% of the Ara9(+/*fxneo*) mice, 55% of the *Ara9*(+/−) mice, and 83% of the *Ara9*(*fxneo/fxneo*) mice. That expression level of ARA9 correlates with the frequency of a phenocopy of the *Ahr*-null allele supports the conclusion that the ARA9 protein is essential for AHR signaling during development.

The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor best known for mediating the adaptive and toxic responses to polycyclic aromatic hydrocarbons and halogenated dibenzo-*p*-dioxins (Poland, 1982; Eisen et al., 1983). Once ligands such as these bind the receptor, the complex translocates to the nucleus in which the AHR dissociates and dimerizes with its transcriptional partner, the aryl hydrocarbon receptor nuclear translocator (ARNT). In the nucleus, the AHR/ARNT dimer binds to cognate "dioxin-responsive elements" in the enhancer regions of responsive genes, resulting in the transcriptional up-regulation of those genes (Reyes et al., 1992; Henry and Gasiewicz, 1993; Hord and Perdew, 1994). The effects mediated by this pathway include the adaptive up-regulation of certain cytochromes P450, as well as toxic endpoints that include thymic involution, hepatocellular damage, and cleft palate (Poland and Knutson, 1982).

It has been shown recently that the AHR also plays an important role in hepatovascular development (Lahvis et al., 2005). In the developing embryo, the ductus venosus (DV) serves as a shunt that connects the umbilical cord blood with blood from the portal vein and the inferior vena cava (IVC) (Schermerhorn et al., 1996). The DV normally closes within a few hours or days of parturition. Although *Ahr*(−/−) mice are viable and fertile, a consistent phenotype observed in these animals is the presence of an open, or patent DV throughout life (Lahvis et al., 2005). Failure of DV closure in *Ahr*(−/−) mouse models causes a decrease in portal blood supply to the liver, thereby limiting nutrients and decreasing overall liver size (Lahvis et al., 2005).

The cytosolic form of the receptor has been shown to be complexed with a dimer of heat shock protein of 90 kDa and either the cochaperone p23 or the aryl hydrocarbon receptor-associated protein-9 (ARA9) (Carver and Bradfield, 1997; Shetty et al., 2003; Hollingshead et al., 2004). ARA9 is also known as the aryl hydrocarbon receptor-interacting protein or the hepatitis B virus X-associated protein 2 (XAP2) (Ma and Whitlock, 1997; Meyer and Perdew, 1999). In an effort to understand whether ARA9 plays a role in the developmental aspect of AHR signal transduction, we used gene targeting techniques to generate a series of hypomorphic alleles at the *Ara9* locus in mice. Hypomorphic alleles at *Ara9* were used because mice harboring an *Ara9*-null allele [i.e., *Ara9*(−/−)] display 100% embryonic lethality as a result of several embryonic heart defects (Lin et al., 2007). Given that *Ahr*(−/−) mice do not display similar congenital heart defects, we conclude that ARA9 plays a role in heart development that is independent of its role in AHR function (Fernandez-Salguero et al., 1996; Schmidt et al., 1996).

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ABBREVIATIONS: AHR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator; IVC, inferior vena cava; ARA9, aryl hydrocarbon receptor-associated protein-9; bp, base pair(s); kb, kilobase(s); TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

Our use of *Ara9* hypomorphic models was based upon two predictions. First, we hypothesized that mice hypomorphic for *Ara9* could be developed that would express enough protein to alleviate the developmental block caused by the essential role of the protein in early cardiac development. Second, if ARA9 was essential for AHR developmental signaling, then mice with a lower level of ARA9 protein expression would also display a phenocopy of the *Ahr*-null animal (i.e., a patent DV). We show here that these criteria can indeed be met. Furthermore, we provide the first evidence that ARA9 is essential for AHR-mediated developmental signaling.

Materials and Methods

Construction of the *Ara9^{fxneo}* Targeting Vector. Creation of the *Ara9^{fxneo}* targeting vector is identical to the creation of Δ*CAra9^{fxneo}* and is described in Fig. 1 and in previous work from our laboratory (Lin et al., 2007). A BamHI restriction enzyme analysis resulting in fragment sizes of 553, 844, and 11,165 bp was performed to confirm the orientation of components of the targeting vector (PL 2008), including the tetratricopeptide repeat domains, Lox P sites, and arms of homology. Embryonic stem cell culture conditions and genotyping were performed using the same methods as reported previously (Lin et al., 2007).

Visualization of the Ductus Venosus. Mice harboring a patent DV were identified by perfusion of the liver with a solution of trypan blue using methods described previously (Walisser et al., 2004a). The status of the DV was confirmed on a subset of livers via time-lapse angiography using a method described previously (Walisser et al., 2004b).

Western Blot Analysis. Western blots were performed using the same methods described previously (Lin et al., 2007).

Statistics. In experimental analysis of multiple comparisons, an analysis of variance was performed, and Tukey's test was used to determine differences, with a $p \leq 0.05$. Statistical analysis of genotype distribution was compared by χ^2 analysis (Devore and Peck, 1986).

Results

Generation and Genotyping of *Ara9^{fxneo}* Mice. Homologous recombination of the *Ara9^{fxneo}* targeting construct (Fig. 1A) into embryonic stem cells was confirmed by Southern blot analysis of BamHI-digested genomic DNA. The wild-type and *Ara9^{fxneo}* recombinant allele gives rise to bands at 2.7 kb and 3.9 kb, respectively (Fig. 1B). Progeny resulting from a heterozygote by heterozygote cross were as follows: 12/52 (23%) were *Ara9*(+/+), 30/52 (58%) were *Ara9*(+/*fxneo*), and 10/52 (19%) were *Ara9*(*fxneo*/*fxneo*) (Fig. 1C). The ratio of the genotypes obtained from χ^2 analysis was not significantly different from expected with a viable allele ($p \leq 1$; $\chi^2 = 0.69$) and indicated adherence to the expected Mendelian ratio of 1:2:1. Gross anatomy and histological examinations suggested that wild-type, heterozygote, and homozygous *Ara9^{fxneo}* animals are outwardly normal (data not shown).

The *Ara9^{fxneo}* Allele Is Hypomorphic. The expression of ARA9 was measured by Western blot analysis of protein extracts from heart, liver, kidney, spleen, and thymus from wild-type [*Ara9*(+/+)] and *Ara9*(*fxneo*/*fxneo*) mice (Fig. 2A). Hypomorphs displayed marked decreases in ARA9 protein expression in all tissues examined. Antibodies raised against either the FK506-binding protein or tetratricopeptide repeat domains (Lin et al., 2007) yielded similar results (data not shown). Based on the ARA9 Western blot gradient, expression of the ARA9 protein from the *Ara9^{fxneo}* allele was estimated to be approximately 10% of the expression of the wild-type allele (Fig. 2B).

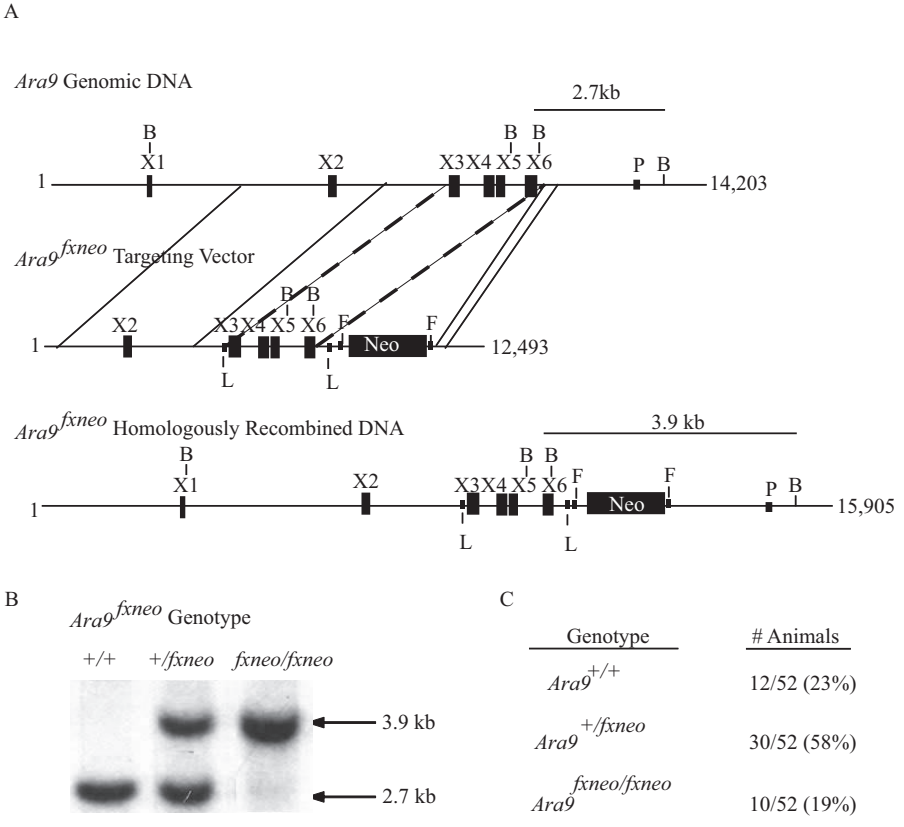


Fig. 1. Targeting vector and Southern blot analysis for *Ara9^{fxneo}* targeting construct. A, genomic DNA of *Ara9*, the targeting vector, and a map of *Ara9^{fxneo}* homologous recombination. B, Southern blot analysis of *Ara9^{fxneo}* embryonic stem cell that has undergone homologous recombination. The wild-type band is present at 2.7 kb, and homologous recombination is depicted at 3.9 kb. C, Mendelian distribution of genotypes in hypomorphic *Ara9* animals. Lines denote regions of homology. B, BamHI; P, probe; X, exon; Neo, Neomycin cassette; L, Lox P; F, Frt site; +/+, wild-type; +/*fxneo*, heterozygote; *fxneo*/*fxneo*, homozygote.

The ARA9 Protein Is Necessary for Closure of the Ductus Venosus. To determine whether *Ara9*^{fxneo} mice display a patent DV similar to that observed in *Ahr*(−/−) mice, we evaluated this phenotype by obtaining serial angiograms of contrast dye flowing through the perfused liver and by trypan blue perfusion (Fig. 3, A and B) (Lahvis et al., 2000; Walisser et al., 2004a). In the progeny from appropriate crosses of heterozygous animals, the angiograms of wild-type littermates revealed that the contrast agent flowed through the portal vein and into the branches of the liver and then into the suprahepatic then infrahepatic IVC (Fig. 3A). In contrast, livers of *Ahr*(−/−) and *Ara9*(*fxneo/fxneo*) mice display portocaval shunting between the portal vein and IVC, indicating the presence of a patent DV (Lahvis et al., 2000). Perfusion assays using trypan blue were performed on a larger number of animals and revealed that *Ara9*(*fxneo/fxneo*) mice display a patent DV at a frequency of 83%. Mice that are *Ara9*(+/−) display a DV frequency of 56%. Mice that are *Ara9*(+/*fxneo*) display a patent DV with a frequency of approximately 10% (Fig. 3B). Wild-type animals [*Ara9*(+/+)] do not display a patent DV. No association was noted between the sex of the animal and persistence of the DV (data not shown). Estimates of DV patency in *Ara9*(−/−) mice could not be obtained because these mice are resorbed or stillborn because of cardiac defects that begin around day E12 (Lin et al., 2007).

The Presence of DV Causes a Significant Decrease in Liver Weight. In addition to and as a result of the patent DV, another common phenotype observed in *Ahr*(−/−) mice is a decreased relative liver weight. To investigate whether this relationship also holds true for the *Ara9* hypomorphic lines, we compared relative liver weights from wild-type, heterozygous hypomorphic, homozygous hypomorphic, and heterozygous null animals. As expected, liver weight in *Ara9*(*fxneo/fxneo*) and *Ara9*(+/−) mice were significantly less than in wild-type and *Ara9*(+/*fxneo*) mice (Fig. 3B).

Discussion

Characterization of the *Ahr*(−/−) phenotype has defined a role for the AHR in a developmental pathway essential for the normal closure of the DV in the developing liver (Lahvis et al., 2000). Based on this observation, it follows that proteins that influence AHR folding, localization, or function may also be essential for AHR-mediated hepatovascular development. The simplest way to test this idea is to create mouse models with null alleles at candidate genes and to examine their AHR-mediated response profile. Unfortunately, most, if not all of the proteins known to be involved in AHR signaling are not amenable to classic gene targeting approaches that use null alleles, because many AHR-associated gene products are essential for more than one independent cellular pathway required for the survival of the animal. A relevant example is the ARNT protein, which is also required for developmental angiogenesis mediated by hypoxia-inducible factors (Maltepe et al., 1997; Chan et al., 1999). For ARNT, we were able to overcome its essential nature and provide evidence for its role in AHR-mediated development by using mice that exhibited decreased expression of ARNT (i.e., hypomorphic expression) (Walisser et al., 2004b). In

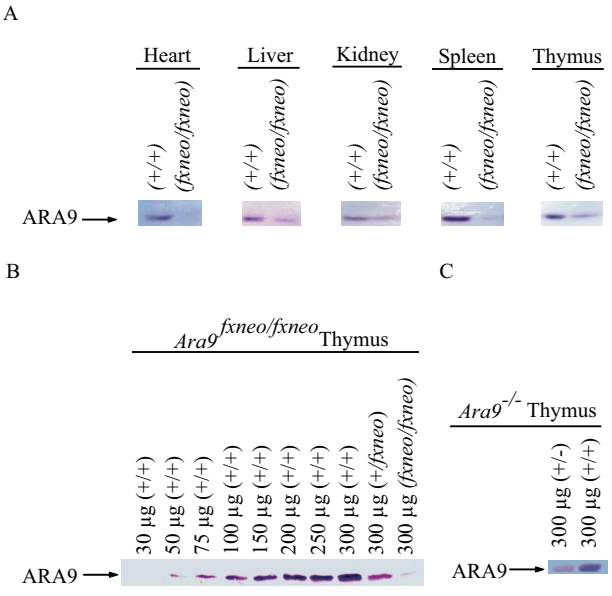
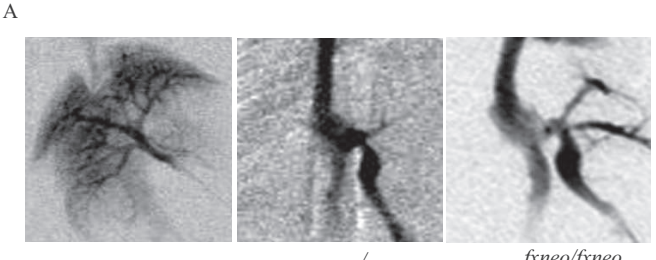


Fig. 2. Western blot analysis of *Ara9*^{fxneo} mice shows decrease in ARA9 protein levels. A, Western blot of *Ara9*^{fxneo} protein comparing wild-type *Ara9*(+/+) and homozygous *Ara9*$^{fxneo/fxneo}$ littermates in heart, liver, kidney, spleen, and thymus. B, Western blot of *Ara9*(*fxneo/fxneo*) and Ara9(+/*fxneo*) protein compared with a wild-type Ara9(+/+) gradient in the thymus. C, Western blot of *Ara9*(+/−) protein compared with wild-type *Ara9*(+/+) in thymus. The ARA9 protein band was detected with a mouse monoclonal FK506-binding protein domain-specific antibody.



B

Genotype	% With DV	Relative Liver Weight
Ara9$^{+/+}$	0% (0/16)	5.42% ± 0.01
Ara9$^{+/fxneo}$	10% (1/10)	5.65% ± 0.01
Ara9$^{+/-}$	56% (5/9)	3.73% ± 0.00
Ara9$^{fxneo/fxneo}$	83% (5/6)	3.98% ± 0.01

Fig. 3. Visualization of ductus venosus and incidence of occurrence in *Ara9* hypomorphic or heterozygous mice. A, radio images showing presence of a ductus venosus in *Ara9*(*fxneo/fxneo*) mouse. The C57BL/6 liver (left) is shown as comparison for a wild-type (well perfused) liver. An *Ahr*(−/−) liver (center) is shown as a representative of a poorly perfused liver with ductus venosus. An *Ara9*(−/−) liver (right) is also shown with a poorly perfused liver with ductus venosus. B, a gradient of ARA9 expression leads to a gradient in the presentation of ductus venosus and liver weight in *Ara9*^{fxneo} and *Ara9*(+/−) mice. The percentage of animals with DV (no. of animals/total animals) in *Ara9*^{fxneo} or *Ara9*(+/−) mice is shown. Liver weight of all mice within a given genotype is represented as a percentage of total body weight ± S.D. A statistical analysis using a one-way analysis of variance shows a significant difference between livers of wild-type and *Ara9*(+/*fxneo*) animals versus *Ara9*(*fxneo/fxneo*) and *Ara9*(+/−) mice (∗, $p < 0.01$).

that model, we found that decreased expression of the ARNT protein allowed for normal embryonic angiogenesis, yet yielded a developmental phenotype that was identical to that observed in the *Ahr*(−/−) model (i.e., a patent DV).

Given our recent study demonstrating that *Ara9*(−/−) mice die in fetal development as a result of double-outlet right ventricle and ventricular septal defect, we chose to use a "hypomorph strategy" similar to that used for ARNT (Walisser et al., 2004b; Lin et al., 2007). To this end, we constructed a series of recombinant alleles at the *Ara9* locus in mice. Our strategy was based on work from this laboratory and others, which has shown that targeted insertion of the *Neo* gene into a locus of interest frequently results in the generation of a hypomorphic allele (Walisser et al., 2004b). Given that *Neo* insertion was an intermediary step in generating a conditional *Ara9* allele, a hypomorphic allele of *Ara9* was constructed and used to characterize the influence of hypomorphic ARA9 expression on AHR-mediated liver development. It was our prediction the generation of a hypomorphic mouse would allow us to bypass embryonic lethality and enable us to study the effect of decreased ARA9 protein expression on AHR-mediated biology.

Using the *Ara9*^{fxneo} allele along with our previously generated *Ara9* allele, we created an allelic series where graded expression of the ARA9 protein could be achieved after appropriate genetic crosses. The expression levels of ARA9 protein ranged from highest to lowest in *Ara9*(+/+), *Ara9*(+/*fxneo*), *Ara9*(+/−), and *Ara9*(*fxneo*/*fxneo*) mice, respectively (Fig. 2A; data not shown). Although our Western blot-derived estimates of ARA9 protein expression from these models provides only a semiquantitative measure of relative protein expression, the data do support an estimate of approximately a 10% expression of ARA9 in the *Ara9*(*fxneo*/*fxneo*) mice, a 50% expression in the *Ara9*(+/−)mice, and a 60% expression in *Ara9*(+/*fxneo*) mice relative to wild-type mice (Fig. 2A).

Examination of a large cohort of *Ara9* mutants supports our two initial predictions. First, we found that mice homozygous for a hypomorphic *Ara9* allele (*Ara9*(*fxneo*/*fxneo*) proceeded through development in normal numbers (Fig. 1), suggesting that 10% of normal expression of ARA9 is sufficient to overcome the block in heart development observed previously in null animals (Lin et al., 2007). Second, we hypothesized that a marked reduction in ARA9 expression would lead to insufficient AHR signaling and that the corresponding mice would display many of the phenotypes of *Ahr*-null animals. Examination of the developmental phenotype, patent DV, was of particular interest to us. To this point, we measured the frequency of the DV and found 83% of the hypomorphic animals to display this developmental phenotype (Fig. 3). In addition to the phenocopy exhibited by *Ara9* hypomorphs and *Ahr*-null animals, additional support for the importance of ARA9 in AHR-mediated DV closure came from the observation of a "dose effect" of ARA9 expression on these endpoints. A slight reduction of ARA9 protein levels by one copy of *Ara9*^{fxneo} (i.e., the *Ara9*(+/*fxneo*) model) led to only a 10% frequency of patent DV. Intermediate expression of ARA9 from the *Ara9*(+/−) model display a patent DV incidence of 56%. Even greater reduction of ARA9 protein from two copies of the hypomorphic allele (i.e., *Ara9*(*fxneo*/*fxneo*) led to an 83% incidence of patent DV. In our examination of hundreds of wild-type mice (*Ara9*), we have never observed a patent DV. In keeping with this, we have observed a 0% frequency of patent DV in our wild-type cohort. Together, these data support the idea that ARA9 plays an important role in AHR-mediated developmental signaling.

Because of the persistence of the DV in ARA9 hypomorphic animals, this model has less power as a tool to study the adaptive and toxic pathways mediated by the AHR. This concept is based upon the observation that the portocaval shunting, which accompanies a patent DV, can lead to aberrant disposition of TCDD and related AHR agonists in many tissues, making a direct comparison between mutant and wild-type responses problematic (Thomae et al., 2004). For example, if we were trying to show a relationship between *Ara9* expression and TCDD toxicity for a given liver endpoint, we would predict a decreased potency in the *Ara9* hypomorphs. However, if this did occur, we would not know whether the attenuation was due to decreased cellular AHR signaling or to a decreased concentration of TCDD in the hepatic parenchyma as a result of shunting. Therefore, to understand the role ARA9 plays in toxic and adaptive signaling, our future plans include crossing the *Ara9*^{fxneo} hypomorph with the FLPeR line (mice containing Flp-recombinase driven by the GTSRosa promoter), thereby allowing for germline excision of the neomycin cassette and creation of a conditional *ARA9* mouse (Dymecki, 1996). These mice can then be crossed to mice where expression of Cre is driven by the Albumin promoter, allowing for specific excision of the *Ara9* allele in hepatocytes (Kellendonk et al., 2000). Such a model will allow us to understand the relationship between hepatic expression of ARA9 and AHR-mediated hepatotoxicity and adaptive metabolism.

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Address correspondence to: Dr. Christopher Bradfield, 1400 University Ave., Madison, WI 53706. E-mail: bradfield@oncology.wisc.edu