Deletion of the Aryl Hydrocarbon Receptor-associated Protein 9 Leads to Cardiac Malformation and Embryonic Lethality*

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The aryl hydrocarbon receptor-associated protein 9 (ARA9, also known as XAP2 or AIP1), is a chaperone that is found in complexes with certain xenobiotic receptors, such as the aryl hydrocarbon receptor (AHR) and the peroxisome proliferator-activated receptor α (PPARα). In an effort to better understand the physiological role of ARA9 outside of its role in xenobiotic signal transduction, we generated a null allele at the Ara9 locus in mice. Mice with a homozygous deletion of this gene die at various time points throughout embryonic development. Embryonic lethality is accompanied by decreased blood flow to head and limbs, as well as a range of heart deformations, including double outlet right ventricle, ventricular-septal defects, and pericardial edema. The early cardiovascular defects observed in Ara9-null mice suggest an essential role for the ARA9 protein in cardiac development. The observation that the developmental aberrations in Ara9-null mice are distinct from those observed for disrupted alleles at Ahr or Ppara indicates that the role of ARA9 in cardiac development is independent of its interactions with its known xenobiotic receptor partners.

The aryl hydrocarbon receptor-associated protein 9 (ARA9, also known as AIP1) is found in association with two mammalian client proteins, the aryl hydrocarbon receptor (AHR) and the peroxisome proliferator-activated receptor α (PPARα) (1–4). The ARA9 protein has also been characterized in association with the hepatitis-B virus X-protein, HBVx. As the result of its interaction with this viral protein, it is also known by the name hepatitis B virus X-associated protein or XAP2 (5).

The ARA9 protein is structurally related to the immunophilin family of proteins, harboring an N-terminal FK506 binding (FKBP) domain and C-terminal tetratricopeptide repeats (TPRs) (1, 3, 6). The FKBP domains of many immunophilins possess peptidyl-prolyl cis-trans isomerase (PPIase) activity that can be blocked by the binding of immunosuppressants such as FK506 (7, 8). Despite the sequence similarity between the N terminus of ARA9 and the FKBP domains of other immunophilins, ARA9 does not appear to possess isomerase activity or affinity for FK506 (1). We postulate that the structural similarity of ARA9 to the immunophilin family is a reflection of a shared capacity to act as a cellular chaperone, and play a role in the folding and localization of client proteins.

The ARA9 protein has been extensively studied in association with the AHR. As a chaperone, ARA9 maintains the AHR in a cytosolic localization, decreases AHR degradation, and increases its ligand binding capacity (9–11). Although little is known regarding the functional role of ARA9 in PPARα and HBVx function, initial data are consistent with ARA9 acting as a chaperone for these proteins as well (4, 5). We hypothesize that in addition to xenobiotic signaling, the ARA9 protein is also a common chaperone to many proteins involved in a variety of essential cellular functions. This idea is supported by results from whole mount in situ staining of embryos that show ARA9 expression as early as embryonic day 9.5 (e9.5). This early developmental expression is well before appearance of the AHR or PPARα, which are first expressed around e13.5 (1, 12–14). In addition, the spectrum of ARA9 protein expression is broader than that of the AHR or PPARα and includes tissues such as the thalamus and neuroepithelium, where AHR and PPARα are not expressed (12, 14, 15). In keeping with this idea, we find that loss of the Ara9 locus in mice leads to a number of phenotypes not found in either the Ahr-/- or Ppara-/- mice. These Ara9-/- specific phenotypes include: lethality during the growth of the embryo accompanied by cardiac malformations including double outlet right ventricle (DORV), ventricular septal defect (VSD), and pericardial edema. Taken in sum, these observations indicate a role for ARA9 in normal mammalian biology, one that is outside of its role as an AHR or PPARα chaperone.

**EXPERIMENTAL PROCEDURES**

**Null Allele Strategy**—The approach used to create a null allele of Ara9 is part of a strategy that is also being used to generate a conditional allele. Our strategy is based on the idea that reversal of exons 4 through 6 within Ara9 reverses the sequence of the C-terminal tetratricopeptide repeats (TPR), resulting in the truncation of the Ara9 protein and creation of a null allele. The resulting allele is referred to as Ara9<sup>ΔCtfneo</sup>. As seen in Fig. 1, the Ara9<sup>ΔCtfneo</sup> targeting construct employed here can easily be modified to construct a conditional allele...
where exons 4–6 remain in-frame and flanked by the LoxP sites for later excision by a recombinase.\textsuperscript{3}

\textbf{Oligonucleotides—}Oligonucleotides were synthesized by Integrated DNA Technologies (IDT, Coralville, IA). Oligonucleotides used in the study are as follows: OL 3163: 5′-GGC GTT GAC ATA ATC TCG TAT AGC ATAT CAT TAT ACG AAG TTA TGA TGG CAC CTC TGC TGC C-3′; OL 3164: 5′-GGC GTT GAC ATA ATC TCG TAT AGT GTC TAT GGC TGT TAT GCG AAG TTA TGA TGG CAC CTC TGC TGC C-3′; OL 3165: 5′-GGC CGG CGG GAA GGC GTC TTA GCA ACC TCC-3′; OL 3166: 5′-GGC CGG CGG GAA GGC GTC TTA GCA ACC TCC-3′; OL 3167: 5′-GGC GTT GAC ATA ACT TCG TAT AAT GTA TGC TAT ACG-3′; OL 3168: 5′-GGC CGG CGG GAA GGC GTC TTA GCA ACC TCC-3′; OL 3169: 5′-GGC CGG CGG GAA GGC GTC TTA GCA ACC TCC-3′; OL 3170: 5′-GGC CGG CGG GAA GGC GTC TTA GCA ACC TCC-3′; OL 3171: 5′-GGC CGG CGG GAA GGC GTC TTA GCA ACC TCC-3′.

\textbf{Construction of the Ara9\textsuperscript{ACfxneo} Targeting Vector—}A 1,761-base pair (bp) region of homology surrounding the exons encoding the TPR domains of Ara9 was PCR amplified from Bacterial Artificial Chromosome (BAC) plasmid 17276 (Genome Systems, St. Louis, MO). Oligonucleotides 3163 and 3164 were used to introduce two LoxP sites flanking the PCR-amplified TPR domains (referred to as the “floxed region” of our targeting vector). To confirm ligation of the floxed region into the pGemTeasy vector (Promega, Madison, WI), we digested the resultant plasmids with SacI to obtain 588-bp and 4,236-bp bands. A 1,600-bp region homologous to the 3′-untranslated region adjacent to exon 6 of Ara9 was PCR-amplified using OL 3165 and 3166 to create the “short arm.” To confirm short arm ligation into pGemTeasy, the construct was digested with EcoRI to obtain 160-bp, 1,515-bp, and 3,018-bp bands. A 4,222-bp region incorporating exon 2 and the flanking intronic region of Ara9 was PCR amplified using OL 3161 and 3494 to create the “long arm.” To confirm long arm ligation into pGemTeasy, the plasmid was digested with EcoRI to obtain 527 bp, 3,018-bp, and 3,684-bp bands. The 1,682-bp SacI fragment containing the short arm was cloned into the SacI site of the targeting construct PL 1169 (pFrtLoxPNeo). The PL 1169 was digested with BamH1 to obtain 553-bp, 844-bp, and 6,685-bp bands. A 1,761-kb KpnI/SalI fragment containing the floxed region was then cloned into the KpnI/SalI sites of the final targeting construct that was designated as PL2008 (i.e. Ara9\textsuperscript{ACfxneo}). To confirm the final ligation of all homologous regions into PL1169, the plasmid was digested with BamH1 to obtain 553-bp, 844-bp, and 11,165-bp bands.

\textbf{Cell Culture Conditions and Treatments—}Cultivation of embryonic stem cells and generation of mouse embryonic fibroblasts were performed using methods described previously (17).

\textsuperscript{3}B. C. Lin, J. Walisser, L. Nguyen, and C. A. Bradfield, manuscript in preparation.

\textbf{Generation of Ara9\textsuperscript{ACfxneo/fxneo} Mice—}Approximately 10 μg of the targeting construct was electroporated into ES cells (Genome Systems). Selection was performed using 200 μg/ml G418 (Sigma-Aldrich) until a control plate with untransfected ES cells displayed 100% cell death (roughly 5–6 days). To confirm homologous recombination, ES clones were screened by Southern blot analysis of BamHI-digested genomic DNA. The probe used was derived from an EcoRI-digested fragment that is complementary to the 3′-untranslated region adjacent to exon 6 of Ara9 (PL 2044). The ES cell clones that displayed homologous recombination were injected into 3.5-day post coital C57BL/6 blastocysts and the resulting chimeras were backcrossed to C57BL/6 mice. Contribution of the ES clones to the germline was determined by Southern blot analysis of BamHI-digested DNA isolated from tail biopsies obtained from the progeny.

\textbf{Genotyping—}To confirm homologous recombination of the Ara9\textsuperscript{ACfxneo} construct, DNA was isolated from proteinase K-digested tail biopsies using phenol-chloroform extraction. Ten micrograms of DNA was digested using BamH1. Genotyping was then confirmed using Southern blot analysis with a probe homologous to the 3′-region past the end of the targeting construct (PL 2044).

\textbf{Animal Care—}The Ara9\textsuperscript{ACfxneo} mice were housed in accordance with guidelines set by the University of Wisconsin Animal Care and Use Committee. Mice were housed in a pathogen-free facility on corn cob bedding with food and water \textit{ad libitum}. Heterozygous female mice were weighed, paired with males at 4 PM, and then separated at 9 AM the next day. We define gestation time to be at e1.5 the day when the mice are separated. At defined gestational time points, animals were weighed as an indicator of pregnancy then sacrificed by cervical dislocation.

\textbf{Histology—}Samples were fixed in 10% (v/v) neutral-buffered formalin overnight to ensure penetration of the fixative. A cavity allowing for dehydration/hydration was created via abdominal puncture. Following overnight fixation, fetuses were dehydrated through serial ethanol washes starting with 0.8% (w/v) NaCl in ethanol, 70% (v/v) ethanol, 85% (v/v) ethanol, 90% (v/v) ethanol, and 100% ethanol. Following dehydration, samples were treated with three washes of 10% (v/v) xylene, then 50:50 (v/v) xylene/paraffin, then washed three times in 60 °C-heated paraffin, and then embedded in fresh 60 °C-heated paraffin. Following embedding, transverse, or sagittal sections (10 μm) were cut through the entire fetus and every 3rd slide was stained with hematoxylin and eosin.

\textbf{Hematoxylin and Eosin Staining—}Slides were dipped in 100% xylene twice and then subjected to consecutive washes of 100% ethanol, distilled water, Meyer’s Hematoxylin, distilled water, Scott’s Solution (23.8 mM NaHCO\textsubscript{3}, 81.1 mM MgSO\textsubscript{4}), distilled water, 80% (v/v) ethanol, eosin, 90% (v/v) ethanol, two washes of 100% ethanol, and three washes of 100% xylene. Coverslips were then sealed onto slides using Micromount (Surgipath, Richmond, IL).

\textbf{Western Blot—}Western blot of ARA9 was performed using the method described previously (9). Affinity-purified polyclonal rabbit ARA9 antibodies were obtained commercially (Quality Controlled Biochemicals, Hopkinton, MA). Antibody 106652 was raised against amino acids 7–26 (RLREDG-
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A. *Ara9* Genomic DNA

![Diagram of *Ara9* Genomic DNA with bands at 2.7 kb and 3.9 kb.]

- **Ar9**
  - Targeting Vector
  - Homologously Recombined DNA

B. *Ar9<sup>ΔCfxneo</sup>* Genotype

- **fxneo<sup>+</sup>/+**
- **fxneo/+**

C. *Ara9* Genotype

- **Ara9<sup>+</sup>/Ara9<sup>ΔCfxneo+</sup>**
- **Ara9<sup>ΔCfxneo</sup>/Ara9<sup>ΔCfxneo</sup>fxneo**

**FIGURE 1. Generation of an *Ara9<sup>ΔCfx</sup>* null allele.** A comparison of genomic *Ara9* DNA (top), *Ara9<sup>ΔCfx</sup>* targeting vector (middle), and the targeted allele, homologically recombined DNA (bottom). Legend: lines indicate arms of homology. Lines drawn from targeting vector to genomic DNA represent short and long arm regions of homology. B, BamH1; P, probe; X, exon; NEO, neomycin cassette; L, LoxP. F, Frt Site; +/+ wild-type (Ara9<sup>+</sup>/+); +/fxneo, heterozygote (Ara9<sup>ΔCfxneo</sup>); B, Southern analysis of genomic DNA isolated from tail biopsies with homologous recombination of the *Ara9<sup>ΔCfxneo</sup>* allele. Presence of homologous recombinants indicates germ-line transmission. Homologous recombinants appear at bands at both 2.7 kb and 3.9 kb. C, genotyping data from heterozygote by heterozygote crosses without neomycin cassette (Ar9<sup>ΔCfx</sup> × Ar9<sup>ΔCfx</sup>). Data show a genotypic ratio of roughly 1:2:0 with 59 wild-type, 127 heterozygotes, and 0 homozygous nulls in a total of 186 pups genotyped at 4 weeks of age. Data indicates that *Ara9<sup>ΔCfx</sup>* mice undergo embryonic lethality. Legend: +/+ wild-type (Ara9<sup>+</sup>/+); +/fxneo, heterozygote (Ara9<sup>ΔCfxneo</sup>); fneom, homozygous null (Ara9<sup>ΔCfxneo<sup>/ΔCfxneo</sup></sup>).

**FIGURE 2. Removal of neomycin cassette from *Ara9<sup>ΔCfx</sup>*</sup> allele.** A. *Ara9<sup>ΔCfx</sup>* targeted allele, homologously recombined and neomycin-excised DNA (top), *Ara9<sup>ΔCfx</sup>* targeted allele (bottom). Legend: B, BamH1; P, probe; X, exon; NEO, neomycin cassette; L, LoxP, Frt site; +/+ wild-type (Ara9<sup>+</sup>/+); +/fxneo, heterozygote (Ara9<sup>ΔCfxneo</sup>); B, Southern analysis of genomic DNA isolated from tail biopsies with homologous recombination of the *Ara9<sup>ΔCfxneo</sup>* and *Ara9<sup>ΔCfx</sup>* allele. Homologous recombinants appear at bands at both 2.7 kb and 3.9 kb. Homologous recombinants with the neomycin cassette excised appear at bands at both 2.7 kb and 3.9 kb. C, genotyping data from heterozygote by heterozygote crosses without neomycin cassette (Ar9<sup>ΔCfx</sup> × Ar9<sup>ΔCfx</sup>). Data show a genotypic ratio of roughly 1:2:0 with 22 wild-type, 32 heterozygotes and 0 homozygous nulls in a total of 55 pups genotyped at 4 weeks of age. Data indicate that mice homozygous for the *Ara9* targeted allele with embryonic lethality regardless of presence of neomycin cassette. Legend: +/+ wild-type (Ara9<sup>+</sup>/+); +/fx, heterozygote with neomycin cassette removed (Ar9<sup>ΔCfx</sup>); +/f<sup>ROSA</sup>, heterozygote with neomycin cassette removed (Ar9<sup>ΔCfx</sup>/f<sup>ROSA</sup>); 0/54 homozygous null with neomycin cassette removed (Ar9<sup>ΔCfx<sup>/ΔCfx</sup></sup>/f<sup>ROSA</sup>).

**RESULTS**

**Generation of *Ar9<sup>ΔCfx</sup>* Heterezygous and Homozygous Mutant**—To create the *Ar9<sup>ΔCfx</sup>* targeting vector, exons 3–6 were inverted to an antisense orientation (Fig. 1A). A neomycin cassette was then inserted following exon 6 and flanked on either side with Frt sites to allow for excision by Flp recombinase (19). Germ-line transmission of the resultant *Ar9<sup>ΔCfx</sup>/f<sup>neo</sup>* allele was confirmed by Southern blot analysis. Following homologous recombination, wild-type mice are represented by a single band at 2.7 kb, while heterozygous mice show bands at 2.7 kb and 3.9 kb corresponding to the wild-type and targeted allele, respectively (Fig. 1B).

**Loss of *ARA9* Leads to Lethality**—Following germ-line transmission of the *Ar9<sup>ΔCfx</sup>* allele, heterozygous littersmates were interbred in an attempt to obtain homozygous *Ar9<sup>ΔCfx</sup>*/*f<sup>neo</sup>* mice. For a viable allele, the Mendelian ratio predicts a 1:2:1 ratio of wild-type to heterozygous to homozygous pups for a single segregating locus. Genotyping of pups from heterozygote by heterozygote crosses at 4 weeks of age revealed 59 wild-type mice *Ar9<sup>+</sup>/+*, 127 heterozygous *Ar9<sup>ΔCfx</sup>*/+ mice, and no homozygous *Ar9<sup>ΔCfx</sup>*/*f<sup>neo</sup>* mice (Fig. 1C). Chi-square analysis indicated that the ratio of the genotypes obtained was significantly different from expected based on Mendelian segregation of a viable allele (p ≤ 0.001, χ² = 54.166). Gross pathology and breeding studies of the heterozygous *Ar9<sup>ΔCfx</sup>*/+ mice appear phenotypically normal and fertile (data not shown).

**Presence or Absence of Neomycin Cassette Does Not Effect Lethality**—To ensure that lethality was not caused by cis-effects from the neomycin cassette, heterozygous mice were mated with transgenic mice expressing Flp-Recombinase driven by the Gt(ROSA)26Sor promoter (“FLPeR” mice) (20). This cross removed the neomycin cassette between the two FRT sites when expressed ubiquitously from the ROSA promoter (Fig. 2A). The resulting animals are designated as *Ar9<sup>ΔCfx</sup>*. Germ-line transmission of the resultant *Ar9<sup>ΔCfx</sup>* allele was confirmed by Southern blot analysis. Wild-type mice are represented by a single band at 2.7 kb, while heterozygous mice show bands at...
2.7 kb and 3.6 kb corresponding to the wild-type and targeted allele with neomycin cassette removed, respectively (Fig. 2B). Following heterozygote by heterozygote crosses of Ara9 CK6/+++ mice analyzed at 4 weeks of age, there were 22 wild-type mice Ara9+/+/, 32 heterozygous Ara9 CK6/+, and no homozygous Ara9 CK6/+++ mice (Fig. 2C). Chi-square analysis indicated that the ratio of the genotypes obtained was significantly different from expected based on Mendelian segregation of a viable allele (p ≤ 0.001, χ² = 16.711). Given that the presence of the neomycin cassette does not appear to alter the lethality, all data and observations for the remainder of this report are combined results from both Ara9 CK6/neo/neo and Ara9 CK6/CK6 mice and the targeted allele will be referred to as Ara9−/−.

Embryonic Lethality Occurs in Both Early and Late Embryonic Development—To investigate the time of lethality in Ara9−/− mice, we examined embryos produced from heterozygote by heterozygote crosses at various time points during gestation. From a total of 156 embryos genotyped at e10−14, there were 52 wild-type (Ara9+/+), 81 heterozygous (Ara9+/−), and 23 homozygous (Ara9−/−) embryos. A chi-square analysis indicated that significant mortality of Ara9−/− animals was associated with the allele as the ratio of the genotypes obtained was significantly different from expected based on Mendelian segregation of a viable allele (p ≤ 0.05, χ² = 6.78). From a total of 89 embryos genotyped at e15−17, there were 30 wild-type (Ara9+/+), 50 heterozygous (Ara9+/−), and 9 homozygous (Ara9−/−) embryos. Chi-square analysis indicated that significant mortality of Ara9−/− animals was associated with the allele as the ratio of the genotypes obtained was significantly different from expected based on Mendelian segregation of a viable allele (p ≤ 0.001, χ² = 13.93). The observation that our genotyping at e10−14 indicated a wild-type/heterozygote/homozygote frequency of 1:1.5:0.4, indicates that the first loss of Arazozygote frequency of 1:1.5:0.4, indicates that the first loss of

Western Blot Reveals a Decrease in ARA9 Protein in Multiple Tissues—Cytosolic extracts were prepared from various tissue samples taken from 8–10 week old wild-type Ara9+/+ or heterozygous Ara9+/−/− mice. All Western blots were carried out on adult animals as embryonic lethal nulls did not provide enough protein to carry out a conclusive Western blot analysis. Samples from heart, lung, kidney, and spleen were then analyzed by Western blot using rabbit reticulocyte lysate translated human Ara9 cDNA as a positive control. The ARA9 protein from tissue samples ran at the same size as control protein and this band was detectable by both an N-terminal, “FKBP”-specific antibody (1066S2) and a C-terminal, “TPR”-specific (R38KEQP) antibody. As predicted for a heterozygous Ara9+/+ mouse, all tissues show an approximate 50% decrease in ARA9 protein levels compared with wild-type controls (Fig. 4, A and B).

Vascular and Cardiac Abnormalities in Ara9−/− Mice—At e10.5, gross morphology of surviving Ara9−/− embryos appeared outwardly normal. Embryos at all stages of gestation were assessed as “alive” if there was detectable movement in response to stimuli of light pressure and bled when the umbilical cord was removed. All Ara9−/− animals with one exception at e18.5, appeared to be alive. By e14.5–15, surviving Ara9−/−
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Embryos displayed a decreased volume of blood in vessels in the head and extremities (forelimbs and hind limbs). The homozygous embryos were markedly paler than heterozygous and wild-type littersmates (Fig. 5). There was no significant difference observed in sizes of pups (data not shown). Histological sections from e13.5–18.5 day embryos were sectioned through entirely and examined for abnormalities. Sections taken from homozygous Ara9^−/− embryos, between e13.5–15 and e18.5 indicated severe heart phenotypes. Microscopic examination of head, lung, liver, and all other major organ sections from wild-type, heterozygous, and null embryos yielded no further obvious abnormalities (data not shown).

In normal physiology, the pulmonary artery has an outlet from the right ventricle and the aorta has an outlet from the left ventricle. However, in 57% (4/7) of all surviving Ara9^−/− embryos between e13.5–14.5, there was a double-outlet right ventricle (DORV) where both the pulmonary artery and aorta display an outlet from the right ventricle (Fig. 6, A and B). In the majority of cases where homozygous null animals had a DORV, there was a concomitant presence of an abnormal opening between the two ventricles. This abnormal opening, referred to as a ventricular septal defect (VSD), presented with a 71% penetrance (5/7) in Ara9^−/− embryos (Fig. 7, A and B). Accompanying these heart defects was pronounced pericardial edema, whereby the pericardial sac is enlarged and filled with fluid. This pericardial edema was seen in 71% (5/7) of the embryos (Fig. 8).

A small number of null animals also exhibited defects with blood vessels on the body surface such as a decreased number and caliber of vessels in homozygous null yolk sacs compared with the wild-type littersmates (Fig. 9A). At e18.5, some homozygous animals also presented with an open abdominal cavity (omphalocele) as well as hemorrhaging, and pecto-chiae along the surface of their bodies near the head and upper torso seen in embryos between e14.5 to e18.5 (Fig. 9B).

DISCUSSION

Relationship to Xenobiotic Receptors—In an effort to test the hypothesis that ARA9 plays a role independent of known xenobiotic receptor-associated pathways, we have generated a null allele at the Ara9 locus in mice and compared its phenotype to that of the previously described Ahr^−/− and Pparα^−/− mice. These studies arose from our interest in the study of AHR signal transduction. Having identified ARA9 as important in AHR biology, we became interested in determining whether ARA9 played a role that was specific to xenobiotic receptor signaling or whether it played a broader role in mammalian develop-
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plays a range of cardiac phenotypes that are not observed in either the Ahr\(^{-/-}\) or Ppar\(^{-/-}\) mouse models (23–25). If ARA9 function is unique to AHR or PPAR\(\alpha\) signaling, one would expect the corresponding null alleles to have significant overlap or be partial phenocopies of each other. Also in support of additional biological roles for ARA9 is the recent observation of its associations with additional mammalian proteins such as the E3 ubiquitin ligase CHIP (26), phosphodiesterase 2A (27), phosphodiesterase PDE4A5 (28), viral protein EBNA-3 (29), and the apoptotic factor survivin (30). These observations coupled with the unique developmental expression patterns of ARA9 suggest that its entire range of client proteins are not yet understood and may be quite broad.

The Role of ARA9 in Heart Development—These results introduce a new player in the field of genes involved in heart formation. This work demonstrates that deletion of Ara9 leads to congenital heart defects such as DORV and VSD (Figs. 6 and 7). Half of all Ara9\(^{-/-}\) animals die before e10.5 and remaining fetuses die around e14.5 and none survive past e18.5. By e14.5, fetuses begin to display a decreased caliber in blood vessels and exhibit decreased flow to extremities such as the head, forelimbs, and hind limbs (Fig. 5). Concurrent with this decrease in blood flow around e14.5 are heart malformations including DORV and VSD as well as the presence of pericardial edema. Pulmonary edema is often seen as a classic sign of fetal cardiac abnormalities, often developing secondary to the effect of the malformation itself (31).

The role of ARA9 in heart development is in keeping with the high levels of ARA9 mRNA in this tissue. Previous Northern blot (5, 6) and microarray analyses (32) demonstrate high levels of Ara9 mRNA in adult heart tissue as compared with other visceral organs. More importantly, databases of developmentally expressed genes reveal that at developmental times as early as e13.5, the primordial heart displays is the site where the ARA9 mRNA is most highly expressed (33). The high level expression of Ara9 in the heart at e13.5, along with cardiovascular abnormalities at e14.5 in the null animals is consistent with an early essential tissue autonomous role for the ARA9 in the developing heart. We postulate that the pathology exhibited in this developing organ in the absence of Ara9 expression is the result of aberrant protein function of a client(s) that requires ARA9 as a chaperone. The identification of such client proteins can now be attempted, given that we are armed with information related to target protein location and temporal expression.
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A. Decreased Yolk sac Vascular Network

B. Hemorrhage and Petechiae

FIGURE 9. Additional vascular abnormalities seen in some but not all Ara9−/− fetuses. Phenotypic comparison of e18.5–19 wild-type and homozygous-null animals. Comparison of abnormalities in wild-type (left) versus homozygous-null littermates (middle and right). A, decreased number and caliber of vessels within the vascular network of the yolk sac in homozygous-null animals. Vessels indicated by red arrow. B, presence of hemorrhage and petechiae along upper torso. Hemorrhage and petechiae indicated by red arrows.

The observation that ARA9 expression shifts to other tissues after birth suggests that ARA9 may take on additional chaperone functions in the lung, kidney, thymus, and spleen (e.g. Fig. 4 and Ref. 6). Given that the loss of ARA9 leads to embryonic lethality, it is difficult to assess the impact of the loss of ARA9 at other tissue sites where this gene may be required in adulthood. We predict that additional pathologies in other tissues will arise in the absence of Ara9 expression. This issue is currently being addressed by our work on a conditional knock-out animal model that has been developed in response to these results.

The study of abnormal cardiac development in Ara9-null animals is important given that congenital heart defects are the most common birth defect in humans, affecting 1 out of every 150 births in the United States (34, 35). Through numerous studies, it is becoming clear that genes involved in the structural development of the heart, specifically defects in turning and formation of chambers cause the majority of these congenital heart defects. In normal development, the linear heart tube loops toward the right, creating a four-chambered organ (36–38). In abnormal development, insufficient looping, misalignment of the linear tube, or incorrect remodeling of the inner curvature of the heart can lead to cardiac abnormalities such as DORV (39–42). Another cause of DORV may be a lack of proper endothelial to mesenchymal transition (EMT) by cells within the cardiac cushions that form the septations between developing heart chambers (43). Failure of septation or incorrect septation may lead to DORV (44, 45).

Concurrent with the presence of DORV in Ara9-null animals, is the presence of a VSD. The presence of a VSD is thought to be a secondary malformation due to an initial defect in the cardiovascular structure, such as a DORV. In keeping with these possible mechanisms, the DORV phenotype is also observed in a number of other loss-of-function mouse models including jumonji (46) and members of the endothelin signaling pathway such as Pdgfra (47), Pitx2 (48), Ece (endothelin-converting enzyme) (49), Tgfβ2 (50), ET-1 (endothelin-1) (51), Sox-4 (52), Egfr/Shp2 (53), Rkrα (54), and Fog-1 (Friend of GATA) (55).

We hypothesize that cardiac defects present in Ara9−/− embryos are in part due to a failure of the heart to rotate properly when transforming from a linear heart tube into a four-chambered organ. Whether the defective rotation of the heart is due to aberrant apoptosis, or abnormal proliferation/migration of cells in the inner curvature or forming septa, a failure to correctly establish a left-right axis, or a failure to complete endothelial to mesenchymal transition remains to be elucidated. It can also be hypothesized that the presence of a DORV causes an imbalanced right ventricular load that is compensated through a disruption in blood flow through the ventricles (56).

One candidate in our hypothesis for a molecular mechanism behind heart malformation in Ara9−/− embryos is the protein survivin. Recent work has shown that loss of ARA9 impairs the expression and function of survivin, an anti-apoptotic factor in endothelial cells (30). Endothelial cell-specific survivin-null animals die early on in embryogenesis around e10–13.5 with e13 tie1-cre/survivin−/− embryos exhibiting outflow tract misalignment (57). Expression of survivin is detected in embryonic mouse tissue, specifically in neural crest-derived cells (58).

In comparison, our examination of gross morphology indicates a lack of blood flow to peripheral blood vessels in Ara9−/− embryos. This may involve an inability to develop new vessels or maintain vascular integrity. The degeneration of the vascular system may have caused the petechiae and hemorrhaging as well as decreased blood flow in the head and limb and decreased vasculature seen in the yolk sac of some null embryos. The survivin protein has also been implicated in angiogenesis through a mediation of VEGF signaling (59).

The Role of ARA9 in Cancer Biology—While this work was in preparation, recent human genetics has revealed an important role for ARA9 in human pituitary cancer (60–63). In this regard, genetic association studies have shown that human populations that are haploinsufficient for the human AIP1 locus (the putative Ara9 orthologue) have a much higher incidence of pituitary adenomas (61, 64, 65). This observation lends support to the idea that the ARA9 protein is important in additional signaling pathways related to cell growth and development. Thus, mice harboring mutations at the Ara9 locus may provide valuable models of a number of important human diseases and developmental defects that extend beyond the range of heart aberrations and into the realm of cancer.

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