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 Pparg 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 been extensively studied 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 2 (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 immunophilin proteins 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 Pparg−/− 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 Ara9ACxneo. As seen in Fig. 1, the Ara9ACxneo 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.

Oligonucleotides—Oligonucleotides were synthesized by Integrated DNA Technologies (IDT, Coralville, IA). Oligonucleotides used in the study are as follows: OL 3163: 5’-GGC GTC GAT AAT ACT TCG TAT AGC ATA CAT TAT ACG AAG TTA TGA TGG CAT CAC TGC TGC C-3’; OL 3164: 5’-GGC GTC GAC ATA ACT TCG TAT AGT GTA TGC TAT ACG AAG TTA TGA TAT AGT AGT TCT GCA AAG CC-3’; OL 3165: 5’-GGC CCG CGG GAA GGC TGG TCA TCA CTG G-3’; OL 3166: 5’-GGC CCG CGG GAA CTT GAC ATC GTG GC-3’; OL 3161: 5’-GGG GTG ACC CAG GCA GAG AGT CTA GTG TG-3’; OL 3494: 5’-GGG GTC GAC CCC TCA CAG TAA AGC CCT C-3’; OL 4172: 5’-CC TTC GAG ACC ATG AGC GGC GCC AAC-3’; OL 4173 5’-CAG TCC GCC ATG GTG AGT CTT CA-3’.

Construction of the Ara92Cfxneo 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 ScaI 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 1,600-bp region homologous to the 3′-untranslated region adjacent to exon 6 of Ara9 was PCR-amplified using OL 3161 and 3164 to create the “long arm.” To confirm long arm ligation into pGemTeasy, the plasmid was digested with EcoRI to obtain 527-bp, 844-bp, and 3,916-bp bands. The 4,222-bp SalI fragment containing the long arm was then cloned into the pGemTeasy vector (Promega, Madison, WI), we digested the resultant plasmid with ScaI 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 ScaII fragment containing the short arm was cloned into the ScaII site of the targeting construct PL 1169 (pFrtLoxPNeo). The PL 1169 was previously described (16). To confirm ligation of the short arm into PL1169, the resultant plasmid was digested with PstI to obtain 197-bp, 2,142-bp, and 4,295-bp bands. The 4,222-bp SalI fragment containing the long arm was then cloned into the corresponding sites of the targeting vector containing the short arm (PL 1849). To confirm the long arm ligation into PL1169 already containing the short arm, we digested the plasmid with EcoRI to obtain 4,107-bp and 6,685-bp bands. A 1,761-kb Kpnl/Sall fragment containing the floxed region was then cloned into the Kpnl/Sall sites of the final targeting construct that was designated as PL2048 (i.e. Ara92Cfxneo). To confirm the final ligation of all homologous regions into PL1169, the plasmid was digested with BamHI to obtain 553-bp, 844-bp, and 11,165-bp bands.

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

Generation of Ara92Cfxneo/fxneo Mice—Approximately 10 µg of the targeting construct was electroporated into ES cells (Genome Systems). Selection was performed with 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 cells 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.

Genotyping—To confirm homologous recombination of the Ara92Cfxneo 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).

Animal Care—The Ara92Cfxneo 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 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 c1.5 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.

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, and 90% (v/v) 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.

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 NaHCO3, 81.1 mM MgSO4), 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).

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.** Ar9 Genomic DNA

| Gene | Targeting Vector | Homologously Recombined DNA |
|------|------------------|----------------------------|
| Ar9  | B X1 X2 X3 X4 X5 | B X1 X2 X3 X4 X5 |
| Ar9<sup><del>Cfx</del></sup> | | |
| Ar9<sup><del>Cfx</del></sup>neo | | |

**B.** Ar9<sup><del>Cfx</del></sup> Genotype

| Gene | Genotype | # Pups/Total Pups |
|------|----------|------------------|
| Ar9  | +/+      | 59/186           |
| Ar9<sup><del>Cfx</del></sup>/+ | 127/186        |
| Ar9<sup><del>Cfx</del></sup>/neo | 0/186           |

**C.** Ar9 Genotype

| Gene | Genotype | # Pups/Total Pups |
|------|----------|------------------|
| Ar9<sup><del>Cfx</del></sup> | +/+      | 22/54            |
| Ar9<sup><del>Cfx</del></sup>/+ | 32/54     |
| Ar9<sup><del>Cfx</del></sup>/fx | 0/54     |

**Results**

**Generation of Ara9<sup><del>Cfx</del>neo</sup> Heterozygous and Homozygous Mutant**—To create the Ara9<sup><del>Cfx</del>neo</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 Ara9<sup><del>Cfx</del>neo</sup>/fxneo 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 Ara9<sup><del>Cfx</del>neo</sup> allele, heterozygous littermates were interbred in an attempt to obtain homozygous Ara9<sup><del>Cfx</del>neo</sup>/fxneo 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 Ara9<sup><del>Cfx</del>neo</sup>/+ mice, and no homozygous Ara9<sup><del>Cfx</del>neo</sup>/fxneo 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, \( \chi^2 = 54.166 \)). Gross pathology and breeding studies of the heterozygous Ara9<sup><del>Cfx</del>neo</sup>/fxneo 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 Ara9<sup><del>Cfx</del></sup> mice. Germ-line transmission of the resultant Ara9<sup><del>Cfx</del></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 3.9 kb.
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+/Cfx/+ mice analyzed at 4 weeks of age, there were 22 wild-type mice Ara9+/+/, 32 heterozygous Ara9+/Cfx/+, and no homozygous Ara9/Cfx/Cfx 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/Cfx/neomox/neomox and Ara9/Cfx/Cfx 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 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 = 7.79). From a total of 64 embryos genotyped at e18–19, there were 24 wild-type (Ara9+/+), 38 heterozygous (Ara9+/−), and 2 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 ≤ 0.05, χ² = 13.93). The observation that our genotyping at e10–14 indicated a wild-type/heterozygote/homozygote frequency of 1:1.5:0.5 is in line with our observation that the first loss of Ara9−/− occurs before this developmental point. The 1:1.5:0.4 ratio falls to 1:1.6:0.3 by e15–17 and even further to 1:1.6:0.08 by e18–19 (Fig. 3).

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 littermates (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 littermates (Fig. 9A). At e18.5, some homozygous animals also presented with an open abdominal cavity (omphalocele) as well as hemorrhaging, and petechiae 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 Ppar^-/- 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 devel-

FIGURE 5. Phenotypic comparison of wild-type, heterozygous, and homozygous-null littermates at embryonic days 14.5–15, or 18.5–19. Wild-type Ara9^+/+(top), heterozygote, Ara9^+/-(middle), and homozygous-null Ara9^-/- (bottom). Null animals appear paler than counterparts and display a decreased caliber of vessels in the head (as indicated by red arrows), forelimbs, and hind limbs.

FIGURE 6. DORV in Ara9^-/- fetuses in comparison to wild-type littermate. Transverse histological samples of e14.5 fetuses visualized by hematoxylin and eosin staining. A, model of a normal heart. The figure was adapted from The Cove Point Foundation (Johns Hopkins University) (top left). Transverse serial sections (running left to right in sequence) of a wild-type heart with flow of blood from aorta to left ventricle and pulmonary artery to right ventricle (top middle). Arrows following the flow of blood are depicted for the first picture of every row. Penetration of DORV in wild-type animals (top right, B, VSD in Ara9^-/- fetuses in comparison to wild-type littermate. Model of a heart with DORV (bottom left). Transverse serial sections (running left to right in sequence) of a null heart with flow of blood from both aorta and pulmonary artery into the right ventricle. Arrows following the flow of blood are depicted for the first picture of every row (bottom middle). Penetration of DORV in homozygous-null animals (bottom). Legend: RA, right atria; LA, left atria; RV, right ventricle; LV, left ventricle; A, aorta; PA, pulmonary artery.
Abnormal septation is indicated by VSD (that extends beyond the realm of xenobiotic receptor signal with the idea that ARA9 plays roles in mammalian biology).

In keeping with our initial prediction, these data are consistent with the idea that ARA9 plays roles in mammalian biology that extends beyond the realm of xenobiotic receptor signal transduction. This conclusion is based on the observations that the Ara9^{-/-} allele presents with embryonic lethality and developmental problems including 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 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 to 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 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/Shape2 (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/survivinlox/lox embryo hearts 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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