A mouse model for hereditary hemorrhagic telangiectasia (HHT) type 2

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Hereditary hemorrhagic telangiectasia (HHT) is an autosomal-dominant disorder characterized by the age-dependent development of focal arteriovenous malformations and telangiectases. HHT type 2 is caused by loss of function mutations in activin receptor-like kinase 1 (ACVRL1 or ALK1). However, the factors that initiate lesion formation and those that influence disease progression remain unknown. Because heterozygous mice contain the appropriate genotype for an animal model of this disorder, mice heterozygous for a loss-of-function mutation in Acvrl1 were carefully examined for an HHT-like phenotype. These mice developed age-dependent vascular lesions in the skin, extremities, oral cavity and in the internal organs (lung, liver, intestine, spleen and brain), similar to those seen in HHT patients. Major histopathological features of the lesions included thin-walled dilated vessels in close proximity to each other, hemorrhage and fibrosis. Similar to HHT patients, the mice also exhibited gastrointestinal bleeding, as evidenced by positive fecal occult blood tests. An Acvrl1+/− mouse with profound liver involvement also displayed a secondary cardiac phenotype, similar to that observed in human patients. The similarity of affected organs, age-dependent penetrance, histological similarity of the lesions and recapitulation of a secondary phenotype suggest that the Acvrl1+/− mice are an appropriate animal model for the identification of additional genetic and environmental factors that cause pathology in HHT type 2 patients. In addition, studies utilizing this animal model can yield valuable information on the role of ALK1 in maintenance of adult vascular architecture including arteriovenous identity.

INTRODUCTION

Hereditary hemorrhagic telangiectasia (HHT) or Osler–Weber–Rendu disease is an autosomal dominant vascular disorder characterized by multisystemic focal vascular lesions that can lead to hemorrhage, stroke, high-output cardiac failure, and death (1,2). The hallmark vascular lesions of HHT are telangiectases and arteriovenous malformations (AVMs). Telangiectases consist of clusters of abnormally dilated thin-walled vessels, and are typically found in the skin and mucocutaneous tissues. Recurrent nosebleeds (epistaxis) from telangiectases in the nasal mucosa are among the earliest and the most commonly recognized symptoms of HHT in humans. An AVM is an abnormal connection between an artery and a vein, made either directly or through a tangle of blood vessels (nidus). In either case, the connection is devoid of intervening capillaries. These lesions are most commonly found in major internal organs, including the liver, lungs and brain of HHT patients.

Penetrance of HHT is age-dependent and nearly complete only by the fourth decade of life (3,4). The age of onset, number and location of lesions are highly variable, even among affected members of the same family. Based on etiology, the disease has been classified into two types. HHT type 1 (OMIM 18730) is caused by loss-of-function mutations in ENDOGLIN (5), whereas HHT type 2 (OMIM 600376) is caused by loss-of-function mutations in ACTIVIN RECEPTOR-LIKE KINASE 1 (ACVRL1 or ALK1) (6). Endoglin and ALK1 are both cell surface receptors for the TGF-β superfamily of ligands (7). ACVRL1, the gene mutated in HHT type 2, encodes a type I TGF-β serine–threonine kinase receptor that is expressed predominantly on endothelial cells (8–11). The range of

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ACVR1 mutations in HHT type 2 patients include nonsense and missense mutations, as well as insertions and deletions resulting in frameshifts (6,12–19). These mutations have been found throughout the ACVR1 coding region, including the extracellular ligand-binding, transmembrane, and the intracellular kinase domains. The nature and location of these mutations suggest that HHT type 2 results from loss of function for ACVR1.

Despite the identification of the genes mutated in HHT patients, questions pertaining to vascular lesion formation in HHT remain unaddressed. As ENDOGLIN and ACVR1 are expressed in all vascular endothelial cells, heterozygosity of the mutated allele results in the reduced expression of the functional protein throughout the vascular system. However, the vascular lesions do not usually appear congenitally but instead develop progressively with age. The age-dependent formation and focal nature of the vascular lesions suggest that critical factors initiate lesion development. Expressivity in HHT is also highly variable, even among family members who share the same mutant allele, suggesting that modifying factors play an important role in disease progression. The genetic and environmental factors that initiate lesion formation and those that influence the disease progression remain unidentified. Their discovery requires the investigation of animal models that recapitulate the disease pathology. Although an animal model for HHT type 1 was described recently (20), one for HHT type 2 has not yet been reported.

In the mouse, two inactivating mutations in the Acvrl1 gene have been generated by gene targeting. One mutation disrupts transcriptional and translational initiation (21), and the other disrupts exon 8 that encodes the kinase subdomain V of ALK1 (22). Embryos homozygous for either mutation die during mid-gestation (E10.5) and display widespread defects in vascular development, including dilation and fusion of arterial and venous capillaries (21,22). A phenotype in the Acvrl1 heterozygous mice has not been previously described. Because such mice possess the appropriate genotype for an animal model of this human autosomal dominant disorder, mice heterozygous for an Acvrl1 null mutation (22) were examined for a disease-like phenotype.

RESULTS

Forty-seven Acvrl1+/− mice (in a predominantly C57BL/6 background) and 47 wild-type littermate controls were allowed to live beyond 1 year to encourage the development and progression of an age-dependent HHT-like phenotype. A smaller number of mice were derived from crosses into other genetic backgrounds (Balb/C and 129/Ola). Live mice were visually inspected for external vascular lesions. During this time, nine Acvrl1+/− mice died spontaneously, or were euthanized due to morbidity. Eight wild-type controls also died spontaneously during the study. At the termination of the study, all living mice were sacrificed, at ages ranging from 12 to 21 months. All mice, including the wild-type controls, were examined for external lesions when alive, and inspected under a stereoscope for both external and internal vascular lesions after death. Evidence of vascular lesions and/or hemorrhage was observed in diverse anatomic locations in 19 Acvrl1+/− mice, six of which exhibited multiple vascular lesions. In contrast, no vascular lesions were detected in any of the wild-type mice (Table 1). Histological examination was carried out on tissue samples containing grossly visible lesions. The affected organs were similar to those seen in human HHT patients (Table 2).

Cutaneous lesions in Acvrl1+/− mice

Vascular lesions in the nailbed were frequently the first external manifestation of the disease phenotype in Acvrl1+/− mice. Discolored, cyanotic nailbeds and hemorrhage into claws were observed in 6/47 Acvrl1+/− mice (Fig. 1A). Claws that appeared bloody upon gross visual inspection were examined histologically. Numerous small vessels were present in abnormal locations, some of which had hemorrhaged. In the most extreme cases, these vessels had altered the adjacent bone, in contrast to unaffected toes (Fig. 1F, H-left, I). Even in the absence of hemorrhage, the pathological abundance of blood vessels in the nailbeds would be sufficient to result in a blood-filled appearance of the claws. In contrast, in unaffected nails, the periosteum and the connective tissue surrounding the bone were relatively thin and clearly demarcated from the bone (Fig. 1H-right).

Dilated vessels were also observed on the palmar surface of the forepaw of 3/47 Acvrl1+/− mice (Fig. 1C). An age-matched wild-type control is shown for comparison (Fig. 1D). The lesions were identified in similar anatomical locations to the telangiectases that are commonly found on the palms of human HHT patients (Fig. 1E). Abnormal vessel dilation was also observed on the external ear of 2/47 Acvrl1+/− mice. Telangiectases on the face and ear lobe are commonly observed in HHT patients (23).

Muco-cutaneous and oro-facial lesions in Acvrl1+/− mice

Upon gross examination, telangiectases were observed on the tongue (Fig. 2A) and histologically, adjacent to the maxilla

<table>
<thead>
<tr>
<th>Age in months</th>
<th>Sex</th>
<th>Location of vascular defects</th>
<th>Number of affected organs</th>
</tr>
</thead>
<tbody>
<tr>
<td>20.7</td>
<td>M</td>
<td>Liver</td>
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</tr>
<tr>
<td>20.6</td>
<td>F</td>
<td>Liver</td>
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<tr>
<td>17.7</td>
<td>M</td>
<td>Gun, nailbed</td>
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<tr>
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<td>M</td>
<td>Nailbed</td>
<td>1</td>
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<tr>
<td>16.0</td>
<td>F</td>
<td>Liver, palm</td>
<td>2</td>
</tr>
<tr>
<td>15.6</td>
<td>F</td>
<td>Intestine, palm</td>
<td>2</td>
</tr>
<tr>
<td>15.6</td>
<td>M</td>
<td>Intestine</td>
<td>1</td>
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<tr>
<td>15.6</td>
<td>M</td>
<td>Nailbed</td>
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<tr>
<td>15.6</td>
<td>F</td>
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<td>1</td>
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<td>14.9</td>
<td>M</td>
<td>Liver</td>
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</tr>
<tr>
<td>14.2</td>
<td>M</td>
<td>Liver, nailbed, palm, tongue, maxilla</td>
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</tr>
<tr>
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<td>F</td>
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<tr>
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<tr>
<td>7.0</td>
<td>M</td>
<td>Skin (ear)</td>
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</tr>
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Table 1. Phenotypes observed among Acvrl1+/− mice
Telangiectases are commonly found in the oral mucosa of HHT patients (Fig. 2C) (24,25). Severe inflammation of the gums was noted in two Acvrl1+/- mice, with one of these exhibiting dilated vessels and cyanosis of the gums.

Lesions in lung, spleen and brain

Although muco-cutaneous telangiectases are common among HHT patients, AVMs in the internal organs are often the most clinically relevant lesions. Vascular lesions were found in the lung, spleen and brain of certain Acvrl1+/- mice.
In an Acvrl1+/−/C0 mouse that exhibited loss of motor control, gross stereoscopic examination of the lungs revealed three hemorrhagic vascular foci (Fig. 3A). Such foci were not evident in any of the wild-type mice (Fig. 3B). HHT patients with pulmonary AVMs have an increased susceptibility to stroke, because the presence of a pulmonary AVM permits blood clots to travel to the brain without being filtered. It is unclear whether the pulmonary vascular phenotype in this Acvrl1+/−/C0 mouse contributed to its morbidity.

A splenic vascular lesion was identified in a 9.1-month old Acvrl1+/−/C0 mouse that died spontaneously. This mouse was of back-cross generation N1 in the 129/Ola background. Nodular splenomegaly had grossly distorted the morphology of the organ (Fig. 3C). Histological examination revealed alternating areas of hemorrhage and fibrosis indicating that recurrent hemorrhage and repair had occurred, in addition to the more recent massive hemorrhage which possibly contributed to the death of the animal (Fig. 3E). This is in sharp contrast to the normal arrangement of the erythroid and lymphoid regions that are readily visible at the same magnification in the spleen of a wild-type mouse (Fig. 3F). A grossly dilated blood vessel was also observed in the cerebellum of an Acvrl1+/−/C0 mouse of back-cross generation N1 in the BALB/c background (Fig. 3G).

**Hepatic vascular lesions**

Hepatic vascular lesions are frequently found in HHT patients, where their severity ranges from sub-clinical to life-threatening. Hepatic vascular lesions were observed in eight of the Acvrl1+/− mice. Focal pin-point hemorrhagic vascular lesions were found in the liver of four asymptomatic Acvrl1+/− mice (Fig. 4A), with hemorrhage into surrounding tissue grossly visible from the surface of the organ. Histological examination of one such lesion indicated the presence of multiple thin-walled dilated vessels around the lesion adjacent to an area of hemorrhage (not shown). The lesion was sub-capsular, and there was evidence of compression and atrophy of adjacent hepatocytes. Immunostaining for von Willebrand factor confirmed the lack of vascular integrity. Externally visible, grossly dilated sinusoidal spaces were found in three other asymptomatic Acvrl1+/− mice (Fig. 4B).

The most severe example of liver pathology was found in an Acvrl1+/− mouse that died spontaneously (age 14 months), where the liver displayed extreme enlargement, distortion and hemorrhage (Fig. 4C). The abnormal lobe contained an intact but abnormally large vessel, areas of hemorrhage, and connective tissue deposition, suggestive of cycles of chronic hemorrhage and repair (Fig. 4E). At higher magnification, multiple, large vessels of variable wall thickness were observed (Fig. 4G). Focally extensive areas with dilated sinusoidal spaces were also evident (Fig. 4I, wild-type for comparison in 4J). Even in an apparently normal lobe of liver from the same animal, abnormally closely positioned dilated vessels were present, with an apparent discontinuity of vessel walls in certain regions (not shown). These pathologies are consistent with those reported in human patients with hepatic AVMs (26–28).
GI tract bleeding in Acvrl1+/− mice

As hemorrhage from gastrointestinal lesions is a common feature of HHT, a rapid and non-invasive method was chosen to monitor the living mice for gastrointestinal hemorrhage. Acvrl1+/− mice (n = 41) and littermate controls (n = 29) of ages ranging from 7 to 17 months were monitored for GI bleeding for five consecutive days using a fecal occult blood test. One month later, the same mice were re-assayed in an identical manner. The Acvrl1+/− cohort showed a significantly greater percentage of total positive test results (53%) than the wild-type controls (21%; P < 0.001; Fig. 5A). On an individual basis, 98% of the heterozygous mice tested positive at least once during the test period, in contrast to 76% of the wild-type controls (P = 0.005). The high frequency of positive tests in the wild-type animals suggests that the assay is highly sensitive, but less specific for hemorrhage from putative gastrointestinal vascular lesions. Thus, a single positive test result cannot by itself be used to identify individual mice with internal hemorrhage caused by the Acvrl1 mutation. However, the two genotypes exhibited even greater differences (P < 0.001) in the number of days for which individual mice tested positive.

Figure 3. Vascular lesions in internal organs of Acvrl1+/− mice. (A–B) Lungs. (A) Multiple foci of hemorrhage were present in the lung of an Acvrl1+/− mouse, indicated by circles. (B) Wild-type lungs lacked such foci. (C–F) Spleen. (C) The spleen of an Acvrl1+/− mouse contained a multi-nodular discolored expansion at one pole of the spleen that roughly doubled the total splenic size, and demonstrated patches of hemorrhage (h). (D) A representative wild-type spleen is shown for comparison. (E) In a section of a region adjacent to the discolored region in the affected Acvrl1+/− spleen, a significant portion of the lesion is characterized by thrombosis (t), fibrous (f) connective tissue repair, and foci of active hemorrhage (h). (F) A section of wild-type spleen at the same magnification shows an orderly arrangement of the lymphoid (ly) and erythroid (e) regions absent in the spleen with lesion. (G–H) Cerebellum. (G) An abnormally dilated vessel was found in the cerebellum of an Acvrl1+/− mouse. (H) A corresponding region from a wild-type mouse in the same magnification lacks a dilated vessel.
Figure 4. Hepatic vascular lesions. (A) The liver of an Acvrl1+/− mouse contained a focal lesion (arrow) that hemorrhaged into surrounding tissue. (B) Sinusoidal capillaries were grossly dilated (circles) in the liver of an Acvrl1+/− mouse. (C) Hepatomegaly, nodularity, and hemorrhage (h) were evident in the abnormal liver lobe (arrow) of another Acvrl1+/− mouse. The other lobes of the liver appeared normal in this mouse, similar to those in a wild-type mouse (D). (E–J) Histopathology of the liver lesion shown in panel C. (E) A low magnification view (Masson trichrome stain) displayed an abnormally large intact vessel (v), and areas of fibrosis (f) and hemorrhage (h). (F) A wild-type liver at the same magnification lacks such features. (G) A higher magnification view demonstrated many abnormally large vessels, arranged juxtaposed to each other, with minimal intervening parenchyma. White arrows indicate relatively thick-walled regions, while black arrows indicate thin walls or apparent discontinuities in the vessel wall. (H) A wild-type liver at the same magnification lacked such vascular structures. (I) Dilated sinusoidal channels (white spaces) were present in the affected lobe. (J) A wild-type liver at the same magnification is shown for comparison.
The majority of the wild-type mice either tested negative, or tested positive only once or twice during the 10 days of testing. In contrast, the majority of the \textit{Acvrl1}\textsuperscript{+/−}/\textit{C0} mice tested positive for 4 or more days in the same period of time (Fig. 5B). Thus, the fecal occult blood test can be used as a screen for GI hemorrhage when used over a period of days.

At necropsy, five \textit{Acvrl1}\textsuperscript{+/−} mice showed visible evidence of intestinal hemorrhage (Fig. 5C), with three showing evidence of multiple sites of hemorrhage. These data on the \textit{Acvrl1}\textsuperscript{+/−} mice are consistent with the high frequency of lesions associated with GI hemorrhage in HHT patients (29,30).

### Secondary phenotypes in \textit{Acvrl1}\textsuperscript{+/−} mice

Because secondary cardiac pathology is commonly encountered in HHT patients with hepatic AVMs, the heart of the \textit{Acvrl1}\textsuperscript{+/−} mouse with the most severe liver pathology (Fig. 4C) was histologically examined. The heart exhibited numerous patches of fibrosis, deposits of connective tissue and foci of necrotic cardiomyocytes, consistent with multiple, recurrent ischemic events and repair (Fig. 6A). Cardiac blood vessels appeared normal in this animal. Cardiac lesions were absent in wild-type mice of comparable ages, as well as in \textit{Acvrl1}\textsuperscript{+/−} mice that lacked large visceral vascular lesions (Fig. 6B), consistent with a hepatic etiology for the secondary cardiac pathology.

### DISCUSSION

Although the genes for HHT types 1 and 2 have been identified, several important questions regarding the disease pathogenesis remain unanswered. The identification of factors that initiate vascular lesion formation, and those that modify disease progression, are among the most crucial. This study illustrates that \textit{Acvrl1}\textsuperscript{+/−} mice, the genetically appropriate animal model for HHT type 2, develop an HHT-like vascular pathology. Thus, these animals represent an important tool for the analysis of disease onset and progression.

HHT patients develop multiorgan vascular defects, with the nasal mucosa, skin, extremities, oral cavity, GI tract, liver, lung and brain being the most commonly affected sites. The \textit{Acvrl1}\textsuperscript{+/−} mice exhibited vascular lesions in the skin, oral cavity, liver, spleen, lung, intestine and brain. The first detectable external manifestation of vascular pathology in the majority of the \textit{Acvrl1}\textsuperscript{+/−} mice was hemorrhage into claws. This phenotype was also the most frequently observed among externally visible lesions. Telangiectases of the nailbed are not uncommon in HHT patients (31).

Histologically, the lesions found in the \textit{Acvrl1}\textsuperscript{+/−} mice consisted of ectatic or dilated vessels with normal or thin walls, in isolation or in clusters, in normal or abnormal locations. Some of these vessels had hemorrhaged. In a few cases, a collection of blood vessels was found in close proximity to each other. Such proximity might increase the potential for abnormal connections similar to that observed with human AVMs (32). In addition, there was organ enlargement and/or fibrosis associated with some of these lesions, also observed in HHT patients (33,34).

High-output cardiac failure is a secondary phenotype commonly encountered in HHT patients with hepatic AVMs (35–38), and in some cases is the first diagnosed symptom (39) or even the primary cause of lethality (40). Cardiac failure occurs due to a hyperdynamic circulatory state resulting from a hepatic arteriovenous shunt (33,41). The ischemic events of the...
heart in the Acvrl1+/− mouse with the enlarged liver may have resulted from anemia due to chronic liver hemorrhage or from high cardiac output due to a hepatic shunt. The extent of destruction of cardiomyocytes in a heart that contained normal vessel morphology suggests that Acvrl1+/− mice with large hepatic AVMs can develop secondary cardiac defects similar to HHT patients. Thus, the Acvrl1+/− mice may also be useful for the study of secondary defects seen in HHT patients.

By 12 months of age or greater, the Acvrl1+/− mice, in the predominately C57BL/6 background, exhibited a penetrance of 40% (19/47 mice) for at least one vascular lesion. Nonetheless, the true penetrance may be higher than this, as these estimates are dependent on the resolution of the methods used for screening. HHT patients are screened for internal lesions using a number of sophisticated imaging techniques including MRI, angio¬graphy, CT and endoscopy. These procedures can detect vascular lesions in a variety of organs, even when the lesion lies deep within the tissue of the organ. While these imaging procedures are becoming increasingly adapted for use with small animals such as mice (42,43), they are cost-prohibitive as a high-volume screening tool.

In contrast, these mutant mice were screened for internal lesions by gross visual inspection of organs upon death. Only organs that appeared grossly abnormal, or those that showed surface vascular lesions, were fixed for serial cross-sectioning and subsequent detailed histopathological examination. This strategy was too laborious and costly to perform for each organ of every mouse. Thus, many if not most of the animals may have harbored additional lesions in these same or other organs.

The need for novel screening modalities is not reserved for visceral lesions. The extent of muco-cutaneous involvement may also be underestimated in this model. Lesions in the oral cavity, very common and easily observed in human HHT patients, cannot be easily visualized on live mice except under anesthesia. These same lesions were more difficult to identify and photograph in dead animals, where the loss of systemic blood pressure caused the dilated vessels of the malformation to rapidly collapse. It is therefore clear that novel approaches to high volume screening will be necessary to plumb the depths of the phenotype in this model. The use of the fecal occult blood test to identify gastrointestinal hemorrhage represents one such example. Novel screening modalities that can be performed on live animals are required to identify age-dependent phenotypes involving the brain and visceral organs, as these are most clinically relevant for HHT.

Even with these caveats, the penetrance of this mutation in this animal model may indeed be much lower than the nearly complete penetrance observed in human HHT. Lower penetrance may be due to inherent anatomic differences between the species. The short life-span of the mice may also contribute to lower penetrance of many age-dependent phenotypes. The mice in a barrier facility may not be exposed to environmental factors that modulate the HHT phenotype, factors that humans routinely encounter. The controlled environment housing the mice allows for specific testing of non-genetic factors that may influence the phenotype. The contribution of environmental effects to the human disease would be much more difficult to determine.

Similarly, this mouse model can be used to identify additional genetic factors that modulate HHT pathogenesis. HHT-like phenotypes were observed in mice harboring the Acvrl1 mutation in a variety of mixed genetic backgrounds, suggesting that the model may be robust to the effects of genetic background. Strain-specific differences in phenotype, if observed, can be used to genetically map and identify genes that modulate the phenotype. Of particular value might be the identification of loci that modulate organ-specificity of the phenotype. For example, hepatic AVMs present one of the most serious complications of HHT. Liver lesions in HHT can range from pin-point telangiectases to nodular hyperplasia and pseudocirrhosis. Hepatic shunts can seriously compromise liver function or may result in secondary defects such as high-output cardiac failure, portal hypertension or portosystemic encephalopathy in HHT patients (27,33,44). The profound liver pathology observed in the some of the Acvrl1 heterozygous mice suggests that this model may be particularly well suited for the investigation of specific factors contributing to hepatic involvement in HHT. Hepatic involvement in the mice may be

Figure 6. Secondary cardiac phenotype in an Acvrl1+/− mouse. (A) A section of the heart (Masson trichrome stain) of the Acvrl1+/− mouse with a major liver lesion exhibited multiple areas of mature fibrosis replacing myocardium. These foci surrounded and extended from coronary vessels suggesting areas of myocardial ischemia and loss. Coronary blood vessels appeared normal in this heart. (B) Left ventricular myocardium from an age-matched wild-type mouse lacked this pathology.
related to the locus involved, as was suggested by the liver phenotype observed for some HHT type 2 families (19).

In summary, the phenotype observed in the Acvrl1 heterozygous mice strikingly resembles the human HHT phenotype in a number of characteristics, including age-dependent penetrance, specific anatomical locations of lesions, wide variation in severity, histological similarity of the lesions, and even the recapitulation of the secondary phenotype of cardiac pathology. Together, these data suggest that the Acvrl1+/− mice are an appropriate animal model of HHT2, and represent an effective tool to study the molecular mechanisms in the pathogenesis of HHT. This information will in turn provide the framework for the design of novel therapeutic approaches uniquely tailored to patients with HHT type 2. In addition, this animal model can yield valuable insights into the role of ALK1 in the maintenance of normal vascular function and arteriovenous identity.

MATERIALS AND METHODS

Mice

Mice heterozygous for the Acvrl1 mutation (Acvrl1+/−) (22) were bred and maintained in a barrier facility in micro-isolator cages with forced ventilation, and automatic watering. The mice were fed a standard diet (Rodent Diet 20, PicoLab) and maintained on a 14 h light cycle. Quarterly examination of sentinel mice indicated that the colony was free from common viral pathogens. All procedures were conducted in accordance with protocols approved by the Duke University Institutional Animal Care and Use Committee.

Mice were genotyped by PCR as previously described (22). Acvrl1+/− mice of a mixed genetic background (C57BL/6:129S1/SvImJ) were back-crossed with C57BL/6 mice for two generations. The founder mice (n = 10), and those that were born in the N1 (n = 23) and N2 (n = 14) back-cross generations were examined for expression of an HHT-like phenotype. Littermates served as wild-type controls. Asymptomatic mice were necropsied at ages ranging from 7 to 21 months, although some of mice died spontaneously or required euthanasia due to morbidity (Table 1). These deaths occurred over the course of the study and resulted in lower numbers of animals examined for the phenotypes measured in older mice.

Although the mice from C57BL/6 background comprised the main experimental group in this study, the Acvrl1 mutation was introgressed into two other inbred strains (Balb/C and 129/Ola) in a regular basis throughout the study for the presence of externally visible vascular lesions and hemorrhage. Mice were evaluated for hemorrhage from the GI tract using the fecal occult blood test (Hemoccult SENSA, Beckman Coulter, CA, USA) with slight modifications of the manufacturer’s instructions. The reaction time was extended to 2 min to allow a more complete color development and the reaction was read on the surface of the smeared strip. All Acvrl1+/− mice over the age of 7 months at the time of testing (n = 41; age range 7–17 months) and wild-type littermate controls (n = 29) were examined daily for five consecutive days. This procedure was repeated with the same mice 30 days later. The test reader was blinded to animal genotype. Data from the two sets were analyzed using Pearson’s χ2 test to test for significant phenotypic differences between the two genotypes.

Terminal examination of external and internal organs

Specimens were collected following spontaneous death or after euthanasia by carbon dioxide asphyxiation. External and internal organs were inspected grossly under a stereoscope (10× magnification) for indications of abnormal vasculature or hemorrhage. Brains were sliced into 2 mm coronal sections and visually inspected for gross internal vascular lesions. Tissues exhibiting vascular lesions or hemorrhage on gross inspection were fixed in neutral buffered formalin for 24 h, processed and embedded in paraffin. Osseous tissues, including paws (for digital nailbeds) and head (for turbinates and other cranial structures) were decalcified using Cal-Ex decalcifying solution (Fisher Scientific, NJ, USA) prior to embedding. Sections (5 μm) were prepared and stained with hematoxylin and eosin (H&E) according to standard procedures. Sections were examined for connective tissue and for evidence of fibrosis using Masson’s trichrome staining. Additionally, vessel walls were delineated using Verhoeff–van Gieson Masson’s stain, which detects the elastin fibers in the internal elastic lamina of arteries. Vascular endothelial cells were detected using immunoperoxidase staining with antisera to von Willebrand factor (rabbit anti-VWF 1:12,000, DAKO, USA). Histological examination was performed by a board-certified veterinary pathologist.

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