Potential Role of Modifier Genes Influencing Transforming Growth Factor- β 1 Levels in the Development of Vascular Defects in Endoglin Heterozygous Mice with Hereditary Hemorrhagic Telangiectasia

Annie Bourdeau,* Marie E. Faughnan,[†]
Merry-Lynn McDonald,* Andrew D. Paterson,[‡]
Ian R. Wanless,[§] Michelle Letarte*

From the Cancer and Blood Program,* The Hospital for Sick Children and Department of Immunology, University of Toronto, Toronto; the Division of Respiratory Medicine,[†] Department of Medicine, St. Michael's Hospital, University of Toronto, Toronto; The Centre for Applied Genomics,[‡] The Hospital for Sick Children and Department of Public Health Sciences, University of Toronto, Toronto; and the Department of Laboratory Medicine and Pathobiology,[§] University of Toronto Health Network, Toronto, Ontario, Canada

Hereditary hemorrhagic telangiectasia (HHT) is an autosomal dominant disorder because of mutations in the genes coding for endoglin (HHT1) or ALK-1 (HHT2). The disease is associated with haploinsufficiency and a murine model was obtained by engineering mice that express a single *Endoglin* allele. Of a total of 171 mice that were observed for 1 year, 50 developed clinical signs of HHT. Disease prevalence was high in 129/Ola strain (72%), intermediate in the intercrosses (36%), and low in C57BL/6 backcrosses (7%). Most mice first presented with an ear telangiectasia and/or recurrent external hemorrhage. One-third of mice with HHT showed severe vascular abnormalities such as dilated vessels, hemorrhages, liver and lung congestion, and/or brain and heart ischemia. Disease sequelae included stroke, hydrocephalus, fatal hemorrhage, and congestive heart failure. Thus the murine model reproduces the multiorgan manifestations of the human disease. Levels of circulating latent transforming growth factor (TGF)- β 1 were significantly lower in the 129/Ola than in the C57BL/6 strain. Intercrosses and 129/Ola mice expressing reduced endoglin also showed lower plasma TGF- β 1 levels than control. These data suggest that modifier genes involved in the regulation of TGF- β 1 expression act in combination with a single functional copy of endoglin in the development of HHT. (*Am J Pathol* 2001, 158:2011–2020)

Hereditary hemorrhagic telangiectasia (HHT), also known as Rendu-Osler-Weber syndrome, is an autosomal-dominant disorder that affects blood vessels and has a prevalence of $\sim 1:8000$.¹ HHT is primarily associated with frequent nosebleeds (epistaxis), telangiectases, and internal vascular lesions.² Patients can develop life-threatening complications such as severe gastrointestinal bleeding and arteriovenous malformations (AVM) (direct connection between a dilated venule and arteriole bypassing the capillary network) of the liver, lung, or brain. Shunting of blood through pulmonary or cerebral AVMs can lead to hypoxemia, stroke, brain abscess, heart failure, and fatal hemorrhage.³

Clinical manifestations of HHT are highly heterogeneous between families as well as within a given family. Genetic and epigenetic factors have been postulated to account for this diversity. Two genes are responsible for HHT, *ENG* (*ENDOGLIN*) mutated in HHT1⁴ and *ACVRL1* (*ACTIVIN RECEPTOR-LIKE KINASE 1*, also known as *ALK-1*), mutated in HHT2.⁵ HHT1 is associated with a higher incidence of pulmonary AVMs than HHT2, which generally has a later onset.⁶ Severity of HHT is not correlated with the type of mutation or its position.⁷ Mutated *ENG* is rarely expressed in HHT1 patients and only as an intracellular species.⁸ The current model for HHT1 is haploinsufficiency, because of reduced levels of functional endoglin at the surface of endothelial cells.^{8–11} Haploinsufficiency in *ALK-1* also seems to be associated with HHT2.¹²

Both endoglin and ALK-1 are components of the transforming growth factor- β (TGF- β) superfamily of receptors

Supported by grant no. NA3434 from the Heart and Stroke Foundation of Ontario, grant no. MT-6247 from the Canadian Institute of Health Research, and an industrial grant from Syn X Pharma Inc.

A. B. was a recipient of a Studentship from the Medical Research Council of Canada; M. E. F. is supported by the Nelson Arthur Hyland Foundation and the Squires Club of Toronto; M. L. is a Terry Fox Research Scientist of the National Cancer Institute of Canada.

Accepted for publication March 7, 2001.

Address reprint requests to Michelle Letarte, Cancer and Blood Program, The Hospital for Sick Children, 555 University Ave., Toronto, Ontario, M5G 1X8, Canada. E-mail: mabl@ SickKids.on.ca.

that are predominantly expressed on vascular endothelium. Endoglin cannot bind ligands of the TGF- β superfamily by itself. However it binds TGF- β 1, TGF- β 3, activin-A, and bone morphogenetic protein-7 and -2 via association with their respective ligand-binding receptors.¹³ Endoglin modulates several cellular responses to TGF- β 1 but its role in regulating effects of other ligands has yet to be demonstrated.^{14,15} ALK-1 is a type I receptor recently shown to bind TGF- β 1 in endothelial cells.¹⁶ Thus the receptor complex for TGF- β 1 on vascular endothelium contains endoglin associated with the ligand binding receptor, T β R-II, and signaling via the type I receptors ALK-1 or ALK-5.

Recently a crucial role for endoglin in angiogenesis was demonstrated in mice deficient in the *Endoglin* (*Eng*) gene, which showed multiple vascular and cardiac defects leading to death in early embryos.^{17–19} From embryonic day 9.0, the primitive vascular plexus of yolk sac failed to remodel into mature vessels causing vascular channel dilation, rupture, and hemorrhage. Internal bleeding was also observed in the embryo implying vessel fragility. Endocardial cushion formation, essential for valve development and heart septation did not occur. Pericardial edema was also observed.¹⁸ The yolk sac defects in *Endoglin* null embryos were similar to those observed in mice lacking *ALK-1*, *TGF- β 1*, *T β R-II*, and *Smad5*.^{16,20–22} We also observed that some *Endoglin* heterozygous mice (referred to as *End*^{+/-} mice) developed external signs of HHT such as telangiectases and bleeds.¹⁸

We now report the full characterization and validation of the murine model of HHT. Phenotypic heterogeneity, including severe visceral involvement, is described for 50 *End*^{+/-} mice with external signs of disease. We also demonstrate that the 129/Ola strain is more susceptible to HHT and has a lower level of plasma TGF- β 1 that is further reduced in *End*^{+/-} mice. Our data suggest that endoglin haploinsufficiency, combined with the effects of modifier genes that regulate TGF- β 1 expression, are responsible for the heterogeneity and severity of HHT.

Materials and Methods

Generation and Breeding of *End*^{+/-} Mice

End^{+/-} mice were generated by homologous recombination using embryonic stem cells of 129/Ola origin.¹⁸ Male chimeras were mated with wild-type 129/Ola (129) (Harlan UK, Bicester, UK) giving rise to 129 inbred progeny, homozygous at all alleles but *Endoglin*. Male chimeras were also mated with C57BL/6 (B6) females (Taconic Farms, Germantown, NY) yielding an F1 progeny, with a heterozygous B6/129 genome. The F1 were backcrossed to wild-type B6 mice, giving an N2 generation, with a 50% probability at any locus of being heterozygous (B6/129) or homozygous for B6 alleles. Intercrosses of *End*^{+/-} F1 mice yielded the F2 generation with at any particular locus a ratio of genotypes of 1:2:1 for 129 homozygous, B6/129 heterozygous, and B6 homozygous, respectively. Mice were kept in ventilated racks in a germ-free facility

and all protocols were approved by the Ethics Committee of the Hospital for Sick Children Laboratory Animal Services.

Genotyping of Mice

The genotype of each mouse was first assessed by β -galactosidase staining made possible by the presence of a *LacZ* reporter gene driven by the *Endoglin* promoter in the targeting construct.¹⁸ At weaning time (3 weeks), an ear punch from each pup was washed in phosphate-buffered saline, fixed in 0.25% glutaraldehyde for 15 minutes, washed three times for 5 minutes, stained overnight at 30°C in X-gal solution, and observed under a microscope. Blue vessels are associated with *End*^{+/-} mice because all *End*^{-/-} embryos die *in utero* at day 10 to 10.5. The *Endoglin* genotype was often confirmed by multiplex polymerase chain reaction using tail DNA.¹⁸

HHT Phenotype Assessment

End^{+/-} and *End*^{+/-} mice were observed daily and all signs of HHT were recorded in a FileMaker Pro data base. The age of onset of external signs was recorded: telangiectases (location, frequency/week), interstitial bleeding (location, severity, frequency/week), breathing capacity, mobility, weight loss, moribund state, and other manifestations such as ear or tail loss. Internal signs were also carefully examined during autopsy. Each mouse was dissected to evaluate dilation of inner skin vessels, interstitial bleeding and its origin. Organs including spleen, liver, intestine, kidneys, lungs, heart, and brain were analyzed for parameters such as size, color, edema, effusion, prominence of vessels, and presence of telangiectases and hemorrhages (focal or diffuse). Other severe consequences from hemorrhage such as hydrocephalus and stroke were also noted. Histological examination allowed to determine more precisely the site and extent of hemorrhage, number and size of vessels, organization of vessel wall components, ischemia, congestion, and infiltration.

Immunohistochemical Staining

Organs from *End*^{+/-} and control mice were immediately embedded in OCT and frozen on isopentane/dry ice. Cryosections (7 μ m) were fixed for 10 minutes in acetone, washed briefly in TBST (0.01 mol/L Tris, pH 7.4, 0.16 mol/L NaCl, 0.2% Tween 20), dipped 5 seconds in 0.1 N HCl to remove endogenous alkaline phosphatase and washed thoroughly in TBST. Sections were then blocked with 5% normal rabbit serum (DAKO, Mississauga, Ontario, Canada) for 20 minutes, blocked sequentially with avidin and biotin solution (Vector Laboratories, Burlington, Ontario, Canada) for 20 minutes, and washed. Sections were incubated at 4°C for 2 hours with optimal concentrations of primary antibodies. These were mAb JC7/18 to endoglin (CD105, purified IgG, 2 μ g/ml; Pharmingen, Mississauga, Ontario, Canada), mAb MEC13.3 to PECAM-1 (CD31, purified IgG, 5 μ g/ml;

Pharmingen), mAb 1A4 to α -smooth muscle cell actin (ascites, diluted 8000-fold; Sigma, Oakville, Ontario, Canada), and nonimmune rat IgG (5 μ g/ml, Sigma). Slides were washed and incubated for 1 hour at 4°C with biotinylated rabbit anti-rat IgG (diluted 400-fold, Vector Laboratories). For α -smooth-muscle cell actin detection, biotinylated polyclonal antibody from the LSAB kit was used (DAKO). The streptavidin-alkaline-phosphatase amplification system (StreptABC/AP, DAKO) was used and the enzymatic reaction was performed as described.¹¹ Some sections were counterstained with 5% neutral red (Sigma). Tissue morphology was assessed with both frozen and paraffin-embedded sections stained with hematoxylin and eosin and/or Masson's trichrome.

Measurement of Plasma TGF- β Levels

Blood was collected from both *End*^{+/-} and littermate controls *End*^{+/+} using heparinized hematocrit tubes by tail bleeds from live mice or at the time of autopsy. A total of 173 mice were analyzed: 129 backcrosses (*n* = 45), B6 backcrosses (*n* = 42), and intercrosses (*n* = 84). The plasma was recovered by centrifugation and kept at -70°C. Plasma was then diluted 1:200 and latent TGF- β was activated by acid treatment with 1 N HCl and lowering to pH 2 for 15 minutes. The samples were then neutralized to pH 7.6 with 1 N NaOH. The TGF- β levels were assessed by enzyme-linked immunosorbent assay using TGF- β 1 Emax ImmunoAssay system with the internal standard provided, following the manufacturer's instructions (Promega, Madison, WI). Two to four dilutions were done per sample, and all measurements were done in triplicate.

Statistical Analysis

Results of the plasma TGF- β levels were analyzed using the statistical software package, SPSS V.5. As the data did not show a normal distribution, the nonparametric Mann-Whitney test was used to compare TGF- β levels between groups. The data are reported as median plus the 25th and 75th percentile values. Group differences with *P* values < 0.05 were considered significant.

Results

End^{+/-} Mice Develop HHT

Mice with a single allele of the *Endoglin* gene were found to spontaneously develop clinical signs of HHT. To characterize onset, progression, and mechanism of disease, we studied 171 *End*^{+/-} mice for a minimum of 25 weeks and a maximum of 52 weeks. Mice who developed early onset of disease and died before 25 weeks were also included. A diagnosis of HHT was made in 50 mice based on their *End*^{+/-} genotype and at least one of these criteria: the presence of telangiectases on the ears, skin, tail, or genitals; external bleeds from nose/mouth, ears, tail, genitals, or intestine; and/or vascular abnormalities in viscera such as lungs, brain, liver, and intestine. In 90%

Mouse ID	Sex	Onset of HHT (weeks)	External signs		Internal signs				Others
			Telangiectases	Bleeds	Dilated vessels	Hemorrhage	Organomegaly	Intest	
129/Ola Backcrosses									
54.78	F	36							kidney artery rupture
55.5	M	13							
69.1	F	9							stroke, left atrial thrombus
95.1	M	35							severe breathing difficulty
107.4	M	7							
Intercrosses									
44.1	M	43							right atrial thrombus
46.7	M	43							
67.1	M	25							hydrocephalus, subgaleal hemorrhage
74.1	M	16							
75.2	M	16							left atrial thrombus
75.71	F	43							stroke, subarachnoid bleed
75.73	F	35							kidney hemorrhage, left atrial thrombus
76.10	M	17							severe breathing difficulty
77.5	M	4							subarachnoid bleed, hydrocephalus
91.10	M	10							paraplegia, subdural hemorrhage
93.3	M	9							descending aorta rupture
93.5	M	8							
111.3	M	2							subarachnoid bleed, hydrocephalus
111.11	M	8							kidney artery rupture
111.140	F	2							subarachnoid bleed, hydrocephalus
C57BL/6 Backcrosses									
62.12	M	37							descending aorta rupture
62.20	M	37							descending aorta rupture
Legend:									
	Ears		Ears		Liver				
	Skin		Nose/ mouth		Lungs				
	Genitals		Gastrointestinal		Heart				
	Tail		Tail/ genitals		Brain				

Figure 1. Autopsy findings in 22 *End*^{+/-} mice with HHT. The external and internal signs of HHT were systematically assessed. External signs such as telangiectases and bleeding were examined daily and internal signs were determined at autopsy and by histological analysis of liver, lungs, heart, and brain. Tissue sections stained with H&E, Masson's trichrome, or with antibodies to endoglin and PECAM-1 were examined. Various parameters were scored as present or absent. Additional important features concerning each animal are also noted.

of cases, telangiectases were the first signs of disease whereas 52% of HHT mice experienced external bleeds. Telangiectases on the ears, and bleeding from nose/mouth and ears were the most frequent external HHT signs in the *End*^{+/-} mice (Figure 1). Disease severity was highly variable; some mice had a mild phenotype whereas others (32%) reached an agonal phase because of rupture of major vessels that caused fatal internal hemorrhage (Figure 1).

Gastrointestinal Bleeds in *End*^{+/-} Mice with HHT

To examine visceral involvement, 22 of 50 *End*^{+/-} mice with life-threatening signs of HHT and/or at an advancing age were sacrificed. Direct microscopic examination of the mesenteric surface of the small intestine revealed a telangiectasia, seen as a network of dilated vessels, in an *End*^{+/-} HHT mouse but not in the *End*^{+/+} littermate control (Figure 2, a and b). Antibodies to endoglin and PECAM-1, specifically stained endothelial cells of arteries, veins, and capillaries in the submucosa in control and *End*^{+/-} HHT mice (Figure 2, c-f). Abnormally dilated arteries and veins were noted within the submucosa and serosa of the HHT mouse (Figure 2, d and f). Levels of expression of endoglin and PECAM-1 were similar in vessels of the control intestine (Figure 2, c and e). However, in the HHT mouse, endoglin-staining intensity was much reduced, compared to PECAM-1 (Figure 2, d and f). This lower level of endoglin on endothelial cells of *End*^{+/-} mice reflects the expression of a single allele. We randomly tested 20 of 50 HHT mice for fecal occult blood in their stools and found 11 positive ones including mouse 44.1 described in Figures 1 and 2. Although we

Figure 2. Intestinal telangiectases in HHT mice. **a:** Microscopic examination of small intestine mesenteric surface showing normal ramification of vessels in an *End*^{+/+} mouse. **b:** In the HHT mouse (44.1), a collection of abnormally dilated vessels forming a telangiectasia is shown (arrow). Cryosections were stained for endoglin (**c** and **d**) and PECAM-1 (**e** and **f**). **c:** Endoglin is expressed on vessels of the submucosa (sm) in the normal mouse. **d:** It is also seen on dilated vessels in the muscularis propria (mp) and the serosa (sr) in an *End*^{+/-} mouse. **e:** PECAM-1 serves as a positive control for endothelial cells in normal vessels. **f:** It is also expressed at normal levels on the dilated vessels of the *End*^{+/-} mouse. Scale bars: 1000 μ m (**a** and **b**), 200 μ m (**c-f**).

could not exclude that positive tests were caused by ingesting blood, there were no external bleeds observed in mouse 93.3 and three others with HHT (Figure 1 and

data not shown). These findings demonstrate the presence of fecal blood as early as 9 weeks and correlate with the presence of microscopic telangiectases on the intestinal surface. Hematocrit values were normal even in older mice with HHT, unlike in elderly human patients where intestinal bleeds lead to chronic anemia.²³

Liver Lesions in *End*^{+/-} Mice with HHT

On autopsy, gross morphology revealed that 48% of *End*^{+/-} HHT mice had liver abnormalities, including prominent vessels, telangiectases in one or several segments, focal or severe hemorrhage, and hepatomegaly (Figure 3a). However, histological examination revealed 80% of dissected mice had vessel enlargement, hepatic congestion, and/or hemorrhage (Figure 1). Endoglin was detected on endothelial cells of all types of vessels including central veins and sinusoidal endothelium in *End*^{+/+} control mice as illustrated in Figure 3b. Liver sections from an *End*^{+/-} HHT mouse showed dilated central veins when compared to control, and signs of hepatic congestion in a case of mild disease (Figure 3c). At lower magnification, a marked increase in the number of vessels was noticeable, especially in the subcapsular region in mild cases of disease (Figure 3e) compared to littermate controls (Figure 3d). As disease progressed, sinusoidal dilation and hepatocellular atrophy were seen near the central veins in severe cases of HHT (Figure 3f).

Figure 3. Dilatation of vessels and focal hemorrhage in the liver of HHT mice. On dissection, gross morphology was assessed (**a**) and cryosections of several lobes of liver were immunostained for endoglin (**b-f**). **a:** Liver of mouse 44.1 with HHT showing focal sites of hemorrhage (arrows) and dilated vessels (arrowheads). **b:** Liver of a control mouse showing endoglin expression on a vein and on normal sinusoidal endothelium. **c:** In an animal with mild disease (54.78), dilation of veins and congestion of the sinusoids disrupts the normal tissue organization. **d:** Edge of a liver lobe showing normal lobular architecture with small vessels. **e:** Liver of mouse 54.78 with congestion and increased number of vessels at the edge of a lobe. **f:** In a more severe case of HHT (91.10), more dilation of vessels, reduced endoglin staining and atrophy of hepatocytes are seen. Scale bars: 100 μ m (**b** and **c**), 500 μ m (**d-f**).

Figure 4. Pulmonary abnormalities in HHT mice. Gross morphology of several lungs was assessed (a–c) and tissue sections were stained with Masson's trichrome (d–g). **a:** Pink colored lung of a healthy mouse. **b:** Lung of HHT mouse 95.1 revealing a focal site of hemorrhage in the right upper lobe (arrow). **c:** Lung of HHT mouse 91.10 with telangiectases (arrow-heads) and severe hemorrhage. **d:** Littermate control lung showing size of normal vessels relative to bronchi. **e:** Dilated pulmonary arteries and veins in the lung of HHT mouse 75.73. **f:** Littermate control illustrating normal lung architecture at higher magnification. **g:** In mouse 75.73, congestion is noted by an increase in red blood cell number. Scale bars: 500 μ m (d and e), 100 μ m (f and g).

Vascular Abnormalities and Hemorrhage in Lungs of $End^{+/-}$ HHT Mice

Several $End^{+/-}$ HHT mice had difficulty breathing, manifested by tachypnea and marked inspiratory effort. Pulmonary involvement was seen, mostly on the ventral aspect of the upper lobes, by gross morphological examination in 33% of sacrificed HHT mice whereas microscopic analysis revealed abnormalities in 50% of cases (Figures 1 and 4). Gross changes included dilated vessels, visible telangiectases, and hemorrhages ranging in severity from focal to diffuse (Figure 4; a, b, and c). Histological sections demonstrated abnormally large vessels with increased thickness of the adventitial layer in lungs of $End^{+/-}$ HHT mice compared to littermate controls (Figure 4, d and e). At higher magnification, congestion of the alveolar capillaries was apparent (Figure 4, f and g). These findings suggest that pulmonary lesions tend to develop more often in the dependent areas of affected lungs, upper lobes in the case of mice and lower lobes in humans.

Cerebral Phenotype in $End^{+/-}$ HHT Mice

Most of the $End^{+/-}$ HHT mice first showed external signs of disease between 7 and 43 weeks, as described in

Figure 1. Animals 77.5, 111.3, and 111.140 were exceptions as they seemed unwell and developed cephalic changes at 2 to 4 weeks of age, before appearance of telangiectasia or external bleeds. These affected mice developed dome-shaped head, limb weakness, kyphosis, lethargy, drowsiness, and emaciation (Figure 5a). On cranial exposure, severe subarachnoid hemorrhage was found, accompanied by an expanded calvarium and underlying brain with hydrocephalus (Figure 5b). Bilateral enlargement of the ventricles with thinning of the cerebral cortex is shown on a cross-section stained with α -smooth muscle cell actin (Figure 5c). Cortical atrophy was likely because of necrosis or apoptosis resulting from elevated intracranial pressure. A higher magnification shows normal smooth muscle cell distribution and endoglin expression on small cerebral vessels (Figure 5, d and e). A fourth case, among the 22 dissected $End^{+/-}$ HHT mice, experienced a subdural hemorrhage followed by hydrocephalus (Figure 1).

Other brain manifestations were seen in 45% of $End^{+/-}$ HHT mice after histological examination (Figure 1). These included focal subdural, subgalial, or subarachnoid hemorrhage and brain infarction. Two HHT mice (95.1 and 75.71) had a stroke manifested by hemiplegia, facial flaccidity, and ptosis. Brain histology revealed a localized subarachnoid hemorrhage with blood infiltration into the cortex, not seen in an unaffected adjacent region (Figure 5, f and g). At higher magnification, lymphocytic infiltration and inflamed vessels with fibrinoid necrosis of the media are seen in the hemorrhagic area. A threefold thickening of adventitia and loosening of muscle bundles were also observed. The neuropile was vacuolated and early traces of infarction were seen (Figure 5h). These hemorrhagic strokes are reminiscent of neurological defects observed in human HHT with cerebral involvement.^{24,25}

Cardiac Changes in $End^{+/-}$ Mice with HHT

Pulmonary and hepatic congestion were observed concurrently in 9 of 22 $End^{+/-}$ HHT mice along with hypertrophy of the myocardium (50 to 500%) suggesting congestive heart failure (Figure 1). Heart sections demonstrated biventricular hypertrophy with dilatation especially of the left atria, and of the coronary arteries (Figure 6). Organizing thrombi such as shown in Figure 6b were seen in the atrium of four mice with HHT and could have caused embolic events to brain and coronary arteries. Ischemic regions associated with muscle necrosis were noted in three animals (Figure 6, a and b). Immunostaining of endoglin confirmed the vascular hypertrophy seen in some animals and revealed large dilated coronary vessels (Figure 6, c and d). Cardiac failure was likely secondary to the high output from the dilated hepatic and possibly pulmonary arterial circuits.

Our findings suggest two major causes of death in $End^{+/-}$ HHT mice: massive hemorrhage occurring after rupture of a major vessel, such as abdominal aorta, renal artery, and cerebral arteries, or chronic congestive heart failure, occurring as a result of high output failure be-

Figure 5. Cerebral lesions in mice with HHT. **a:** An $End^{+/-}$ mouse (111.140) showing signs of hydrocephalus. **b:** Massive hemorrhage within the subarachnoid space is revealed on lifting the skin over the cranium. **c:** Cryosection immunostained with α -smooth muscle cell actin shows severe bilateral dilatations of the ventricles with compression and atrophy of the surrounding brain. **d** and **e:** Higher magnification view showing normal expression of smooth muscle cell actin and endoglin in the cerebral vasculature. **f:** Formalin-fixed section of a normal brain region from HHT mouse 75.71 (with a stroke), stained with Masson's Trichrome adjacent to **(g)** an area with subarachnoid hemorrhage (**arrows**). **h:** Higher magnification shows the abnormal vessels with fibrinoid necrosis and lymphocytic infiltration in the lesion of this HHT mouse. Scale bars: 1000 μ m (**c**), 500 μ m (**d** and **e**), 200 μ m (**f** and **g**), 50 μ m (**h**).

cause of pulmonary, hepatic, or coronary vascular abnormalities.

TGF- β 1 Plasma Levels Influenced by 129 Background Genes

For analysis of phenotype/genotype correlations, the age of onset and the various HHT manifestations were recorded. Seventy-two percent of $End^{+/-}$ mice on the 129 background observed for 41.8 ± 12.6 weeks, and 36% of $End^{+/-}$ F2 intercrosses observed for 43.6 ± 8.9 weeks, developed HHT whereas only 7% of B6 backcrosses (N2) did when observed for an even significantly longer time of 48.7 ± 14.3 weeks ($P = 0.002$). The age of onset was highly variable. It ranged from 1 to 37 weeks in 129 mice, 14 to 43 weeks in F2 intercrosses, and 37 to 51 weeks in B6 backcrosses (Figure 7). These data indicate an earlier onset and higher susceptibility to HHT in 129 than B6

strain and intermediate age of onset and disease prevalence in F2 intercrosses, suggesting that the 129 background contributes some disease modifier alleles.

TGF- β 1 has multiple effects that include the regulation of its own production. Endoglin as a component of the receptor complex, is capable of modulating several responses to TGF- β 1.²⁵ We ascertained whether plasma TGF- β 1 levels were different in the two strains studied and whether endoglin reduction could further alter these levels. Initial studies revealed no detectable active TGF- β 1 in the plasma. We thus measured levels of total TGF- β 1 (latent plus active, after acid treatment) in plasma from 173 $End^{+/+}$ and $End^{+/-}$ mice of 129 strain and B6 background (backcrosses and intercrosses). As the distribution of TGF- β 1 in the various groups did not follow a normal distribution, the median and the 25th and 75th percentiles are shown for each group in Table 1. The nonparametric Mann-Whitney test revealed that TGF- β 1

Figure 6. Cardiac abnormalities in *End*^{+/-} mice with HHT. Tissue sections from *End*^{+/-} and *End*^{+/-} mice were stained with Masson's trichrome (**a** and **b**) and anti-endoglin (**c** and **d**). **a:** Normal heart from an *End*^{+/-} mouse. **b:** Abnormal heart from mouse 75.73, showing right and left ventricular hypertrophy, and left atrial enlargement with thrombus formation. **c:** Coronary vessels stained for endoglin in a normal littermate control. **d:** Dilation of coronary vessels in the heart of HHT mouse 75.2. Scale bars: 1000 μ m (**a** and **b**), 200 μ m (**c** and **d**).

plasma levels were significantly lower in *End*^{+/-} 129 than in both *End*^{+/-} B6 ($P = 0.025$) and *End*^{+/-} intercrosses ($P = 0.003$). This implies that the 129 mouse strain has lower circulating levels of TGF- β 1 than the B6 strain. This observation is further supported by comparing TGF- β 1 levels of *End*^{+/-} 129 mice with those of *End*^{+/-} B6 backcrosses ($P = 0.001$). Because the intercrosses have a mixed genome, a trend but no significant difference was achieved by comparing *End*^{+/-} 129 mice and *End*^{+/-} intercrosses ($P = 0.077$). Furthermore, expression of a single allele of *Endoglin* seems to lead to a further reduction in the level of circulating TGF- β 1 because *End*^{+/-} intercrosses had lower levels than *End*^{+/-} littermate controls ($P = 0.001$). These results indicate that the lower

Figure 7. Prevalence of HHT in *End*^{+/-} mice. *End*^{+/-} mice generated by backcrosses on 129 ($n = 18$), intercrosses of C57BL/6 (F2; $n = 33$) and backcrosses on C57BL/6 (N2; $n = 42$) were observed for a period of at least 25 weeks (except those dying of HHT before this period) and up to 52 weeks. The graph represents the age of onset of HHT based on external signs in each group of *End*^{+/-} mice. A two-sample test of proportion was done at 52 weeks and P values were significant: *, $P = 0.004$; **, $P = 0.0001$ compared to 129/Ola backcrosses.

level of plasma TGF- β 1 seen in 129 mice combined with the effect of reduced endoglin in *End*^{+/-} could contribute to the development of HHT.

Discussion

We have developed the first animal model of HHT. Mice with a single copy of the *Endoglin* gene can display a multiorgan vascular phenotype similar to the human disease supporting haploinsufficiency as the underlying cause of the disease. However, mice of the 129 strain developed HHT at a much younger age and with greater severity than the B6 mice whereas the F2 intercrosses showed an intermediate phenotype. This suggests that HHT is a complex disorder implying modifier genes. Levels of plasma TGF- β 1 were lower in the 129 strain than in the B6 strain and in *End*^{+/-} mice relative to *End*^{+/-} mice. These data suggest that modifier alleles, some of which are present in the 129 strain and involved in the regulation of TGF- β 1, contribute to the heterogeneity and severity of HHT.

The murine model of HHT reproduces the human disease. For example, cutaneous telangiectases are found in 90% of *End*^{+/-} mice with HHT (ears mostly) and present in 80 to 90% of people (nasal and labial mucosae, skin of face and hands).¹ It is more appropriate to compare visceral involvement in murine HHT to that of human HHT1, because pulmonary and cerebral AVMs are much more frequent in HHT1 than HHT2.^{6,10,26} Although AVMs could not be directly visualized, multiple abnormally dilated vessels along with focal and diffuse hemorrhage were found in the lungs of 50% of *End*^{+/-} mice with HHT, which is similar to that observed in HHT1 patients. Intracranial hemorrhage was seen in 30% of mice with HHT and occurred at 2 to 4 weeks of age in 3 of 22 cases, before any external sign of disease. Similarly, fatal hemorrhage because of rupture of a cerebral AVM has been reported in infants and newborns with HHT^{24,27} and confirmed in a newborn with HHT1.¹¹ Hydrocephalus was observed in 8% of mice with HHT but has only been reported in one human HHT case.²⁸ It likely occurred subsequent to the massive subarachnoid hemorrhage, as documented for several human cases of hydrocephalus.^{29,30} Liver abnormalities such as dilated vessels, congestion, and/or hemorrhage were seen in 80% of HHT mice, based on histological examination. Case reports of HHT have also described disseminated intrahepatic telangiectases, which might be present but remain asymptomatic in many patients. Serious liver complications, seen in 20% of HHT patients include heart failure, portal hypertension, biliary disease, ascites, and nodular transformation.³¹⁻³⁶ The murine model of HHT will help elucidating the mechanisms that lead to the initiation and progression of vascular abnormalities. A telangiectasia arises from the dilation of a postcapillary venule and direct fusion with an arteriole, bypassing the capillary network.³⁷ This implies that regulation of the normal angiogenic process of vessel branching is altered in HHT. The 50% reduction in endoglin observed in endothelial cells of all vessels of individuals with an *Endoglin*

Table 1. TGF- β 1 Plasma Levels in *End*^{+/-} Mice

Strain of mice	Number of mice	TGF- β 1 plasma levels		
		Median (ng/ml)	25th centile (ng/ml)	75th centile (ng/ml)
<i>End</i> ^{+/+} 129/Ola backcross	24	64	37	117
<i>End</i> ^{+/-} 129/Ola backcross	21	55	46	82
<i>End</i> ^{+/+} C57BL/6 backcross	16	99	74	155
<i>End</i> ^{+/-} C57BL/6 backcross	26	88	68	125
<i>End</i> ^{+/+} intercross	27	115	82	164
<i>End</i> ^{+/-} intercross	59	76	50	108

The Mann-Whitney test shows a significant difference between group 1 and both groups 3 ($p = 0.025$) and 5 ($P = 0.003$), between groups 2 and 4 ($P = 0.001$) and between groups 5 and 6 ($P = 0.001$).

mutation^{8,10,11} and in mice engineered to express a single allele of the gene must predispose vessels to dilation. However, additional genetic and environmental factors seem necessary to trigger the development of vascular abnormalities as suggested by their heterogeneity in human and mice with a single functional copy of endoglin.

Our observations that *End*^{+/-} mice on a 129 background develop disease whereas those on the B6 background do not, suggest that allelic variations between strains are responsible for the marked differences in vascular phenotype. To confirm the presence of a genetic modifier and exclude the influence of an unrecognized environmental factor, intercrosses of *End*^{+/-} heterozygous F1 offspring were examined. The F2 mice showed intermediate disease prevalence, consistent with the inheritance of modifier alleles from both strains. Thus a single *Endoglin* allele is necessary but not sufficient to cause disease, because a large proportion of *End*^{+/-} mice do not display clinical signs, when observed for 1 year. The 129 mice are high angiogenesis responders compared to B6 and other inbred strains of mice,³⁸ supporting the presence of genetic factors that control angiogenic potential and blood vessel homeostasis. In particular, the majority of wild-type 129/Ola mice were shown to have reduced numbers of peripheral vessels in liver and lungs and to exhibit natural large intrahepatic connections between portal and hepatic veins, when compared to B6 mice.^{39,40} This might in part also explain the high susceptibility of this strain to hepatic manifestations of HHT.

Lower levels of plasma TGF- β 1 observed in 129/Ola mice might be indicative of a vascular system susceptible to dilation. Circulating TGF- β 1 serves as a prognostic marker for several diseases. Elevated levels have been associated with the pathogenesis of chronic fibrotic and autoimmune diseases, atherosclerosis, and carcinogenesis, whereas deficient levels have been reported in stenosis of major coronary vessels.⁴¹ Our observations that *End*^{+/-} mice have reduced levels of plasma TGF- β 1 suggest that HHT is not only associated with propensity to vasodilation but also with less circulating TGF- β 1. Our preliminary data show that umbilical vein endothelial cells derived from newborns with an *Endoglin* mutation, produce significantly less TGF- β 1 than do normal ones (M Letarte, ML McDonald, S Vera, Hospital for Sick Children, Toronto, unpublished 2000). This suggests that endothelial cells contribute to the production of plasma TGF- β 1.

TGF- β 1 also acts by autocrine and paracrine mechanisms that are likely to play a role in the pathology of HHT. TGF- β 1 can stimulate the activity of its own promoter, and this autoregulation might explain the prolongation of secretion and autocrine action of TGF- β 1 after an initial stimulus.⁴² If endothelial cells from *End*^{+/-} mice secrete less TGF- β 1 than normal cells, we can propose that the level of endoglin controls this autoregulatory pathway. Less endoglin would then lead to reduced autocrine effects of TGF- β 1. This factor plays a crucial role in vascular homeostasis by regulating the synthesis of extracellular matrix proteins that stabilize interactions between endothelial, mesenchymal, and smooth muscle cells of the vessel wall.^{20,43} A decrease in both local and circulating TGF- β 1 levels, will lead to unstable cellular interactions in the vessel wall, dilated vessels, and vascular abnormalities. Such alterations could impair other angiogenic regulatory mechanisms and lead to deterioration of the vascular network associated with the progression of HHT. Reduced TGF- β 1 levels must play a role in vascular remodeling of cerebral and pulmonary human AVMs, that leads to extremely dilated and tortuous vessels with variable thickness of smooth muscle cells, disorganized adventitia, and active angiogenesis.¹¹ Such changes were also observed in mice at advanced stages of the disease. A dysregulation in the mechanisms responsible for maintaining interactions between intimal, medial, and adventitial layers of vessels is thus the likely cause of the progression and expansion of vascular lesions.

Our data demonstrate that one or more variable genetic loci in the mouse exert a profound modifying influence on the HHT phenotype. The variable expression within families also suggests an important role for genetic modifiers. Consistent with this hypothesis, we propose that the co-inheritance of mutated *Endoglin* and specific modifier alleles, predisposes to severe manifestations including the formation of cerebral, pulmonary, and hepatic AVMs. Factors such as increased blood volume and cardiac output combined with hormonal changes such as those observed during pregnancy could then precipitate the growth of AVMs.²⁶ In the absence of modifier genes, mild disease can still occur, and factors such as environment, blood pressure, oxygenation, and shear forces could influence the location of telangiectases.

Modifier alleles that modulate phenotypic outcome in a strain-dependent manner have been reported for several genes including those that control embryonic lethality in

the absence of TGF- β 1.⁴⁴ We can speculate that distinct modifier alleles might contribute to liver, lung, and brain involvement in HHT. Molecules implicated in vascular development and more specifically in the TGF- β pathway such as T β R-II, ALK-1, ALK-5, and downstream signaling Smads are candidates for genetic mutations or polymorphisms that could affect HHT1. ALK-1, the product of the gene mutated in HHT2, was shown recently to bind TGF- β 1 in endogenous endothelial cells and to be present in a receptor complex in association with T β R-II and endoglin.¹⁶ A model was proposed whereby normal angiogenesis would require a balance between activation by TGF- β 1 of ALK-1- and ALK-5-signaling pathways with their respective Smads.¹⁶ Endoglin, which can be present in either the ALK-1 or ALK-5 receptor complex, could thus modulate endothelial cell responses to TGF- β 1 via both pathways. Thus modifier genes contributing to the pathophysiology of HHT may code for molecules that participate in the TGF- β receptor complex or downstream molecules activated on signaling through this complex.

Murine HHT mimics the human disease and provides an excellent model for studying the mechanisms responsible for initiation and progression of this complex vascular disorder. We have demonstrated that endoglin is essential for maintenance of vascular homeostasis as expression of a single allele can lead to abnormal vessels. Our results clearly show genetic variation in susceptibility to disease and suggest that characterization of the murine modifier genes could lead to the identification of susceptibility alleles influencing severity of HHT in human.

Acknowledgments

We thank S. Kaw for technical assistance; L. Morikawa for invaluable help in tissue section preparation; the staff of the Lab Animal Services at the Hospital for Sick Children who helped in the daily assessment of the murine HHT phenotype; and Dr. M.G. Sirois for allowing use of microscopes and computers for data analysis.

References

1. Plauchu H, de Chadarevian JP, Bideau A, Robert J-M: Age-related clinical profile of hereditary hemorrhagic telangiectasia in an epidemiologically recruited population. *Am J Med Genet* 1989, 32:291-297
2. Shovlin CL, Guttmacher AE, Buscarini E, Faughnan ME, Hyland RH, Westermann CJ, Kjeldsen AD, Plauchu H: Diagnostic criteria for hereditary hemorrhagic telangiectasia (Rendu-Osler-Weber syndrome). *Am J Med Genet* 2000, 91:66-67
3. Shovlin CL, Letarte M: Hereditary haemorrhagic telangiectasia and pulmonary arteriovenous malformations: issues in clinical management and review of pathogenic mechanisms. *Thorax* 1999, 54:714-729
4. McAllister KA, Grogg KM, Johnson DW, Gallione CJ, Baldwin MA, Jackson CE, Helmbold EA, Markel DS, McKinnon WC, Murrell J, McCormick MK, Pericak-Vance MA, Heutink P, Oostra BA, Haitjema T, Westerman CJJ, Porteous ME, Guttmacher AE, Letarte M, Marchuk DA: Endoglin, a TGF- β binding protein of endothelial cells, is the gene for hereditary haemorrhagic telangiectasia type 1. *Nat Genet* 1994, 8:345-351
5. Johnson DW, Berg JN, Baldwin MA, Gallione CJ, Marondel I, Yoon SJ, Stenzel TT, Speer M, Pericak-Vance MA, Diamond A, Guttmacher AE, Jackson CE, Attisano L, Kucherlapati R, Porteous ME, Marchuk DA: Mutations in the activin receptor-like kinase 1 gene in hereditary haemorrhagic telangiectasia type 2. *Nat Genet* 1996, 13:189-195
6. Berg JN, Guttmacher AE, Marchuk DA, Porteous ME: Clinical heterogeneity in hereditary haemorrhagic telangiectasia: are pulmonary arteriovenous malformations more common in families linked to endoglin? *J Med Genet* 1996, 33:256-257
7. Shovlin CL, Hughes JM, Scott J, Seidman CE, Seidman JG: Characterization of endoglin and identification of novel mutations in hereditary hemorrhagic telangiectasia. *Am J Hum Genet* 1997, 61:68-79
8. Pece N, Vera S, Cymerman U, White Jr RI, Wrana JL, Letarte M: Mutant endoglin in hereditary hemorrhagic telangiectasia type 1 is transiently expressed intracellularly and is not a dominant negative. *J Clin Invest* 1997, 100:2568-2579
9. Pece-Barbara N, Cymerman U, Vera S, Marchuk DA, Letarte M: Expression analysis of four endoglin missense mutations suggests that haploinsufficiency is the predominant mechanism for hereditary hemorrhagic telangiectasia type 1. *Hum Mol Genet* 1999, 8:2171-2181
10. Cymerman U, Vera S, Pece-Barbara N, Bourdeau A, White Jr RI, Dunn J, Letarte M: Identification of hereditary hemorrhagic telangiectasia type 1 in newborns by protein expression and mutation analysis of endoglin. *Pediatr Res* 2000, 47:24-35
11. Bourdeau A, Cymerman U, Paquet ME, Meschino W, McKinnon WC, Guttmacher AE, Becker L, Letarte M: Endoglin expression is reduced in normal vessels but still detectable in arteriovenous malformations of patients with hereditary hemorrhagic telangiectasia type 1. *Am J Pathol* 2000, 156:911-923
12. Abdalla SA, Pece-Barbara N, Vera S, Tapia E, Paez E, Bernabeu C, Letarte M: Analysis of ALK-1 and endoglin in newborns from families with hereditary hemorrhagic telangiectasia type 2. *Hum Mol Genet* 2000, 9:1227-1237
13. Barbara NP, Wrana JL, Letarte M: Endoglin is an accessory protein that interacts with the signaling receptor complex of multiple members of the transforming growth factor- β superfamily. *J Biol Chem* 1999, 274:584-594
14. Lastres P, Letamendia A, Zhang H, Rius C, Almendro N, Raab U, López LA, Langa C, Fabra A, Letarte M, Bernabéu C: Endoglin modulates cellular responses to TGF- β 1. *J Cell Biol* 1996, 133:1109-1121
15. Guerrero-Esteo M, Lastres P, Letamendia A, Perez-Alvarez MJ, Langa C, Lopez LA, Fabra A, Garcia-Pardo A, Vera S, Letarte M, Bernabeu C: Endoglin overexpression modulates cellular morphology, migration, and adhesion of mouse fibroblasts. *Eur J Cell Biol* 1999, 78:614-623
16. Oh SP, Seki T, Goss KA, Imamura T, Yi Y, Donahoe PK, Li L, Miyazono K, ten Dijke P, Kim S, Li E: Activin receptor-like kinase 1 modulates transforming growth factor-beta 1 signaling in the regulation of angiogenesis. *Proc Natl Acad Sci USA* 2000, 97:2626-2631
17. Li DY, Sorensen LK, Brooke BS, Urness LD, Davis EC, Taylor DG, Boak BB, Wendel DP: Defective angiogenesis in mice lacking endoglin. *Science* 1999, 284:1534-1537
18. Bourdeau A, Dumont DJ, Letarte M: A murine model of hereditary hemorrhagic telangiectasia. *J Clin Invest* 1999, 104:1343-1351
19. Arthur HM, Ure J, Smith AJ, Renforth G, Wilson DI, Torsney E, Charlton R, Parums DV, Jowett T, Marchuk DA, Burn J, Diamond AG: Endoglin, an ancillary TGF β receptor, is required for extraembryonic angiogenesis and plays a key role in heart development. *Dev Biol* 2000, 217:42-53
20. Dickson MC, Martin JS, Cousins FM, Kulkarni AB, Karlsson S, Akhurst RJ: Defective haematopoiesis and vasculogenesis in transforming growth factor- β 1 knock out mice. *Development* 1995, 121:1845-1854
21. Oshima M, Oshima H, Taketo MM: TGF- β receptor type II deficiency results in defects of yolk sac hematopoiesis and vasculogenesis. *Dev Biol* 1996, 179:297-302
22. Chang H, Huylebroeck D, Verschueren K, Guo Q, Matzuk MM, Zwijsen A: Smad5 knockout mice die at mid-gestation due to multiple embryonic and extraembryonic defects. *Development* 1999, 126:1631-1642
23. Vase P, Grove O: Gastrointestinal lesions in hereditary hemorrhagic telangiectasia. *Gastroenterology* 1986, 91:1079-1083
24. Kikuchi K, Kowada M, Sasajima H: Vascular malformations of the brain in hereditary hemorrhagic telangiectasia (Rendu-Osler-Weber disease). *Surg Neurol* 1994, 41:374-380

25. Kadoya C, Momota Y, Ikegami Y, Urasaki E, Wada S, Yokota A: Central nervous system arteriovenous malformations with hereditary hemorrhagic telangiectasia: report of a family with three cases. *Surg Neurol* 1994, 42:234–239
26. Shovlin CL, Winstock AR, Peters AM, Jackson JE, Hughes JM: Medical complications of pregnancy in hereditary haemorrhagic telangiectasia. *Q J Med* 1995, 88:879–887
27. Roy C, Noseda G, Arzimanoglou A, Harpey JP, Binet MH, Vaur C, Caille B: Rendu-Osler disease revealed by rupture of a cerebral arterial aneurysm in a newborn. *Arch Fr Pediatr* 1990, 47:741–742
28. Quickel Jr KE, Whaley RJ: Subarachnoid hemorrhage in a patient with hereditary hemorrhagic telangiectasia. *Neurology* 1967, 17:716–719
29. Sheehan JP, Polin RS, Sheehan JM, Baskaya MK, Kassell NF: Factors associated with hydrocephalus after aneurysmal subarachnoid hemorrhage. *Neurosurgery* 1999, 45:1120–1127
30. Levy EI, Scarrow AM, Firlik AD, Kanal E, Rubin G, Kirby L, Yonas H: Development of obstructive hydrocephalus with lumboperitoneal shunting following subarachnoid hemorrhage. *Clin Neurol Neurosurg* 1999, 101:79–85
31. Martini GA: The liver in hereditary haemorrhagic teleangiectasia: an inborn error of vascular structure with multiple manifestations: a reappraisal. *Gut* 1978, 19:531–537
32. Buscarini E, Buscarini L, Danesino C, Piantanida M, Civardi G, Quaretti P, Rossi S, Di Stasi M, Silva M: Hepatic vascular malformations in hereditary hemorrhagic telangiectasia: doppler sonographic screening in a large family. *J Hepatol* 1997, 26:111–118
33. Weik C, Greiner L: The liver in hereditary hemorrhagic telangiectasia (Weber-Rendu-Osler disease). *Scand J Gastroenterol* 1999, 34:1241–1246
34. Bjørø K, Schrumpf E, Elgjo K, Kolmanskog F: Monstrous ascites in hereditary haemorrhagic telangiectasia. *Scand J Gastroenterol* 1995, 30:92–94
35. Wanless IR, Gryfe A: Nodular transformation of the liver in hereditary hemorrhagic telangiectasia. *Arch Pathol Lab Med* 1986, 110:331–335
36. Garcia-Tsao G, Korzenik JR, Young L, Henderson KJ, Jain D, Byrd B, Pollak JS, White Jr RI: Liver disease in patients with hereditary hemorrhagic telangiectasia. *N Engl J Med* 2000, 343:931–936
37. Braverman IM, Keh A, Jacobson BS: Ultrastructure and three-dimensional organization of the telangiectases of hereditary hemorrhagic telangiectasia. *J Invest Dermatol* 1990, 95:422–427
38. Rohan RM, Fernandez A, Udagawa T, Yuan J, D'Amato RJ: Genetic heterogeneity of angiogenesis in mice. *FASEB J* 2000, 14:871–876
39. Coulson PS, Wilson RA: Portal shunting and resistance to *Schistosoma mansoni* in 129 strain mice. *Parasitology* 1989, 99:383–389
40. Elsaghier AA, McLaren DJ: *Schistosoma mansoni*: evidence that vascular abnormalities correlate with the 'non-permissive' trait in 129/Ola mice. *Parasitology* 1989, 99:377–381
41. Roberts AB: Molecular and cell biology of TGF- β . *Miner Electrolyte Metab* 1998, 24:111–119
42. Kim SJ, Angel P, Lafyatis R, Hattori K, Kim KY, Sporn MB, Karin M, Roberts AB: Autoinduction of transforming growth factor beta 1 is mediated by the AP-1 complex. *Mol Cell Biol* 1990, 10:1492–1497
43. Folkman J, D'Amore PA: Blood vessel formation: what is its molecular basis? *Cell* 1996, 87:1153–1155
44. Bonyadi M, Rusholme SA, Cousins FM, Su HC, Biron CA, Farrell M, Akhurst RJ: Mapping of a major genetic modifier of embryonic lethality in TGF β 1 knockout mice. *Nat Genet* 1997, 15:207–211