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Haploinsufficiency of the murine *Col3a1* locus causes aortic dissection: a novel model of the vascular type of Ehlers–Danlos syndrome

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Aims The vascular type of Ehlers–Danlos syndrome (EDS IV) is an autosomal-dominant disorder characterized by thin translucent skin and extensive bruising. Patients with EDS IV have reduced life expectancy (median 45–50 years) due to spontaneous rupture of arteries (particularly large arteries) or bowel. EDS IV results from mutation of the *COL3A1* gene, which encodes the pro-α_1 chains of type III collagen that is secreted into the extracellular matrix, e.g. by smooth muscle cells. A mouse model of EDS IV produced by targeted ablation of *Col3a1* has been of limited use as only 10% of homozygous animals survive to adulthood, whereas heterozygous animals do not die from arterial rupture. We report a novel, exploitable model of EDS IV in a spontaneously generated mouse line.

Methods and results Mice were identified by predisposition to sudden, unexpected death from dissection of the thoracic aorta. Aortic dissection inheritance was autosomal-dominant, presented at an early age (median, 6 weeks) with incomplete penetrance, and had a similar sex ratio bias as EDS IV (2:1, male:female). Molecular genetic analysis demonstrated that the causal mutation is a spontaneous 185 kb deletion, including the promoter region and exons 1–39, of the *Col3a1* gene. As in EDS IV, aortic dissection was not associated with elevated blood pressure, aneurysm formation, or infection, but may result from aberrant collagen fibrillogenesis within the aortic wall.

Conclusion This novel, exploitable mouse line that faithfully models the vascular aspects of human EDS IV provides an important new tool for advancing understanding of EDS IV and of aortic dissection in general.

Keywords Vascular type of Ehlers–Danlos syndrome • COL3A1 • Mouse

1. Introduction

The vascular type of Ehlers–Danlos syndrome (EDS IV) is an autosomal-dominant connective tissue disorder associated with mutations in the gene encoding the pro-α_1 chains of type III collagen (*COL3A1*). COL3A1 is widely distributed in the skin, blood vessels, and ligaments.[1–3] In particular, type III collagen synthesized by aortic smooth muscle cells[4] is the predominant form of collagen in the aortic media.[5] EDS IV is characterized by hyper-mobility of the joints, fragile, rupture-prone blood vessels, and bruising. Life expectancy is low (<50 years), with death caused by spontaneous rupture of bowel or arteries (particularly large arteries).[6] There are no

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current treatments, with management restricted to surveillance, avoidance of risk, and surgical treatment of complications.

Pre-clinical investigation of EDS IV has been hampered by the lack of suitable animal models. A previous attempt to generate such a model through gene-targeted ablation of *Col3a1* was of limited success, despite homozygous mutant animals displaying both bruising and aortic dissection.[7] This model was difficult to exploit because heterozygous mice (with a 50% reduction in tissue collagen III) were not subject to death from arterial rupture, whereas homozygotes had an average survival rate of 5% at weaning age with most dying within 48 h of birth. The unsuitability of this mutant contrasts with a mouse model of osteogenesis imperfecta, a condition caused by mutations in genes (*COL1A1* or *COL1A2*) encoding collagen type I which, in humans, is associated with bone fractures. Homozygous mice with a mutated allele of the first intron of the *Col1a1* gene are predisposed to aortic rupture in adulthood.[8] The development of this model has made it possible to investigate the role of risk factors, including aneurysm formation,[9] in the small number of patients with osteogenesis imperfecta that die from rupture of major blood vessels.[10,11]

The availability of a usable model of EDS IV would provide an important resource in clarifying the mechanism underlying aortic rupture and determining the factors that contribute to dissection. Here, we describe a novel, exploitable model of EDS IV that arose serendipitously during an unrelated gene-targeting study.[12]

2. Methods

2.1 Generation of animals for genetic mapping

Sudden, unexpected death without prior morbidity was observed in two independent mouse colonies (on a mixed 129Ola:C57BL/6J background) derived from a single aliquot of a 129Ola-derived embryonic stem (ES) cell line during an unrelated gene-targeting experiment focused on the role of the *Mro* gene in sexual development.[12] Animals were genotyped as previously described[12] and inheritance of wild-type *Mro* alleles was confirmed in all cases. Post-mortem examination established that death resulted from thoracic aortic dissection. Animals ($n = 22$) displaying sudden death with haemothorax and histological confirmation of aortic dissection were entered for a single-nucleotide polymorphism (SNP)-based, low-resolution genome scan using Pyrosequencing (Qiagen) to identify a region of 129Ola-derived DNA common to all affected animals. This was followed by high-resolution SNP mapping to refine the size of the critical region on chromosome 1. Finally, DNA sequencing of PCR amplicons surrounding an SNP, shown to be deleted in affected animals, was undertaken to define the exact limits of the deletion and clone the deletion breakpoints. Details of primers/SNPs used for genome-wide scan are available on request.

2.2 Breeding of transgenic mice

All experiments utilized mice derived from an enclosed colony maintained through heterozygote–heterozygote intercrosses following two generations of backcrossing to C57BL/6J (thus 25% 129Ola:75% C57BL/6J). Genotyping was exploited to completely exclude targeted *Mro* alleles from the colony in backcross generation one. Heterozygotes (+/*Col3a1*$^{\triangle}$) were interbred to produce a population of WT (+/+), heterozygous (+/*Col3a1*$^{\triangle}$), and homozygous (*Col3a1*$^{\triangle}$/*Col3a1*$^{\triangle}$) mice in the ratio of 1:2:0. Sex ratios were Mendelian. All animals estimated to have died no more than 12 h previously were autopsied, and tissue samples were taken for analysis. All mice were bred under standard conditions of care and used under licensed approval from the UK Home Office (30/2381: A.G.; 30/2253: L.B.S., and 60/3867: P.W.F.H.). The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.3 Genotyping

Mice carrying the deletion were identified by genotyping from genomic DNA collected during ear marking at weaning (3 weeks); initially using Quantitative PCR (Q-PCR) employing an ABI 7900 Sequence Detection System and the Roche Universal Probe Library (Roche, Welwyn, UK) according to the manufacturer's instructions. Primer sets for *Col3α1* were designed for quantification of DNA within the deleted region. Q-PCR identified +/*Col3a1*$^{\triangle}$ carriers by the detection of a 50% reduction in amplification product when compared with +/+ littermates (normalized to mouse *gapdh*). Following cloning of the deletion breakpoint, genotyping was carried out through PCR amplification from genomic DNA using primers (see Supplementary material online, Methods) which amplify a 174 bp product spanning the deletion breakpoint in +/*Col3a1*$^{\triangle}$ carriers. PCR amplification of a 1.3 kb amplicon between exons 4 and 5 of *Col3α1* was used to confirm inheritance of the wild-type allele. Genomic alignments and base-pair numbering relate to Build NCBI m37, Ensembl release 59.37 L of the mouse genome.

2.4 Post-mortem examination

Detailed necropsies were performed on 14-week-old +/*Col3a1*$^{\triangle}$ male ($n = 4$) and female ($n = 4$) and age- and genetic background-matched wild-type male ($n = 4$) and female ($n = 3$) mice (see Supplementary material online, methods).

2.5 Electron microscopy

Aortas from 4-week +/*Col3a1*$^{\triangle}$ ($n = 3$) and age-matched +/+ mice ($n = 3$) were prepared for transmission electron microscopy as described.[13] Sections (1 μm) were stained with Toluidine Blue to allow selection of suitable areas for investigation. Ultrathin (60 nm) sections from selected areas were stained with uranyl acetate and lead citrate and then viewed in a Phillips CM120 Transmission electron microscope (FEI UK Ltd, Cambridge, England).

2.6 Picrosirius Red staining

Collagen content of the aortic wall was assessed by staining 5 μm sections of the thoracic aorta from 4- to 5-week-old +/*Col3a1*$^{\triangle}$ ($n = 7$) and +/+ ($n = 5$) mice with Picrosirius Red.[14] Digital images obtained from light microscopy were analysed using Image J software (National Institute of Mental Health, Bethesda, MD, USA). Collagen content was calculated as a percentage of the total medial area. Analysis was performed by a single individual (E.M.) blinded to genotype.

2.7 High-resolution ultrasound

Using a Vevo 770 imaging system with a 704 probe (VisualSonics, Toronta, Canada), ultrasound analysis was performed in anaesthetized +/*Col3a1*$^{\triangle}$ and +/+ mice (4 males:4 females of each genotype) at 4 and 12 weeks of age. Scans were performed to analyse cardiac structure and function, and aortic structure and blood flow[15] (see Supplementary material online, methods).

2.8 Systolic blood pressure measurements

Systolic blood pressure was measured by tail-cuff plethysmography in conscious, restrained mice from 4 weeks of age.[16]

2.9 Myography

The descending aorta from 14-week +/*Col3a1*$^{\triangle}$ (3 males:3 females) and +/+ mice (2 males:3 females) was isolated for functional assessment *in vitro*, as described previously.[17]

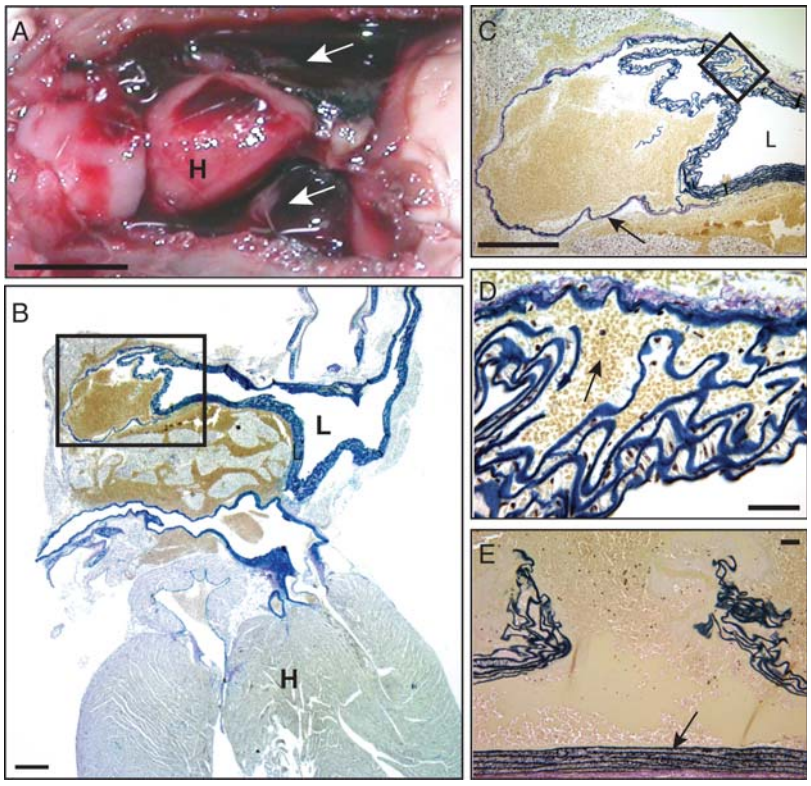

Figure 1 Phenotypic presentation is characterized by haemothorax resulting from acute aortic dissection. (*A*) Partially dissected tissues showing the tongue, cranial mediastinum, and lungs showing clear evidence of haemothorax (the primary phenotypic presentation following sudden death in this mouse colony) coating the lungs (arrow). Bar = 1 cm. (*B*) Longitudinal section through the aortic root from an affected mouse showing aortic dissection (boxed); for orientation, the heart (H) is included in the image (L = aortic lumen). Bar = 1 mm. (*C*) Magnification of the site of dissection [boxed area in (*B*)] clearly shows haemorrhage into the aortic wall and mediastinum, with the external wall of the aorta dissected away from the main structure (arrowed) (L = aortic lumen). Bar = 1 mm. (*D*) Magnification [boxed area in (*C*)] shows the disruption of the elastic lamellae with erythrocytes visible within the aortic wall (arrow). Bar = 100 μm. (*E*) Longitudinal section through the descending thoracic aorta taken from a second affected animal. The site of dissection is clearly visible, characterized by complete disruption of the elastic lamellae. The opposing wall of the vessel is unaffected (arrow). Bar = 100 μm. (All samples: Elastic van Gieson stain.)

2.10 Statistical analysis

Data were analysed using GraphPad Prism version 5 (GraphPad Software Inc., San Diego, CA, USA) using Student's unpaired *t*-test. Values are expressed as mean ± SEM. χ^2 tests were used where appropriate.

3. Results

During an unrelated gene-targeting experiment focused on the role of the *Mro* gene in sexual development,[12] seven mice in the experimental colony died spontaneously without premonitory signs of illness. At post-mortem, the common pathology was haemothorax with haematoma in the mediastinum and surrounding the lungs and heart (*Figure 1A*). Histological analysis of aortas confirmed the cause of death in all cases as acute aortic dissection with rupture of the ascending or descending thoracic aorta, but with no evidence of aneurysm. One mouse exhibited intramural dissection without rupture in the abdominal aorta in addition to rupture of the descending thoracic aorta. Acute aortic rupture was subsequently confirmed in a further 56 animals (*Table 1*). Aortic dissection typically produced a large pseudo-lumen filled with extravasated blood, with clear evidence of involvement of the medial and adventitial layers (*Figure 1B*). Intramural haemorrhage was associated with separation of elastic layers in the media of the aorta with breakages in elastic laminae also evident (*Figure 1C–E*). Examination of haematoxylin- and eosin-stained tissue sections revealed no evidence of inflammatory cells within the aortic wall.

Table 1 Penetrance of the aortic dissection phenotype in +/Col3a1$^{\triangle}$ mice

Mice in colony	+/*Col3a1*$^{\triangle}$	+/*Col3a1*$^{\triangle}$ deaths	Penetrance
473	225	63	28%

3.1 Aetiology of aortic dissection phenotype

The spontaneous aortic dissection phenotype exhibited an autosomal-dominant mode of inheritance (data not shown). The majority of affected animals presented at a relatively young age, with 77% of deaths occurring between 4 and 10 weeks of age

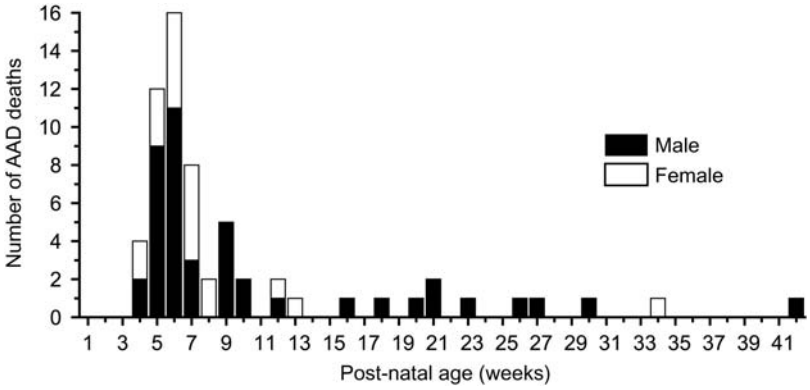

Figure 2 Affected animals die prematurely, with evidence of a sex ratio bias. A comparison of age at death in confirmed cases of aortic dissection reveals 77% of deaths occur between 4 and 10 weeks of age. Peak incidence of death is similar in both sexes; however, beyond 13 weeks, while the frequency of deaths is reduced, the majority of deaths that do occur are male animals (10 males:1 female).

(*Figure 2*). Furthermore, a significant sex ratio bias was observed with male mice twice as likely to die as females (43 males:20 females; $\chi^2 = 21.6$ $P = <0.001$).

3.2 Genetic mapping aortic dissection phenotype

Genotyping of affected animals unequivocally ruled-out targeting of the *Mro* locus[12] as a causal factor underlying the aortic dissection because no affected animal had inherited the targeted allele. This suggested that an independent mutation that had arisen spontaneously was responsible for the deaths in the colony. The aortic dissection phenotype was observed in two independent mouse colonies, both of which were derived from a single aliquot of a 129Ola-derived ES cell line. This strongly suggested that the causal mutation arose within the ES cells prior to independent line generation and thus that the causal mutation was within the 129Ola genome. We exploited the mixed 129Ola:C57BL/6J genetic background of the colony to undertake an SNP-based genome scan on 22 mice in which aortic dissection had been confirmed. This low-resolution genetic mapping identified a single shared 129Ola-derived haplotype in every affected individual, spanning 13 cM (27 Mb) of a region of proximal chromosome 1. Using SNP assays on three informative markers spanning this region (rs4222168, rs3656719, and rs13475846), we demonstrated that the 129Ola-derived allele of the central SNP marker was unexpectedly absent in all affected individuals, suggestive of either a double recombination event spanning this SNP or a deletion of a region of chromosome 1 in all affected animals (*Figure 3A*).

Using classical Mendelian inheritance analysis, we calculated that the shared haplotype across the three SNPs could not have occurred at the frequency observed through independent double recombination events in so many animals in such a small colony (data not shown). In contrast, a hypothetical deletion of a proportion of chromosome 1 in the 129Ola genome surrounding rs3656719 was able to explain the empirical data completely, without invoking requirements for multiple double recombination events. Confirmation of the predicted deletion arose from DNA sequencing of PCR products in the region of the deleted rs3656719 SNP and flanking region, which unequivocally demonstrated that the aortic dissection phenotype resulted from a deletion of 185 kb, encompassing exons 1–39 of the *Col3a1* gene in addition to 145 kb of upstream DNA (*Figure 3B–D*). This allele was named *Col3a1*$^{\triangle}$.

3.3 Inheritance of the *Col3a1*$^{\triangle}$ allele

Genotyping litters ($n = 3$) pre-natally failed to identify *Col3a1*$^{\triangle}$/*Col3a1*$^{\triangle}$ embryos, indicating that inheriting a *Col3a1*$^{\triangle}$/*Col3a1*$^{\triangle}$ genotype was embryonic lethal with 100% penetrance earlier than 9.5 days post conception (dpc) ($\chi^2 = 11.4$, 2 df, $P < 0.01$). Genotyping of litters ($n = 15$) post-weaning, confirmed this observation, as inheritance of the *Col3a1*$^{\triangle}$ allele significantly diverged from expected Mendelian ratios ($\chi^2 = 35.514$, 2 df, $P < 0.0001$). Expected numbers were observed for hemizygous (+/*Col3a1*$^{\triangle}$, $n = 67$) and wild-type (+/+, $n = 38$) littermates; but no mice homozygous for the deletion (*Col3a1*$^{\triangle}$/*Col3a1*$^{\triangle}$) were ever identified. Genetic and phenotypic comparison revealed that all animals dying from aortic dissection were +/*Col3a1*$^{\triangle}$, but not all +/*Col3a1*$^{\triangle}$ animals underwent a dissection event. Further analysis on a larger data set established that the aortic dissection trait exhibited a penetrance of 28% (63/225 +/*Col3a1*$^{\triangle}$ animals) in the experimental population, suggesting that the condition had a complex genetic aetiology (*Table 1*). Further to this, preliminary investigations involving backcrossing for two generations to two further strains (C3HHeH and 129Svev) suggest that the phenotype remains penetrant (albeit incompletely) on other genetic backgrounds (data not shown).

3.4 Phenotypic/physiological analyses

Phenotypic and physiological analyses were undertaken at a range of ages across the peak age of aortic dissection and beyond. Owing to the reduced penetrance of the phenotype, we were acutely aware that from 10 weeks of age onwards, our comparisons would be between animals that, despite their differing genotypes, were unlikely to undergo aortic dissection. Our analyses of +/*Col3a1*$^{\triangle}$ mice are thus split into: (i) causal investigation of the dissection event in younger animals and (ii) investigation of the physiology of 'protected' older individuals.

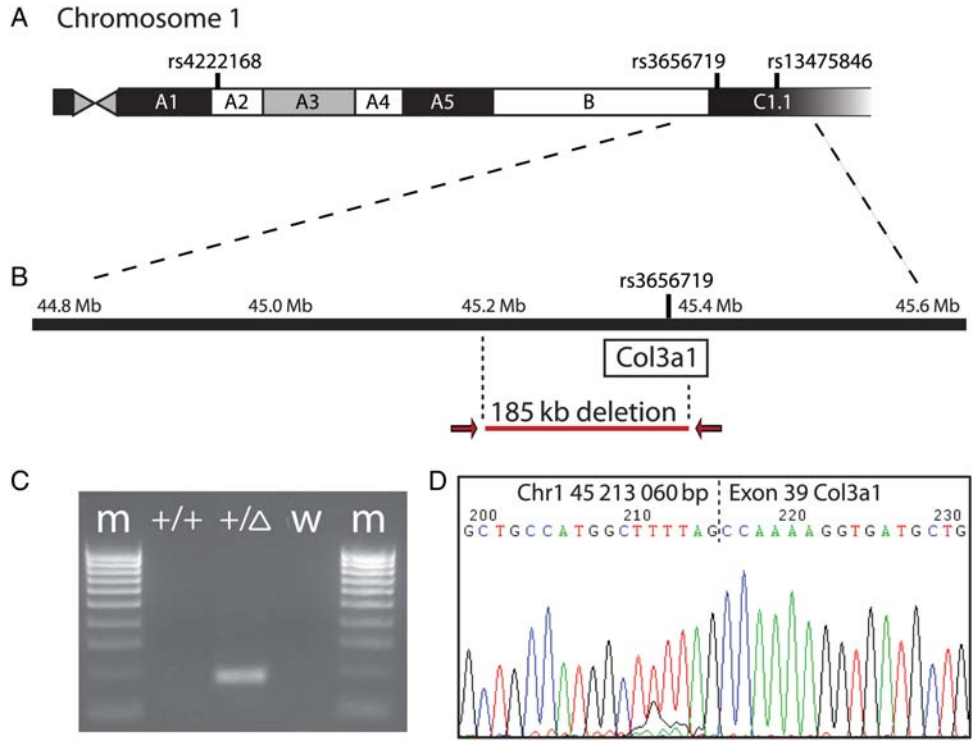

Figure 3 The aortic dissection phenotype results from a 185 kb deletion of chromosome 1 spanning the putative regulatory region and exons 1–39 of the procollagen gene *Col3α1*. (*A*) Schematic representation of the proximal end of mouse chromosome 1 highlighting the location of the three informative SNP markers initially used to haplotype the region. (*B*) Magnified view of a section of chromosome 1; the absence of the allele from the 129Ola genome at SNP rs3656719 in all affected animals was suggestive of a deletion in this region. (*C*) PCR amplification using primers ostensibly 185 kb apart (marked on schematic as red arrows) produces an amplicon of 172 bp in affected animals. (*D*) DNA sequencing of this amplicon identifies the deletion breakpoints in chromosome 1 and confirms the deletion to be 185 kb in size, including 145 kb of the putative regulatory region and exons 1–39 of the *Col3a1*gene. The allele is named *Col3a1*$^{\triangle}$.

3.5 Gross phenotype

Body weight of +/*Col3a1*$^{\triangle}$ mice was not significantly different from +/+ littermates at 4 and 12 weeks of age ($n = 5–7$ per group, data not shown). +/*Col3a1*$^{\triangle}$ individuals were indistinguishable from +/+ littermates upon gross inspection at all ages, and no premonitory signs of illness could be identified prior to aortic dissection, which in two cases occurred within an hour of inspection.

3.6 Functional causes of aortic dissection

Quantitative RT–PCR analysis was undertaken on cDNA derived from total RNA extracted from aortas of 5- to 7-week-old +/+ and +/*Col3a1*$^{\triangle}$ animals ($n = 7$ per group), with a view to identifying early molecular changes that may underlie a dissection event. Analyses of all splice variants of the closely linked *Col5a2* gene revealed no difference in gene expression, suggesting that the deletion does not physically impact upon gene expression in the local environment. Furthermore, examination of gene expression of members of the Tgfβ signalling pathway, which has previously been implicated in cases of thoracic aortic dissection,[18] also revealed no significant difference in gene expression between +/+ and +/*Col3a1*$^{\triangle}$ animals (see Supplementary material online, *Figure S1*).

High-resolution *in vivo* ultrasound was used to determine whether there was any evidence of aneurysm formation or aortic dilatation in the +/*Col3a1*$^{\triangle}$ mice. Comparison of +/+ ($n = 8$; four males, four females) and +/*Col3a1*$^{\triangle}$ pups ($n = 8$; four males, four females) was performed immediately after weaning (age 4 weeks) and repeated when the mice reached the age of 12 weeks. The measurements of left ventricular (LV) mass, Doppler-derived A- and E-wave velocities at the lateral mitral annulus, aortic root dimensions (in mm), and Doppler velocities and dimensions at the ascending aorta, aortic arch, and descending aorta identified no differences between +/+ and +/*Col3a1*$^{\triangle}$ mice (*Table 2*). One of the +/*Col3a1*$^{\triangle}$ animals assessed aged 12 weeks died from aortic dissection while under anaesthetic during the ultrasound procedure.

To determine whether blood pressure was a factor in spontaneous aortic dissection, systolic blood pressure was also measured in the +/+ and +/*Col3a1*$^{\triangle}$ mice used for ultrasound investigations ($n = 8$; four males, four females for each group). Serial measurements were made in conscious, restrained mice using tail-cuff plethysmography at the ages of 4, 8, 12, and 14 weeks. Systolic blood pressures were similar in the +/+ and +/*Col3a1*$^{\triangle}$ mice at all ages except 14 weeks, at which time blood pressure was significantly (+/+ 110.4 ± 3.3 mmHg vs. +/*Col3a1*$^{\triangle}$ 122.0 ± 3.6 mmHg; $P = 0.03$) elevated (by ~12 mmHg) in +/*Col3a1*$^{\triangle}$ mice compared with +/+. Further analysis indicated that this was mainly due to a significant ($P = 0.04$) increase in systolic blood pressure in male mice; an apparent elevation of systolic blood pressure in females was not statistically

Table 2 Echocardiographic comparison of mice carrying the mutant allele (+/Col3a1$^{\triangle}$) with wild-type (+/+) controls

Age	4 weeks			12 weeks		
Parameter	+/+	+/*Col3a1*$^{\triangle}$	*P*-value	+/+	+/*Col3a1*$^{\triangle}$	*P*-value
LV mass (mg)	62.2 ± 6.1	57.5 ± 6.7	0.60	90 ± 4	100 ± 95	0.32
E-wave (mm/s)	714 ± 72	825 ± 68	0.28	655 ± 91	806 ± 178	0.38
A-wave (mm/s)	450 ± 78	492 ± 71	0.69	308 ± 35	491 ± 102	0.07
Annulus (mm)	1.13 ± 0.038	1.12 ± 0.06	0.86	1.42 ± 0.04	1.37 ± 0.04	0.40
SV (mm)	1.22 ± 0.05	1.26 ± 0.06	0.59	1.56 ± 0.05	1.48 ± 0.03	0.24
STJ (mm)	1.24 ± 0.05	1.23 ± 0.06	0.91	1.59 ± 0.03	1.49 ± 0.05	0.11
Doppler (AA Dec)	847 ± 100	912 ± 81	0.61	1270 ± 326	927 ± 106	0.37
Doppler (AA Asc)	838 ± 70	833 ± 111	0.97	1005 ± 102	970 ± 18	0.77
DA (mm)	1.07 ± 0.06	1.06 ± 0.06	0.92	1.35 ± 0.07	1.33 ± 0.13	0.88
Arch (mm)	1.09 ± 0.06	1.19 ± 0.06	0.22	1.43 ± 0.05	1.36 ± 0.03	0.31
AscAo (mm)	1.23 ± 0.06	1.22 ± 0.08	0.95	1.55 ± 0.07	1.50 ± 0.08	0.65

No significant differences were detected in any of the parameters measured in mice at the age of 4 weeks or, subsequently, at 12 weeks ($n = 8$ +/+, $n = 8$ +/*Col3α1*$^{\triangle}$). SV, sinal valve; STJ, sinotubular junction; DA, decending aorta; AscAo, ascending aorta. Data are mean ± SEM, where $n = 8$ (four males, four females) per group.

significant (see Supplementary material online, *Table S1*). It is notable that this increase in blood pressure was detected some time after the ages (4–9 weeks) at which the maximum incidence of death was observed.

3.7 Structural causes of aortic dissection

Staining with Picrosirius Red (see Supplementary material online, *Figure S2*) demonstrated that medial collagen content was lower ($P = 0.01$) in thoracic aortas from +/*Col3a1* (16.7 ± 1.1%; $n = 7$) compared with +/+ (22.2 ± 1.6%; $n = 5$). An apparent reduction of collagen content in the aortic adventitia of +/*Col3a1*$^{\triangle}$ mice did not achieve significance (64.8 ± 3.8 vs. 71.4 ± 1.3%; $n = 5–7$; $P = 0.19$).

Since no obvious structural abnormalities were detected in aortas of +/*Col3a1*$^{\triangle}$ mice, transmission electron microscopy was performed to assess whether ultrastructural abnormalities could be detected. Aortas from 4-week-old +/*Col3a1*$^{\triangle}$ mice showed clear abnormalities compared with +/+ controls (*Figure 4*; $n = 3$ per genotype). Aortas taken from +/+ animals displayed continuous elastic laminae with a consistent width (*Figure 4*); conversely, elastic lamellae in +/*Col3a1*$^{\triangle}$ animals displayed reduced electron density when compared with +/+ animals (all sections were 60 nm thick) along with variable width and a disrupted architecture. Evidence of disruption to the elastic lamellae and tearing of the smooth muscle layer was evident in all +/*Col3a1*$^{\triangle}$ animals examined, which in one animal had resulted in shearing of the smooth muscle layer and entry of erythrocytes into the aortic media (*Figure 4D* and *E*). Shearing of the smooth muscle layer was associated with structural abnormalities in collagen fibres (*Figure 4F* and *G*), which unlike in +/+ mice failed to form correctly.

3.8 Aortic function in adult +/*Col3α1*$^{\triangle}$ animals

Evidence of changes in aortic function was sought in 14-week-old +/*Col3a1*$^{\triangle}$ mice using *ex vivo* isometric myography [+/+, $n = 5$ (2 males:3 females); +/*Col3a1*$^{\triangle}$, $n = 6$ (3 males:3 females)]. Contractile responses of aortas from +/+ mice to both 5-hydroxytryptamine (5-HT) and phenylephrine were increased by removal of the endothelium (*Tables 3* and *4*). In contrast, contractile responses of intact aortas from +/*Col3a1*$^{\triangle}$ mice were larger than in +/+ controls (although this difference only achieved significance for 5-HT). Removal of the endothelium did not augment contraction in aortas from +/*Col3a1*$^{\triangle}$ mice and, consequently, contractile responses were similar in denuded aortas from +/+ and +/*Col3a1*$^{\triangle}$ mice. Histological analysis (Haematoxylin & Eosin) of aortic sections from the adult +/*Col3a1*$^{\triangle}$ mice used for functional analysis did not identify any structural abnormalities compared with age matched controls (not shown).

To establish whether premonitory changes associated with the mutation could be detected in any other body system, we undertook a comprehensive pathological screen on previously asymptomatic animals of both sexes at 16 weeks of age [+/+, $n = 7$ (4 males:3 females); +/*Col3a1*$^{\triangle}$, $n = 8$ (4 males:4 females)] (see Supplementary material online, Methods for tissues examined). Gross and gravimetric differences were not identified between +/*Col3a1*$^{\triangle}$ and control mice of either gender. Furthermore, no histopathological changes were identified in any of the +/*Col3a1*$^{\triangle}$ mice.

4. Discussion

This investigation has demonstrated that a spontaneous deletion of a region of chromosome 1 has serendipitously resulted in generation of a unique and exploitable mouse model of acute aortic dissection, with similarities to EDS IV. Detailed genetic analysis has confirmed that this is due to a deletion resulting in generation of a predicted null allele of the *Col3a1* gene, the causal locus underlying EDS IV in humans (OMIM #130050).

The most common presenting features in adults with EDS IV are gastrointestinal or organ rupture (~70%). Arterial rupture in the thorax or abdomen accounts for ~50% of the cases and often occurs spontaneously (but may be preceded by aneurysm or dissection) (Pepin MG & Byers PH, gene review at http://www.ncbi.nlm.nih.gov/bookshelf/br.fcgi?book=gene&part=eds4). The only gene associated with this condition in humans is the *COL3A1* gene, with mutations acquired by autosomal-dominant inheritance (~50%) or *de novo* mutation (~50%). *Col3a1* has been targeted previously in

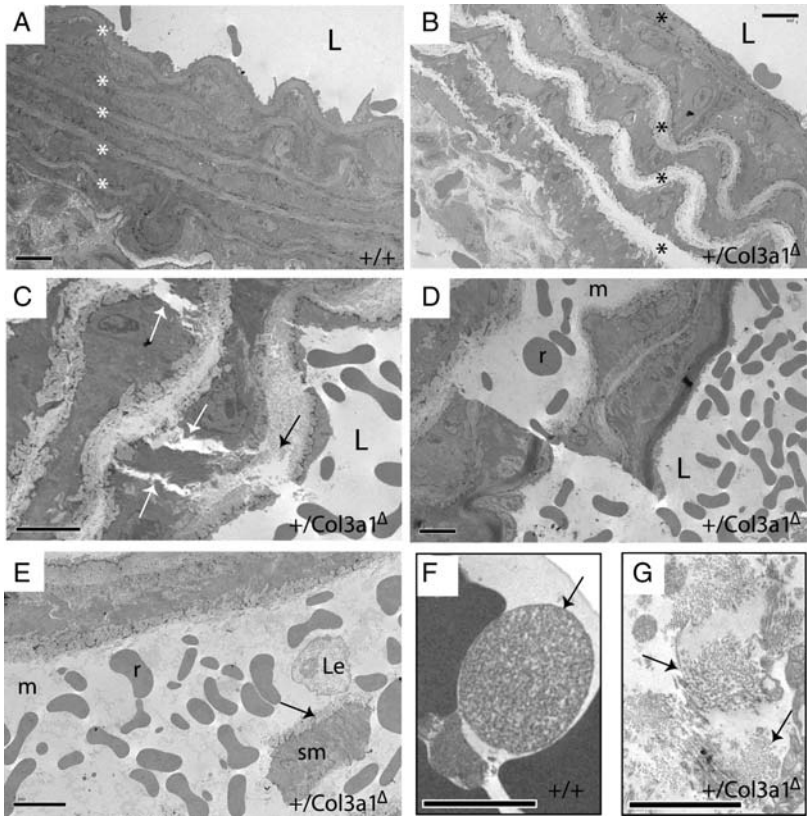

Figure 4 Aortas of 4-week-old +/*Col3a1*$^{\Delta}$ mice display evidence of ultrastructural abnormalities. The ultrastructure of aortas of 4-week-old +/+ and +/*Col3a1*$^{\Delta}$ animals ($n = 3$ per group) were examined using transmission electron microscopy. Aortas taken from +/+ animals display continuous elastic laminae (*) with a consistent width (*A*). Conversely, elastic lamellae in +/*Col3a1*$^{\Delta}$ animals (*) display reduced electron density when compared with +/+ animals (all sections were 60 nm thick) along with variable width and a disrupted architecture (*B*). Evidence of disruption to the elastic lamellae (black arrow) and tearing of the smooth muscle layer (white arrows) is evident in all +/*Col3a1*$^{\Delta}$ animals examined (*C*), which in one animal had resulted in shearing of the smooth muscle layer and entry of erythrocytes into the aortic media (*D* and *E*) (arrow in *E* = elastic lamellae). Shearing of the smooth muscle layer is associated with structural abnormalities in collagen fibres (arrow), which unlike in +/+ mice (*F*), fail to form correctly (arrows) (*G*). L, aortic lumen; m, aortic media; r, erythrocytes; sm, smooth muscle; Le, leucocyte (*A*–*E* bar = 5 μm; *F* and *G*, bar = 2 μm).

an attempt to model the human syndrome in rodents.[7] Genetic inactivation of both copies of the *Col3a1*gene produced mice with many of the features of EDS IV, including death from aortic and gastrointestinal rupture. Unfortunately, investigations using these animals are hampered by the very early death (~90% did not reach adulthood) of homozygotes. Furthermore, unlike EDS IV cases, there was no evidence of autosomal-dominant inheritance, with a lack of any phenotype in heterozygotes.

In contrast, the spontaneously derived line described here presents with only the vascular features of EDS IV. There was no evidence of the skin fragility, bruising, or gastrointestinal rupture associated with the condition in humans and reported in the *Col3a1* knockout.[7] Indeed, no structural abnormalities were detected in any of the organs of older animals subjected to detailed pathology. However, the presentation of unheralded, acute aortic rupture is consistent with EDS IV. Furthermore, inheritance of the aortic dissection trait is autosomal-dominant in the *Col3a1*$^{\Delta}$ model, and unlike the *Col3a1* knockout, death occurs in the +/*Col3a1*$^{\Delta}$ mice over an age range that allows investigation both of the features of the disease and of the potential triggers of dissection. Thus, we have generated, for the first time, an observable and exploitable model for the study of this lethal disease.

The apparent increase in penetrance of the aortic dissection phenotype in the +/*Col3a1*$^{\Delta}$ mutant over the knockout model,[7] such that heterozygous animals display aortic dissection while no phenotype was originally reported in the knockout heterozygotes, is intriguing. More recent evidence confirms that heterozygotes from the original *Col3a1* knockout do not exhibit life-threatening vascular events or gross vascular lesions.[14] They do, however, have reduced levels of aortic collagen and correspondingly reduced aortic wall strength. Furthermore, histological investigation indicates the presence of minor aortic lesions that appear between 2 and 14 months of age and are more common in males than in females.[14] Autosomal-dominant inheritance or spontaneous mutation in a single copy of *COL3A1* resulting in haploinsufficiency has been linked[19,20] (although not exclusively)[21] with vascular complications in EDS IV patients, and thus, the +/*Col3a1*$^{\Delta}$ mutant may more closely recapitulate the human condition than the previously generated knockout.[20] Given the nature of the deletion in the *Col3a1*$^{\Delta}$ mutant, which generates a predicted null allele, along with its mixed genetic background, the

Table 3 Functional consequences of the Col3a1$^{\triangle}$ mutation: agonist-selective enhancement of aortic contraction

Drug	Endothelium	E_{max} (mN/mm), $n = 6$			Sensitivity ($-\log EC_{50}$), $n = 6$		
		+/+	+/*Col3a1*$^{\triangle}$	*P*-value	+/+	+/*Col3a1*$^{\triangle}$	*P*-value
5-HT	Intact	7.5 ± 0.4	11.6 ± 1.3*	**0.01**	6.70 ± 0.87	7.09 ± 0.22	0.16
	Denuded	10.5 ± 1.4	11.0 ± 1.9	0.80	6.85 ± 1.08	7.33 ± 0.14*	**0.03**
PhE	Intact	5.0 ± 1.2	6.6 ± 1.1	0.24	7.28 ± 0.80	7.36 ± 0.17	0.77
	Denuded	7.7 ± 1.8	6.1 ± 1.4	0.58	7.24 ± 0.96	7.84 ± 0.73	0.38

Data are mean ± SEM for *n* mice. Comparisons were made using Student's unpaired *t*-test. E_{max}, maximum contraction; 5-HT, 5-hydroxytryptamine; PhE, phenylephrine.
*$P < 0.05$ compared with +/+ littermates (highlighted in bold).

Table 4 Functional consequences of the Col3a1$^{\triangle}$ mutation: aortic relaxation is unaffected in *Col3a1*$^{\triangle}$ mice

Drug	Endothelium	E_{max} (% relaxation), $n = 6$			Sensitivity ($-\log IC_{50}$), $n = 6$		
		+/+	+/*Col3a1*$^{\triangle}$	*P*-value	+/+	+/*Col3a1*$^{\triangle}$	*P*-value
ACh	Intact	81.0 ± 3.6	77.8 ± 7.7	0.72	7.27 ± 1.90	7.30 ± 0.41	0.92
	Denuded	7.0 ± 2.3	3.5 ± 1.0	0.21	—	—	—
SN	Intact	96.4 ± 1.8	99.6 ± 2.8	0.37	7.99 ± 0.15	8.41 ± 0.44	0.41
	Denuded	95.5 ± 1.8	95.8 ± 2.1	0.91	7.65 ± 0.33	7.99 ± 0.47	0.56

Data are mean ± SEM for *n* mice. Comparisons were made using Student's unpaired *t*-test. E_{max}, maximum relaxation; ACh, acetylcholine; SN, sodium nitroprusside. Responses to vasorelaxants were obtained after contraction with a submaximal concentration of 5-hydroxytryptamine.

most obvious interpretation for this discrepancy is the presence of genetic modifiers that increase the penetrance of aortic dissection in the +/*Col3a1*$^{\triangle}$ mutant when compared with the knockout mouse. Again, this is similar to aortic disease in the human population where genetic predisposition can impact upon the penetrance of disease phenotypes. This unique characteristic of the *Col3a1*$^{\triangle}$ model has important pragmatic and strategic implications. The pragmatic impact is the serendipitous development of a practicable model of aortic dissection for onward study. The more intriguing strategic conclusion is that this colony now provides an opportunity to identify the genetic modifiers underlying the variability in penetrance, something that could have significant impact on study of the human condition, because such genes would naturally become potential targets for therapeutic intervention. Further experiments involving breeding to different genetic backgrounds, in combination with genome-wide association study analysis, could address this possibility.

The histological presentation of overtly normal vascular structure, but with ultrastructural evidence of abnormal development of collagen fibrils, is consistent with similar models resulting from genetic manipulation of genes encoding different forms of collagen.[7,8] In mice carrying the *Col3a1*$^{\triangle}$ mutation, the causes of dissection in the subpopulation exhibiting acute, unheralded death was unclear. Phenotypic analysis indicated that these animals were not hypertensive at the age the majority of deaths occur, do not develop aneurysm or dilatation of the ascending aorta *in vivo*, and have little evidence of vascular dysfunction. It is proposed that this model will prove useful in increasing our understanding of the role of interactions between collagens in regulation of arterial structure. This is likely to clarify the mechanism of conditions associated with arterial dissection, particularly EDS IV, but with potential relevance to a variety of pathologically related conditions.

The demonstration of normal arterial structure in un-dissected aortas of the +/*Col3a1*$^{\triangle}$ mice is consistent with previous models of deletions in the *Col3a1*[7] or *Col1a1*[8] genes. Furthermore, the aetiology of the dissection described in *Col3a1*$^{\triangle}$ mice was similar to that observed in previous models, presenting with separation of the medial elastic laminae resulting in the formation of a blood-filled pseudo-lumen. This is suggestive of an abnormality in the stability of the layers between the elastic laminae. The ultrastructural demonstration of abnormal collagen fibril development is, therefore, consistent with this pathology and in line with results obtained from previous models. Both *Col3a1* knockout and *Col1a1* mutant mice demonstrated reduced collagen content of the aorta accompanied by abnormal fibril development in the media and adventitia.[7,8] It is apparent, therefore, that the stability of the aortic wall depends upon the normal development of collagen fibrils and this requires appropriate contributions from the different types of collagens that form these structures.

The reasons for physiological and phenotypic analysis of the +/*Col3a1*$^{\triangle}$ mice were three-fold. First, the ability to identify at-risk individuals would simplify the selection of animals for subsequent analysis and allow elective harvesting in advance of dissection; secondly, evidence of structural and functional changes in the aorta were necessary for comparison with the human syndrome; and thirdly, the identification of predictors of dissection would help in clarifying the factors that contributed to rupture in those mutants that developed symptoms and, consequently, help identify potential therapeutic targets. The demonstration that dissection was not associated with elevated systolic blood pressure, even under the stress stimulus of physical restraint during plethysmography, suggests neither chronic nor acute hypertension is a predicting factor. This is consistent with clinical features of the syndrome in humans (OMIM #130050), as is the demonstration by ultrasound that neither aortic

dilatation nor aneurysm formation was evident in +/*Col3a1*$^{\triangle}$ mutants. Indeed, ultrasound investigations indicated that function of the cardiovascular system was unimpaired in these animals. Magnetic resonance imaging investigation of *Col1a1* mutants also reported that dissection occurred without the development of an aneurysm, wall defects, or a preceding rupture.[9] The acute death of otherwise healthy animals during preparation for anaesthesia and ultrasound examination suggests a stress-related contribution to dissection, but further work would be required to confirm this relationship.

Despite the evidence that aortic ultrastructure was altered, with inconsistencies in the structure of elastic laminae, in +/*Col3a1*$^{\triangle}$ mice, functional investigations demonstrated that contractility of these vessels, in the absence of the endothelium, was not altered. This suggests that function of the medial smooth muscle cells was not affected by impaired development of collagen fibrils. There was, however, evidence of small agonist-dependent endothelial cell dysfunction. In mouse and rat aorta, the endothelium mediates a physiological antagonism of contraction probably by a combination of basal and stimulated release of nitric oxide.[17,22,23] It is notable that the physiological antagonism of contractile responses was impaired in aortic rings from +/*Col3a1*$^{\triangle}$ mutants, possibly as a result of altered receptor activity in the endothelium.[24] There was no evidence, however, that this contributed to aortic dissection. Whether this mild endothelial dysfunction is a cause, or consequence, of the mild hypertension seen in older mice remains to be established.

In conclusion, we have shown that spontaneous deletion in the promoter region and exons 1–39 of the murine *Col3a1* locus has produced a relevant and exploitable model of the vascular dissection in EDS IV. Further investigation of this model should help to clarify the causes of, and risk factors for, aortic dissection in EDS IV and will help in the search for improved treatments for this condition, including the potential to identify protective genetic factors. Furthermore, this model will also help to establish the importance of the interaction between collagen III and other collagen subtypes in the maintenance of arterial stability and, thus, may shed light on the pathogenesis of vascular dissection associated with other conditions.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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