

Col4a1 mutation in mice causes defects in vascular function and low blood pressure associated with reduced red blood cell volume

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Collagen type IV is the major structural component of the basement membrane and COL4A1 mutations cause adult small vessel disease, familial porencephaly and hereditary angiopathy with nephropathy aneurysm and cramps (HANAC) syndrome. Here, we show that animals with a Col4a1 missense mutation (Col4a1^{+/Raw}) display focal detachment of the endothelium from the media and age-dependent defects in vascular function including a reduced response to nor-epinephrine. Age-dependent hypersensitivity to acetylcholine is abolished by inhibition of nitric oxide synthase (NOS) activity, indicating that Col4a1 mutations affect vasorelaxation mediated by endothelium-derived nitric oxide (NO). These defects are associated with a reduction in basal NOS activity and the development of heightened NO sensitivity of the smooth muscle. The vascular function defects are physiologically relevant as they maintain in part the hypotension in mutant animals, which is primarily associated with a reduced red blood cell volume due to a reduction in red blood cell number, rather than defects in kidney function. To understand the molecular mechanism underlying these vascular defects, we examined the deposition of collagen type IV in the basement membrane, and found it to be defective. Interestingly, this mutation also leads to activation of the unfolded protein response. In summary, our results indicate that mutations in COL4A1 result in a complex vascular phenotype encompassing defects in maintenance of vascular tone, endothelial cell function and blood pressure regulation.

INTRODUCTION

The basement membrane (BM) is a specialized extracellular matrix structure that compartmentalizes tissues, provides structural support and influences cell behaviour (1). Collagen type IV is the major structural component of the BM and in vertebrates, six different collagen type IV alpha chains [$\alpha 1(\text{IV})$ – $\alpha 6(\text{IV})$] have been described which produce three different collagen type IV protomers [$\alpha 1. \alpha 1. \alpha 2(\text{IV})$, $\alpha 3. \alpha 4. \alpha 5(\text{IV})$ and $\alpha 5. \alpha 5. \alpha 6(\text{IV})$] (2).

The most abundant isoform, $\alpha 1. \alpha 1. \alpha 2(\text{IV})$, contributes to the formation of many BMs and its deficiency causes embryonic lethality in mouse (3). COL4A1 mutations have recently

been identified as a cause of familial porencephaly, adult small vessel disease, haemorrhagic stroke and a new syndrome called HANAC (hereditary angiopathy with nephropathy aneurysm and cramps) [for review, see (4)]. In mice deficiency in Col4a1 and Col4a2 and missense mutations in Col4a2 lead to haemorrhaging during development (3,5) underscoring the important structural role of Col4a1 and Col4a2 in the vasculature.

In the vascular wall, the BM borders the vascular smooth muscle cells (VSMCs) and separates them from the endothelium, which lines the vessel lumen (1). The vascular endothelium plays a crucial role in vasodilation through generating

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vasodilator agents including nitric oxide (NO), prostaglandins I₂ and E₂ and endothelium-derived hyperpolarizing factor (EDHF) (6). In large conduit vessels such as the descending aorta, the NO-cGMP pathway predominates whereby endothelium-derived NO activates soluble guanylate cyclase in the VSMC to generate cGMP, ultimately leading to smooth muscle relaxation (7,8). The resulting vasodilation can be blocked by NOS inhibitors, such as L-N^ω-nitroarginine methyl ester (L-NAME). Clinically, endothelial cell (EC) dysfunction affects blood pressure control (9,10) and promotes atherosclerosis (11).

Recently, we have characterized an allelic series of *Col4a1* mouse mutants which display a spectrum of eye phenotypes combined with glomerulopathy (12,13). In one of these mutants, the *Col4a1*^{+/^{Raw} mutation (K950E) changes a lysine residue to glutamic acid at a Y residue of the Gly-X-Y collagen repeat in the collagen domain (12). *Col4a1*^{+/^{Raw} animals are characterized by retinal arteriolar silvering due to focal BM defects (12,13). However, the described function of *Col4a1* in angiogenesis (for review, see (1)), which can be mediated through integrin signalling (14), suggests additional functional roles in the vasculature.}}

To gain further insight into the role of *Col4a1* in the vasculature, the vascular phenotypes of *Col4a1*^{+/^{Raw} mice were explored including a structural and functional analysis of descending aortae. Mutant animals display haemorrhagic stroke and in descending aortae focal detachment of endothelial cells from the media occurs. Mutant animals have altered VSMC and EC function which affects the NO-cGMP pathway. Blood pressure analysis revealed hypotension in mutant animals that is associated with a reduced blood volume and is maintained in part by the described vasodilation defects. We also show that the *Col4a1*^{+/^{Raw} mutation results in defective deposition of collagen type IV in the BM of descending aortae and leads to activation of the unfolded protein response (UPR). These results show for the first time that defects in the BM in general, and collagen type IV in particular, affect the maintenance of vascular tone, endothelial cell function and blood pressure regulation.}}

RESULTS

Col4a1^{+/^{raw} mutation results in perinatal cerebral haemorrhages and reduced viability}

Analysis of newborn *Col4a1*^{+/^{Raw} pups revealed mild cerebral haemorrhaging in mutant pups (Fig. 1A) with more severe haemorrhaging being observed in pups that died shortly after birth (Fig. 1B). The perinatal cerebral haemorrhaging is associated with reduced viability at weaning as we only detect 19.8% *Col4a1*^{+/^{Raw} animals compared to the expected 50% ($\chi^2 = 56.6$, one degree of freedom, $P < 0.0001$) (Fig. 1C).}}

Mutations in *Col4a1* results in ultrastructural changes in the vessel wall

Patients with *COL4A1* mutations can suffer from aneurysm formation of the carotid artery (15) suggesting that *COL4A1* mutations may affect the structure of large calibre vessels.

We therefore performed histopathological and ultrastructural analysis on descending aortae of 6-month-old *Col4a1*^{+/^{Raw} animals which revealed no gross morphological defects and normal total collagen and elastin deposition was observed (Fig. 1D–F, Supplementary Material, Fig. S1). However, ultrastructurally *Col4a1*^{+/^{Raw} mice displayed areas of focal detachment of the intact endothelium from the underlying media (Fig. 1G and H).}}

Col4a1^{+/^{Raw} animals have defects in vascular smooth cell function}

To analyse if the structural BM defects affect vascular function, myography was performed on rings from descending aortae of 3, 6 and 11–15-month-old animals. VSMC function, assessed by contraction in response to KCl containing-physiological salt solution (K-PSS) (125 mM KCl), was impaired in *Col4a1*^{+/^{Raw} aortae (Fig. 2A; $n = 6$, $P = 0.001$). This indicates reduced contractile strength independent of endothelial function (16) and suggests that BM defects may weaken the vessel thereby increasing the susceptibility to cerebral haemorrhaging; observed in patients and mice with *COL4A1* mutations.}

Reduced contractile responses to the adrenoceptor agonist, nor-epinephrine (NE), were observed in 6-month (Fig. 2B) and 11–15 month old mutant animals (Supplementary Material, Fig. S2B) [two-way analysis of variance (ANOVA) $P < 0.0001$] but were normal in 3-month-old animals (Supplementary Material, Fig. S2A). These data would suggest that an age-dependent defect in VSMC function develops between 3 and 6 months of age after the onset of reduced vessel wall strength.

Mutations in *Col4a1* affect endothelial cell function and NO-cGMP-mediated vasodilation

Endothelial cell-mediated vasodilation was assessed in 3-, 6- and 11–15-month-old animals through the vasodilation responses to acetylcholine (ACh) on NE (EC₈₀)-precontracted aortic rings. Endothelium-dependent vasorelaxation was not different between 3- and 6-month-old Wild Type (WT) and mutant animals (Supplementary Material, Fig. S2C and D). However, in contrast, aortae of 11–15-month-old mutant animals displayed significantly increased vasodilation to ACh (two-way ANOVA; $P < 0.0001$; Fig. 2C), indicating that this age-dependent phenotype becomes significant after 6 months. The response to ACh was mediated through the NO-cGMP pathway as blockade of NOS activity by L-NAME abolished ACh-induced vasodilation both in *Col4a1*^{+/^{Raw} and in WT aortic rings (Fig. 2C).}

Because changes in NO generation can be accompanied by alterations in the response of the smooth muscle cells to NO (17), aortic rings were exposed to the NO donor, sodium nitroprusside (SNP). No differences were observed in 3- and 6-month-old mutant and WT animals (Supplementary Material, Fig. S2E and F). In contrast, a significant increase in SNP-induced vasodilation was observed in the 11–15-month-old animals (Fig. 2D), confirming the development of increased NO sensitivity in smooth muscle. These results show that *Col4a1* mutations can affect NO-mediated

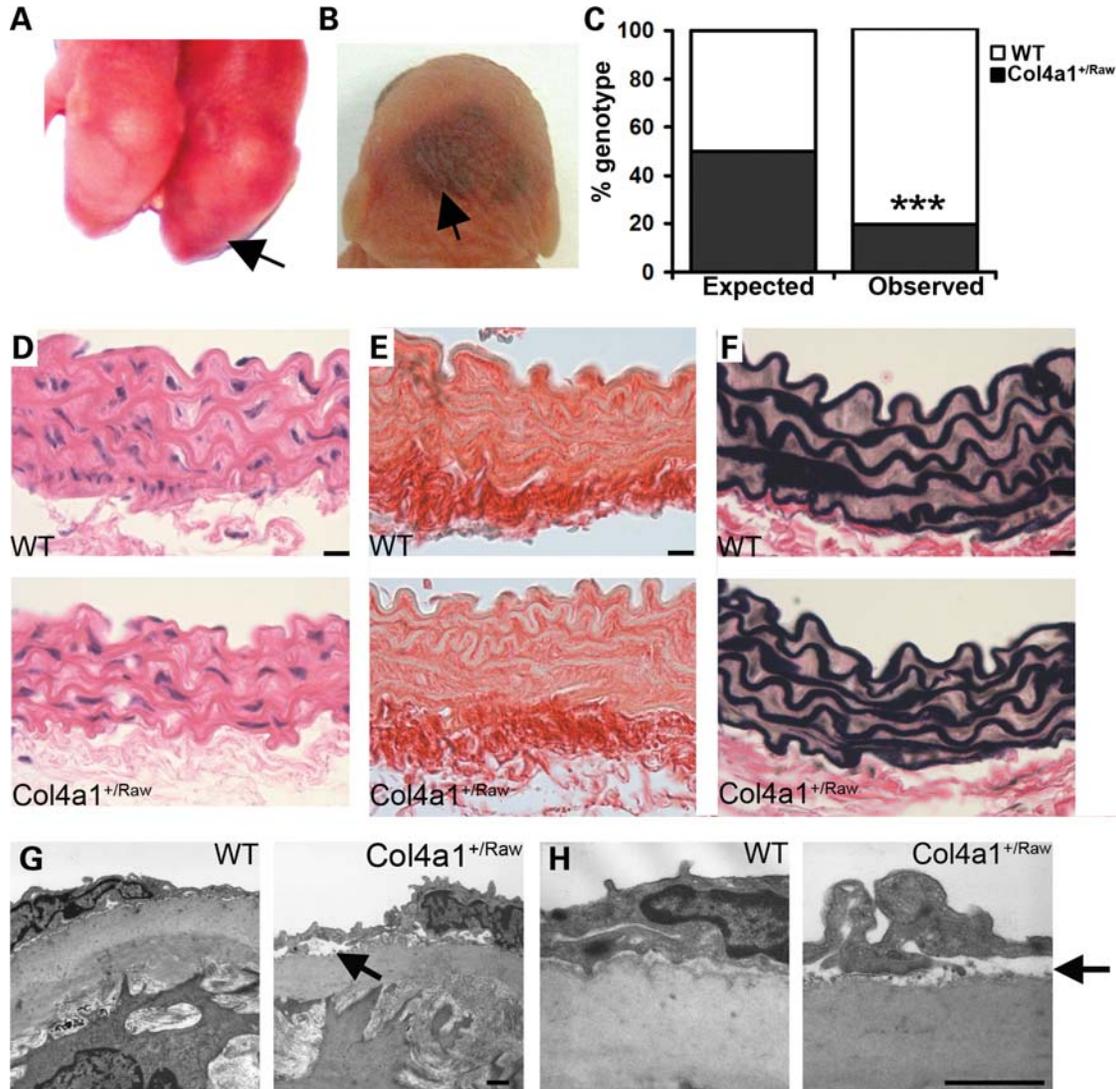


Figure 1. Vascular phenotypes of *Col4a1*^{+/*Raw*} mice. (A and B) Mild and more severe cerebral haemorrhaging in *Col4a1*^{+/*Raw*} pups (arrow). (C) Predicted (50%) and observed (19.8%) percentage of *Col4a1*^{+/*Raw*} animals (31/156) (black) at weaning ($\chi^2 = 56.6$, one degree of freedom, $P < 0.0001$). (D) Lack of gross histological phenotype in mutant aortae. (E) Normal total collagen and elastin (F) deposition in mutant animals (data shown for 6-month-old animals, see Supplementary Material for 3- and 9-month-old animals). (G) Focal endothelial detachment in aortae from *Col4a1*^{+/*Raw*} animals (arrow). (H) Higher magnification of EM analysis. Size 1 μm (G and H), 10 μm (D–F).

vasodilation and NO sensitivity, suggesting an upregulation of the NO-cGMP pathway.

The observed vasodilation defects are specific to the NO-cGMP pathway as the response to the β_2 adrenoceptor agonist, isoprenaline, which induces cAMP-mediated vasodilation (18), was similar between WT and mutant animals (Fig. 2E). These results also indicate that the smooth muscle of mutant vessels is able to maintain tension.

Col4a1 mutant animals have reduced basal nos function

To further investigate the NO-cGMP pathway, we assessed basal NOS function in 12-month-old aortae by measuring the constriction induced by L-NAME (19). Mutant aortae showed a 40% reduction in L-NAME-induced contraction ($P < 0.05$) (Fig. 3A) indicating that mutant animals have

reduced basal NOS function. This reduction in activity is independent of changes in eNOS mRNA and protein expression as shown by quantitative RT-PCR and western blot analysis (Fig. 3C and D). Similarly, the increased NO sensitivity of the smooth muscle is independent of changes in expression of cGMP-dependent protein kinase 1 (Fig. 3B) and soluble guanylate cyclase, respectively (Fig. 3D).

Collagen type IV influences vascular homeostasis

Vascular resistance is one of the key mechanisms involved in blood pressure regulation, which can be altered through alterations in vasodilation (9) and/or vasoconstriction (20). To investigate if the structural defects of *Col4a1*^{+/*Raw*} animals affect vascular homeostasis, the systolic blood pressure of 4–6- and 14-month-old mice was measured by tail plethysmography.

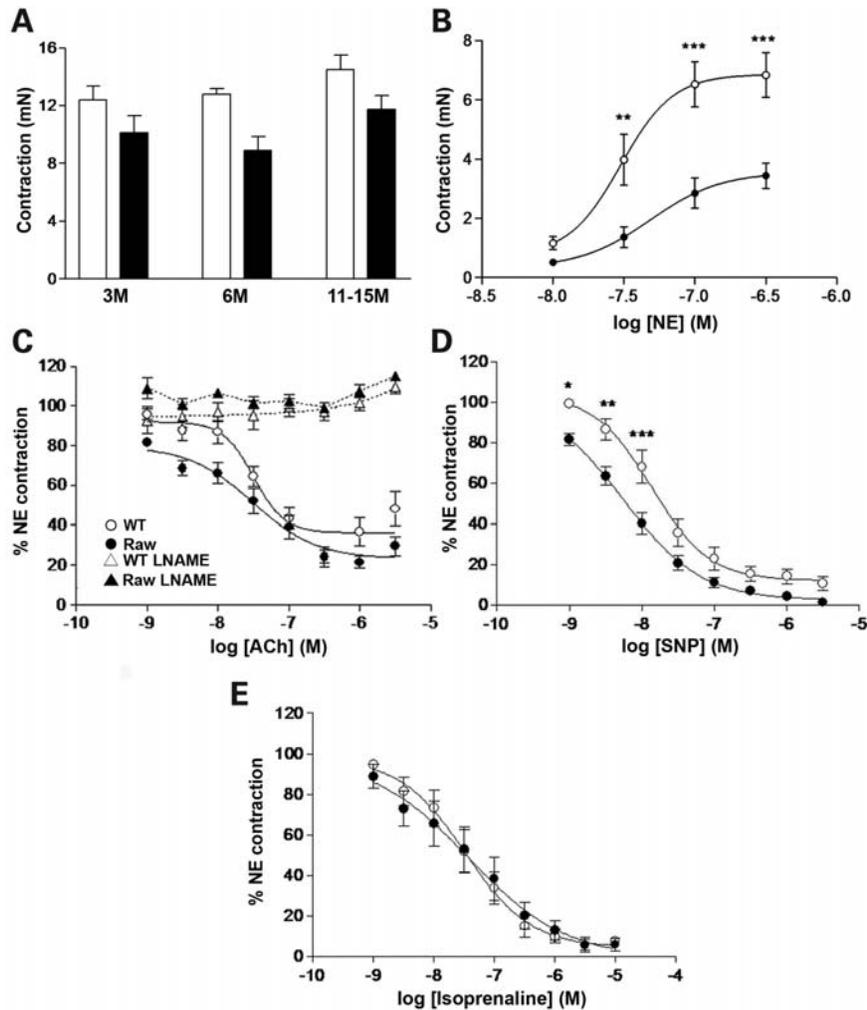


Figure 2. Assessment of vascular function. (A) Mutant animals (black bars) have reduced amounts of contraction (mN) in K-PSS ($P = 0.0012$ two-way ANOVA, $n = 6$ for 3- and 6-month-old animals, $n = 10$ for 11–15 months old). (B) Reduced contraction to nor-epinephrine in 6-month-old mutant animals ($n = 6$) (two-way ANOVA $P < 0.001$, 6 months). (C) 11–15-month-old mutant animals display increased vasodilation to low concentrations of ACh (two-way ANOVA, $n = 10$, $P < 0.0001$). Incubation of vessels in L-NAME (labelled +L-NAME) before administration of ACh abolishes vasodilation. (D) Vasodilation to SNP in 11–15-month-old mutant animals shows increased relaxation (two-way ANOVA, $n = 10$, $P < 0.0001$). (E) Vasodilation to isoprenaline in 11–15-month-old animals ($n = 6$). WT (open circles), *Col4a1*^{+Raw} (filled circles). Error bars indicate standard error of measurement. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ post hoc test.

This revealed that in both cohorts *Col4a1*^{+Raw} animals have a reduction of 20–25 mmHg in their systolic blood pressure ($P < 0.001$ for both cohorts) (Fig. 4A and B) indicating that mutations in collagen type IV can result in hypotension.

Our results differ from previous reports in which animals with a deletion of exon 40 in *Col4a1* were normotensive using tail plethysmography (21). It is known that different mutations in the same collagen alpha chain can affect the displayed phenotypes and phenotype severity (12,22). Moreover, tail cuff recording of blood pressure in mice can be imprecise, particularly in hypotensive states, and is best confirmed using direct measurement (23). We therefore measured the blood pressure of *Col4a1*^{+Raw} animals using direct arterial cannulation. This confirmed that the blood pressure was 20–25 mmHg lower in mutant animals and extended the observation to animals at 40 days of age (Fig. 4C).

The different ages of onset of the hypotension and vasodilation defects data indicate that the vasodilation defects do not cause the hypotension. However, once established, the vasodilation defects may be important for maintaining the hypotension by reducing vascular resistance. Consequently, L-NAME was administered chronically to 14-month-old animals and systolic blood pressure measurements were collected before and during treatment. Two weeks of L-NAME treatment resulted in an increase in blood pressure in WT animals of 8.8 mmHg compared to 20.5 mmHg in mutant animals ($P < 0.01$) (Fig. 4D). Consequently, the treatment resulted in a partial rescue of the hypotension with systolic blood pressure of 121.9 and 113.1 mmHg for WT and mutant animals, respectively. These data confirm the hypothesis that the vasodilation defect is important for the maintenance of the hypotension.

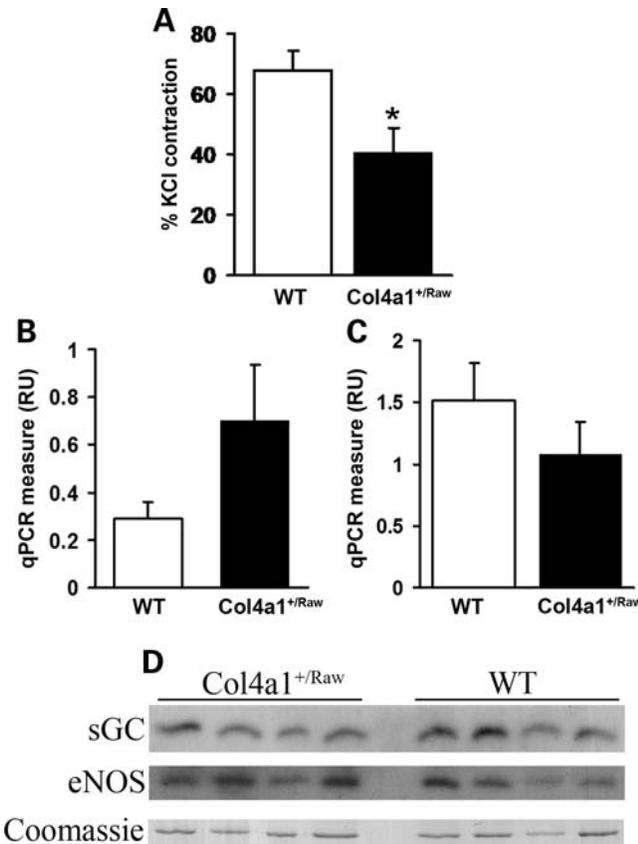


Figure 3. Reduced basal NOS function in *Col4a1*^{+Raw}. (A) Contraction obtained by administration of L-NAME as measure of amount of NO in 11–15 month animals expressed as percentage of contraction obtained with KCl. ($n = 14$ WT, $n = 11$ *Col4a1*^{+Raw}, t -test, $P < 0.05$). (B) qRT-PCR for PRKG1 (cGMP-dependant protein kinase I). Expressed as relative units to 18S RNA levels ($n = 6$). (C) eNOS mRNA levels in 12-month-old WT (white) and *Col4a1*^{+Raw} (black) ($n = 6$) animals as determined by qRT-PCR relative to 18S RNA levels ($P = 0.17$ t -test). (D) eNOS, and sGC protein expression levels in aortae of 11–15-month-old animals. Predominant band of coomassie gel is given as loading control (entire gel is provided in Supplementary Material, Fig. S3). Error bars indicate standard error of measurement. * $P < 0.05$ t -test.

Hypotension in *Col4a1* mutant animals is associated with a reduced blood volume

Since the hypotension is evident before the onset of the vascular function defects, we investigated alternative causes of low blood pressure. Given the pivotal role of the kidney in blood pressure homeostasis (24), sodium excretion was analysed in 40-day-old animals (Fig. 5A) which showed a marked reduction in sodium clearance in mutant animals compared to WT ($P < 0.01$), indicating that renal sodium wasting does not maintain the hypotension. There was a significant increase in plasma aldosterone in mutant animals (Fig. 5B), suggesting activation of the renin–angiotensin–aldosterone system (RAAS) which would promote sodium retention in the distal nephron.

Given the close relation between blood volume and blood pressure, we calculated the blood volume in 40-day to 8-month-old animals following measurement of plasma volume and hematocrit. These analyses revealed a ~26%

reduction in blood volume in mutant animals (Fig. 5C, $P < 0.01$) which is consistent with activation of the RAAS and increased circulating aldosterone levels. However, *Col4a1*^{+Raw} animals have normal plasma volume (Fig. 5D) and the reduction in blood volume reflects a lower average hematocrit of 38% compared to 45% for WT animals (Fig. 5E, $P < 0.001$). These data indicate that the lower blood volume is caused by a decrease in packed cell volume. The decreased packed cell volume is due to a reduction in red blood cell (RBC) number in mutant animals. Measurement of RBC number showed an 11% reduction in RBC number in mutant animals (Fig. 5F, $P < 0.01$) as *Col4a1*^{+Raw} animals have 8.55×10^6 RBC/ μ l compared to 9.50×10^6 RBC/ μ l for WT animals. Thus, the RBC volume reflects a reduction in RBC number.

Col4a1 mutation result in activation of the unfolded protein response

To further characterize the molecular consequences of the *Col4a1*^{+Raw} mutation and how it may lead to the vascular phenotypes, immunostaining was performed on descending aortae from 12-month-old animals. This revealed interrupted staining for the $\alpha 1(\text{IV})$ network compared to continuous laminin staining, suggesting a defective deposition of collagen type IV and altered BM composition (Fig. 6A).

In the endoplasmic reticulum (ER), three collagen type IV alpha chains interact and form a collagen type IV protomer. Mutations in proteins that affect protein folding can lead to ER stress which may lead to activation of the UPR (25). The presence of swollen ER vesicles and increased expression of ER chaperones in *Col4a1* mutant animals (12,26) suggests the occurrence of ER stress. To investigate if this occurs within the vasculature and leads to UPR activation, quantitative RT-PCR analysis was performed to assess *Bip* (also known as GRP78) mRNA expression levels, a marker for unfolded protein response (UPR) activation. This revealed a 1.7-fold increased *Bip* mRNA expression level in mutant animals (Fig. 6B, $P < 0.05$ t -test) indicative of ER stress and UPR activation.

DISCUSSION

The identification of *COL4A1* mutations in patients with familial porencephaly (27–29) and HANAC syndrome (15) combined with vascular phenotypes in mice (for review, see (4)) underlines the important structural role of type IV collagen in the vasculature. However, the role of collagen type IV in angiogenesis implies that collagen type IV and the BM possess additional non-structural roles (1). Here, we describe that a *Col4a1* mutation affects vascular function and homeostasis thereby highlighting a potential novel functional role for type IV collagen.

The *Col4a1*^{+Raw} mutation affects both the capillaries and large calibre vessels, which in neonates results in the occurrence of cerebral haemorrhaging. Although in another *Col4a1* animal model these haemorrhages can lead to the occurrence of porencephaly (28), no evidence of porencephaly was observed in the *Col4a1*^{+Raw} animals (12). This milder

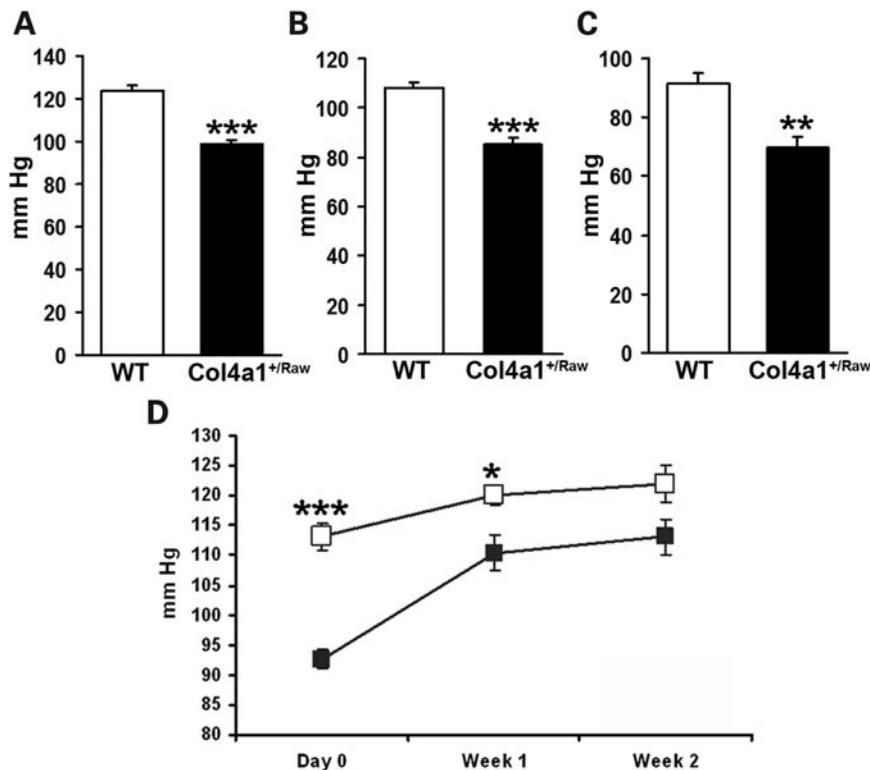


Figure 4. Collagen type IV and blood pressure regulation. (A) Systolic blood pressure of 4–6-month-old animals [*Col4a1*^{+/*Raw*} 99 mmHg (*n* = 6), WT 124 mmHg (*n* = 5), *P* < 0.001 *t*-test]. (B) Systolic blood pressure of 14-month-old animals (*Col4a1*^{+/*Raw*} 85.1 mmHg, WT 108.1 mmHg, *n* = 6, *P* < 0.001, *t*-test). (C) Mean arterial blood pressure of 40-day-old animals [*Col4a1*^{+/*Raw*} 70 mmHg (*n* = 5), WT 91 mmHg (*n* = 7), *P* < 0.01, *t*-test]. (D) Systolic blood pressure of 14-month-old animals before and during treatment with L-NAME. Measurements were collected at day 0, day 7 and day 14 of treatment (WT, *n* = 9, *Col4a1*^{+/*Raw*}, *n* = 7). **P* < 0.05, ***P* < 0.01, ****P* < 0.001 *t*-test.

phenotype has also been observed in the eye (12) and is probably due to the less severe effects of a mutation affecting a lysine residue at the Y position of the Gly-X–Y repeat compared to the effects of a Gly mutation at the Gly position.

In descending aortae, the mutation results in the focal separation of the endothelium from the media. These structural defects are associated with a complex functional phenotype affecting both VSMC and EC function. The reduced contraction in K-PSS in *Col4a1*^{+/*Raw*} animals indicates a reduction of contractile strength independent of endothelial function (16). These data confirm that the structural BM defects weaken the vessel which would increase the susceptibility to cerebral haemorrhaging observed in patients and mice with *Col4a1* mutations (15,21,27–31).

In addition to a reduction in contractile strength, *Col4a1*^{+/*Raw*} animals develop a late onset VSMC phenotype and complex EC phenotype. Basal endothelial activity is reduced as shown by a blunted contraction in response to L-NAME (19). However, stimulated NO generation is increased as illustrated by the increased sensitivity to ACh which is abolished by L-NAME-mediated inhibition of NOS, indicating that the mutation affects NO-mediated vasodilation. Moreover, the vessels develop an increased response to the NO donor, SNP, suggestive of heightened NO sensitivity of the smooth muscle. Taken together, these results suggest an upregulation of the downstream relaxative mechanism, possibly in response

to reduced basal NO production as observed in eNOS-deficient animals (32,33). Our data suggests that the sensitization of this pathway does not compensate the endothelial dysfunction under basal conditions but becomes apparent upon endothelium stimulation. In addition, these results support the hypothesis that VSMCs have the capacity to increase their NO sensitivity as a protective measure to counter the influence of partially reduced endothelial function. This hypothesis is also supported by the heightened NO sensitivity of heterozygous eNOS-deficient animals (17). In eNOS-deficient animals, the increased NO sensitivity is mediated through elevated activity of soluble guanylate cyclase (32) and cGMP-dependent protein kinase (PKG1) (33). It is likely that a similar mechanism occurs in *Col4a1*^{+/*Raw*} animals as no changes in expression levels were observed for sGC, eNOS and PKG1.

The chronic administration of L-NAME and partial rescue of the hypotension indicates that the vasodilation defects contribute to the maintenance of the low blood pressure. However, they are unlikely to be causal as the hypotension precedes the onset of the vasodilation defect. Renal salt wasting causes low blood pressure in several genetic disorders [for review, see (24)] but in this analysis sodium excretion was reduced in *Col4a1*^{+/*Raw*} animals. Thus, the kidney is adapting appropriately to low blood pressure by activation of the RAAS (24).

Interestingly, we showed that the hypotension is associated with a reduction in blood volume due to a decreased packed

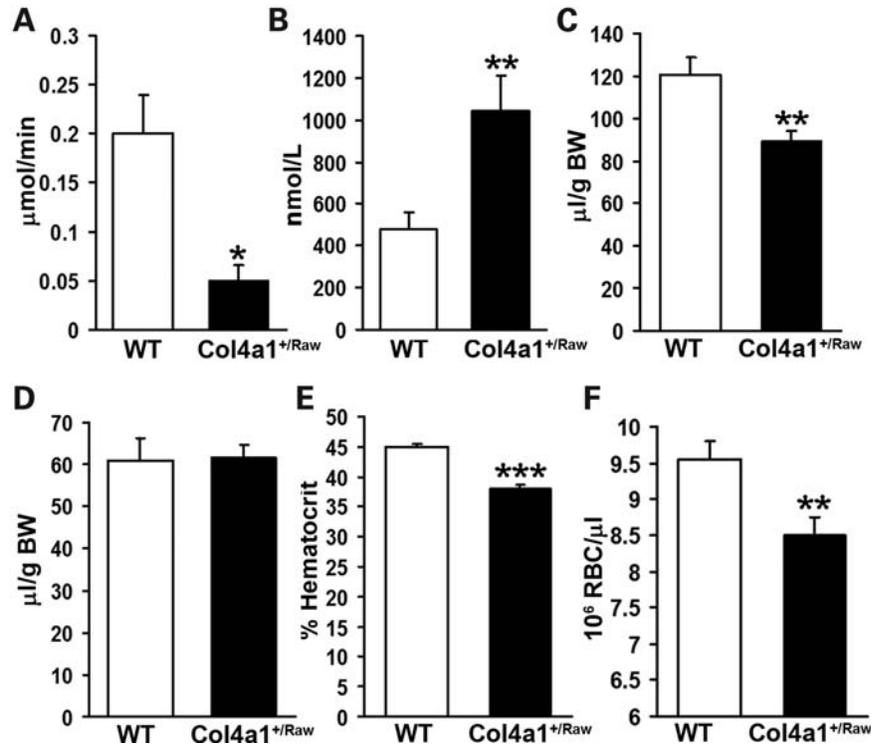


Figure 5. Low blood pressure is associated with reduced blood volume. (A) Sodium excretion in 40-day-old animals [*Col4a1*^{+/*Raw*} 0.05 μmol/min (*n* = 5), WT 0.2 μmol/min (*n* = 7), *P* < 0.05 *t*-test]. (B) Plasma aldosterone levels in 40-day-old animals [*Col4a1*^{+/*Raw*} 1044 nmol/l (*n* = 5), WT 476 nmol/l (*n* = 7), *P* < 0.01 *t*-test]. (C) Blood volume in animals [*Col4a1*^{+/*Raw*} 88.9 μl/g body weight (*n* = 10), WT 120 μl/g body weight (*n* = 13), *P* < 0.01 *t*-test]. (D) Plasma volume of animals. (E) Reduced hematocrit in mutant animals [*Col4a1*^{+/*Raw*} 38% (*n* = 15), WT 45% (*n* = 19), *P* < 0.001 *t*-test]. (F) Reduced red blood cell number in *Col4a1*^{+/*Raw*} animals [*Col4a1*^{+/*Raw*} 8.55 × 10⁶ RBC/μl (*n* = 10), WT 9.50 × 10⁶ RBC/μl (*n* = 12), *P* < 0.01 *t*-test]. Error bars indicate standard error of measurement. **P* < 0.05, ***P* < 0.01, *****P* < 0.001 *t*-test.

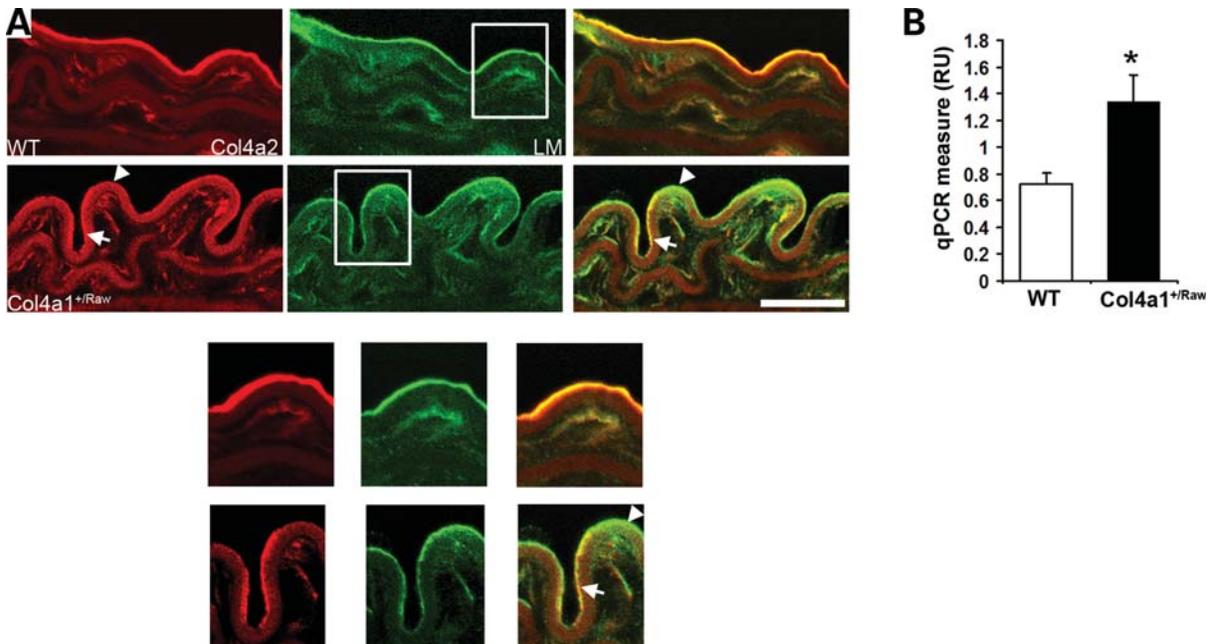


Figure 6. *Col4a1*^{+/*Raw*} mutation results in UPR activation. (A) Interrupted staining for *Col4a2* in vascular BM of descending aortae from mutant animals in contrast to continuous laminin (LM) staining (arrow indicates presence of *Col4a2* in BM. Arrowhead indicates absence of *Col4a2* as indicated by green staining in merged image). Size 20 μm. Detail (white square) is provided below (A). (B) Increased *Bip* mRNA expression levels in mutant animals. Expressed as relative units to 18S RNA levels (*P* < 0.05 *t*-test, *n* = 9).

cell volume caused by a reduction in RBC number. The reduced hematocrit and RBC number are supported by other mouse models with *Col4a1* mutations (5) although no vascular function and blood pressure regulation was measured in these mice. These results suggest that the *Col4a1* mutation is associated with hypotension at least in part due to a reduction in blood volume and that the hypotension is maintained at least in part by the vasodilation defect.

The mechanism underlying the reduced RBC number remains unknown. Because *Col4a1* mutations lead to haemorrhaging (our data and (21,28)), chronic haemorrhaging may underlie this phenotype. Given that no severe haemorrhaging has been observed in mutant animals (van Agtmael T, 2009), this would be caused by multiple microbleeds. Alternatively, the *Col4a1* mutation may affect erythropoiesis as blood progenitor cells need to transmigrate a BM before being released into the peripheral blood and these cells express collagen type IV degrading gelatinases (34). Thus, this migration may be affected due to mutations in type IV collagen. In addition, erythroid differentiation requires interaction with extracellular matrix proteins such as fibronectin, mediated through integrin alpha 4 beta 1 (35), but erythroid progenitor cells also express integrin receptors which can bind the BM protein laminin (36). Further research is required to determine the mechanism by which the *Col4a1* mutations lead to a reduction in RBC number and blood volume.

One important question is how a mutation affecting a BM protein can affect endothelial cell function and vasodilation. ER stress and UPR activation can be triggered by mutations that affect protein folding. The triple helical domain of collagen type IV contains numerous (>20) interruptions which are thought to be important for its flexibility and function as that their location within the alpha chain is highly conserved (37). The sequences flanking the interruptions are atypical suggesting that they may play an important role at the molecular level related to triple helix stability (38). The *Col4a1*^{+/^{Raw} mutation changes a lysine residue to glutamic acid at the Y position of a Gly-X-Y triplet that flanks a major interruption (12). Although most mutations in collagen proteins affect glycine residues, the effects of the *Col4a1*^{+/^{Raw} mutation combined with the inherent instability of the interruption may severely affect protein folding. Our data show that the *Col4a1*^{+/^{Raw} mutation leads to UPR activation, a coordinated response activated by and aimed at alleviating ER stress (25). It is thus tempting to suggest that the defective deposition of collagen type IV in the BM is an indication of reduced secretion with intracellular retention of collagen type IV causing ER stress. This is supported by analysis of other *Col4a1* mutant mouse models that revealed intracellular retention of *Col4a1*, increased chaperone expression and presence of swollen ER vesicles (12,26,28). UPR activation has also been observed in response to mutations in collagen type X (39) where it results in cellular reprogramming. Chronic ER stress is a pathologic mechanism that may lead to diseases such as diabetes (40). Thus, our data raise the interesting hypothesis that the vascular defects may be associated with and possibly due to UPR activation. In this context, it is interesting to note that ER stress and UPR activation has been observed in endothelium in areas prone to atherosclerosis (41), in which endothelial cell dysfunction plays an important}}}

role and is one of the earliest markers. The identification of UPR activation due to *Col4a1* mutations is also of clinical relevance as modulation of UPR activation provides an attractive target for intervention. Indeed, UPR activation has recently been modified successfully in a mouse model of diabetes (40).

In conclusion, we have identified that mutations in collagen type IV are associated with UPR activation and defects in vascular function and blood pressure regulation. As collagen type IV is part of the BM, it is tempting to speculate that other BM proteins and their receptors may have similar roles in vascular biology. In this regard, it is interesting to note that endostatin, a proteolytically cleaved fragment of collagen XVIII (42), induces endothelium-dependent vasorelaxation (43), although the role of collagen XVIII itself has not been investigated. Finally, it is now important to assess these phenotypes in patients with *COL4A1* mutations in order to gain a complete understanding of the spectrum of disease phenotypes and processes affected by *COL4A1* mutations.

MATERIALS AND METHODS

Animals

Studies were performed under guidance from the UK Home Office. Animals were backcrossed for five generations on a Bl/6 background and control animals were WT littermates.

Ultrastructural analysis

Tissue were collected from three adult animals (3 and 6 months old) and processed as previously described (12).

Histopathology

Aortae were fixed overnight in 10% neutral buffered formalin or 4% paraformaldehyde and embedded in paraffin wax. Sections were stained with Haematoxylin–Eosin, Sirius Red or Verhoeff stain using standard protocols ($n = 3$).

Systolic blood pressure analysis

Systolic blood pressure was measured using tail cuff plethysmography on restrained conscious animals using an IITC model 129 analyser (Woodland Hills, CA, USA). All animals underwent three periods of training prior to recording measurements. Per animal, the data were generated from the average of four readings taken on three independent occasions.

in vivo studies

Mice were anaesthetized with Inactin (50 mg/kg IP) and prepared surgically for renal function experiments as described (44). Mice were infused IV with isotonic saline at a rate of 200 μ l/h/10 g body weight. After a 40 min equilibration period, Mean Arterial Blood Pressure (MABP) was recorded for 30–40 min on a Powerlab, sampling at a rate of 2 Hz and urine was collected for 1 h. Following this, Evans Blue (1 μ l/g of a 0.5% solution w : v) was injected for measurement of plasma volume: blood volume was calculated from this and hematocrit (44). At the end of the experiment, a 500 μ l blood

sample was taken for measurements of aldosterone. Sodium concentration in urine and plasma was measured by ISE (Roche). RBC number was determined using blood samples collected from the tail or cheek vein and blood counts were performed using a coulter counter (Beckman). Statistical analysis was done by Student's *t*-test.

Myography

Aortic rings (2 mm) were prepared for myography on a wire myograph (DMT myographs, Denmark) in PSS (NaCl, KCl, MgSO₄, NaHCO₃, KH₂PO₄, EDTA, glucose, CaCl₂), contracted three times in K-PSS (PSS plus 125 mM KCl) and contracted in 30 μM NE followed by a dilation in 1 μM ACh. The following concentration–response curves were performed: NE (10⁻⁸ M, 3 × 10⁻⁶ M), ACh (10⁻⁹ M, 3 × 10⁻⁶ M), SNP (10⁻⁹ M, 3 × 10⁻⁶ M) and isoprenaline (10⁻⁹ M, 3 × 10⁻⁵ M). Dilation response curves were performed following contraction (80% of maximum determined by NE concentration–response curve) with NE. ACh concentration–response curves were performed following incubation (15 min) with 200 μM L-NAME. Basal NO levels were determined by measuring contraction following incubation (12 min) with 200 μM L-NAME after contraction with NE (30% of maximum). The amount of contraction was expressed as a percentage of the KCl contraction. All drugs were from Sigma (Dorset, UK). Statistical analysis was performed using two-way ANOVA followed by post hoc test.

RT-PCR

RNA was isolated using Tri-Reagent (Sigma, UK). First strand synthesis was performed using AMV-RT (Roche Applied Science) and quantitative real time PCR analysis was performed in triplicate using TaqMan Gene Expression (Applied Biosystems) assays on ABI System 7700 (eNOS) or using Stratagene Brilliant Sybrogreen QPCR (Prkg1, *Bip*) on MJ Research Chromo4 Thermal Cycler. mRNA levels were corrected using 18S RNA mRNA levels and statistical analysis was performed using Student's *t*-test.

Immunoblotting

Protein samples were extracted from descending aorta in the presence of RIPA buffer containing EDTA protease (Roche Applied Science) and phosphatase inhibitors (Phostop Roche) using standard protocols. Primary antibodies eNOS (1/1000 BD Transduction Laboratories) and sGC (1/2000 Sigma). Protein levels were corrected for coomassie staining of a predominant protein band and measured using Biorad GS700 Image Analyser software analysis. Statistical analysis was performed using unpaired *t*-test.

Immunohistochemistry

Cryosections were fixed for 10 min in acetone followed by antigen retrieval using 0.1 M HCl/KCl for 10 min and standard protocols. The laminin (1/1000) antibody was a kind gift from Dr Ulrike Mayer and the H22 antibody against *Col4a2* (1/100) from Dr Yoshikazu Sadu.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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