Dominant mutations of Col4a1 result in basement membrane defects which lead to anterior segment dysgenesis and glomerulopathy

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Members of the type IV collagen family are essential components of all basement membranes (BMs) and define structural stability as well as tissue-specific functions. The major isoform, α1.α1.α2(IV), contributes to the formation of many BMs and its deficiency causes embryonic lethality in mouse. We have identified an allelic series of three ENU induced dominant mouse mutants with missense mutations in the gene Col4a1 encoding the α1(IV) subunit chain. Two severe alleles (Bru and Svc) have mutations affecting the conserved glycine residues in the Gly-Xaa-Yaa collagen repeat. Bru heterozygous mice display defects similar to Axenfeld–Rieger anomaly, including iris defects, corneal opacity, vacuolar cataracts, significant iris/corneal adhesions, buphthalmos and optic nerve cupping, a sign indicative of glaucoma. Kidneys of Bru mice have peripheral glomerulopathy characterized by hypertrophy and hyperplasia of the parietal epithelium of Bowman’s capsule. A milder allele (Raw) contains a mutation in the Yaa residue of the collagen repeat and was identified by a silvery appearance of the retinal arterioles. All phenotypes are associated with BM defects that affect the eye, kidney and other tissues. This allelic series shows that mutations affecting the collagen domain cause dominant negative effects on the expression and function of the major collagen IV isoform α1(IV), and pathological effects vary with the individual mutations.

INTRODUCTION

Anterior segment dysgenesis (ASD) is a collection of disorders that affect the anterior eye structures, iris, lens, cornea and drainage networks, and includes Axenfeld’s, Rieger’s and Peter’s anomalies, aniridia, iridogoniodygenesis and posterior embryotoxon (1). Axenfeld–Rieger (AR) anomaly is characterized by iridocorneal adhesions, corneal opaqueness and various iris defects including tears, hypoplasia and eccentric pupil. Importantly, 50% of AR patients develop glaucoma (2). AR is genetically heterogeneous, and some causative genes have been identified, including PITX2 and FOXC1 (3–6). However, linkage of AR to other loci (reviewed in 2) and the absence of mutations in known ASD genes in patients (reviewed in 1) indicate that multiple genes underlie AR. The genetic heterogeneity of ASD is paralleled by inter- and intra-familial phenotypic variability (1).

Mouse models are an excellent tool to analyse gene function, and ENU (N-ethyl-N-nitrosourea) mutagenesis generates single base pair changes that can accurately model human disease-causing mutations (7). As a part of an ENU mutagenesis project for dominant eye phenotypes, we identified two
mutants, Raw (retinal arteriolar wiring) and Svc (small with vacuolar cataracts), with overlapping eye phenotypes (8). Raw displays a silvery appearance of the retinal arterioles and Svc has vacuolar cataracts, small body size, bruising at birth and some arteriolar silvering (See Figure 2 in 8).

Interestingly, both Raw and Svc co-localize in a 10 Mb region on chromosome 8 between markers D8Mit124 and D8Mit155. This was coincident with the location of a third mutant, Bruised (Bru) (9). Bru homozygous mice are embryonic lethal, and heterozygotes have a phenotype similar to Svc, including bruising at birth, small body size and various eye defects (10). Although Bru was found among the offspring of an ENU-mutagenized male, it was reported to be caused by a visible deletion of cytogenetic band A1.3 on chromosome 8 (9). The overlapping phenotypic features and mapping data suggested that these mutants may be allelic (8).

We show here that three mouse mutants, Raw, Bru and Svc, are an allelic series due to point mutations in the gene Col4a1. These mutations result in glomerulopathy and symptoms similar to defects seen in AR anomaly. The observed phenotypes are associated with generalized basement membrane (BM) defects but show a high degree of tissue-specific variability. A genotype–phenotype correlation exists, as mutations affecting the crucial glycine residues in the Gly-Xaa-Yaa repeat result in more severe pathologies. These mutations define a spectrum of diseases caused by mutations of the major collagen IV isoform.

RESULTS

Bru, Raw and Svc are caused by Col4a1 mutations

Bru was reported to be caused by a deletion of cytogenetic band A1.3 on chromosome 8 (9), which is contained in the Raw and Svc candidate region between D8Mit124 and D8Mit155 (8). To determine the extent of any deletion, offspring from Bru and M.m. castaneus inter-cross matings were genotyped using microsatellites spanning the Raw and Svc candidate region (D8Mit149, D8Mit333, D8Mit157, D8Mit285, D8Mit173, D8Mit141 and D8Mit60). Offspring carrying the Bru chromosome had heterozygous genotypes for all markers, indicating that Bru was not caused by a large deletion (data not shown). A genome scan was therefore undertaken which mapped the Bru mutation proximal of D8Mit259 on chromosome 8 ($\chi^2 = 6.23$, one degree of freedom, P-value < 0.05).

The co-localization of the Bru and Raw/Svc candidate regions and overlapping phenotypes suggested that the mutations may be allelic. Further mapping reduced the Raw/Svc candidate region to a 7 Mb interval between SNP WI_WGS_8_6825671 (http://www.jax.org/phenome) and D8Mit60. Owing to the absence of additional informative markers, we performed candidate gene sequence analysis. Examination of Col4a1, which encodes the α1 chain of type IV collagen, α1(IV), identified missense mutations in all three alleles. For Raw mice, a mutation was detected in exon 34 (Fig. 1A) whereas in Svc and Bru, we detected a base pair

Figure 1. Raw, Svc and Bru are caused by mutations in the gene Col4a1. (A) Sequence analysis of Col4a1 exons identified a G-A mutation in Raw (arrow). (B and C) Sequence analysis of Svc (B) and Bru (C) identified an A-G and G-T mutation, respectively (arrow). The sequence trace also shows a polymorphism (square) in Bru between the two background strains 101 and C57BL/6. (D) In Svc, a highly conserved glycine residue has been replaced by an aspartic acid (G1064D) (arrow). (E) Bru mice have a glycine to tryptophan mutation (G627W) (arrow). (F) The mutation in Raw substitutes a lysine residue by glutamic acid (K950E) (arrow). The lysine residue is conserved in vertebrates, whereas it does not seem to be conserved in C. elegans. Mm, hs, m, gg and ce indicates Mus musculus, Homo sapiens, Rattus norvegicus, Gallus gallus and C. elegans, respectively. C. elegans Col4a1 sequence is from EMB-9 and Col4a2 is LET-2 sequence (22). (The amino acid numbering is based on NCBI Col4a1 Seq. accession no. NP_034061.)
change in exons 37 and 26, respectively (Fig. 2B and C). All base pair changes segregated with their respective phenotypes and were absent in a panel of wild-type (WT) laboratory strains, including the strains of origin (BALB/c for Raw and Svc and C3H/101 for Bru). Sequence analysis of α1(IV) cDNA from Raw and Bru showed the presence of both mutant and WT alleles, indicating that the mutant alleles are expressed and suggesting that mutant proteins may be produced (data not shown).

All mutations are located in the central collagen domain of α1(IV) which consists of multiple Gly-Xaa-Yaa repeats separated by short interruptions of non-collagenous sequences. All three mutations affect residues of the Gly-Xaa-Yaa repeat. In Svc and Bru, the mutations substitute the critical glycine residues for an aspartic acid (G1064D) and tryptophan (G627W) residue, respectively (Fig. 2D and E). Both these amino acid changes are predicted to result in severe detrimental effects on collagen triple helix formation and stability. Although the Svc mutation is located in the most 3’-terminal homogenous collagenous domain, the Bru mutation is located centrally in a short collagenous domain flanked by interruptions and may disturb the folding of this domain. In contrast, the Raw (K950E) mutation causes a positively charged lysine residue, located at the Yaa position, to be replaced by a negatively charged glutamic acid. Comparative sequence analysis shows that this lysine residue is conserved throughout evolution in vertebrates (Fig. 1F). As this mutation is located in the first Gly-Xaa-Yaa repeat following a major interruption, it may affect the structure at this highly flexible site. The correlation of an allelic series of mutants with the base pair changes in Col4a1 indicates that these mutations are causative of the described phenotypes.

**Mutations in Col4a1 result in ASD**

Signs of ASD are apparent in Bru mice at or shortly after weaning. By 3 months, half of the Bru eyes (8/16) display buphthalmos (bulging eyes) (Fig. 2A), a possible sign of glaucoma, and ~20% of the eyes display corneal opacity (3/16) (Fig. 2B). Additionally, some animals have malformed pupils, which fail to constrict on light stimulation (Fig. 2A). Histopathological analysis on 3-month-old mutant animals

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**Figure 2.** (A) Bru animals have buphthalmos, abnormal pupil which fails to constrict upon light stimulation (arrow). (B) corneal opacity, (C) vacuolar cataracts (arrow), (D) iris/corneal adhesion (arrow), (E) degeneration of the ganglion cell layer (arrow) and (F) optic nerve cupping (arrow). (G) In Bru, the glomeruli have irregular linings of parietal epithelium of Bowman’s capsule (arrow). Hyperplastic nuclei are clustered in interrupted rows of three or more with intervening gaps. The peri-glomerular interstitium has an increased cellularity in cells with fusiform nuclei consistent with fibroblasts. Scale bar: (C, D and F) 50 μm; (E) 25 μm; (G) 10 μm.
showed that *Bru* eyes can have vacuolar cataracts (2/7 animals), retinal detachment (4/7 animals) and peripheral iridocorneal adhesion (anterior synchiae) (5/7 animals) (Fig. 2C and D). The adhesions may result in glaucoma by preventing proper drainage of the aqueous humour, leading to aqueous accumulation and possibly increased intra-ocular pressure (11). This may be the cause of the observed atrophy of the ganglion cell layer (3/7 animals) and cupping of the optic disc (3/7 animals) (Fig. 2E and F), a sign suggestive of glaucoma. No obvious defects were observed in *Raw* eyes, which could explain the arteriolar silversing (data not shown).

As mutations in other collagen IV genes, *COL4A3-COL4A5*, result in kidney defects characteristic of Alport syndrome (AS) (12), the kidneys of *Raw* and *Bru* mice were examined histopathologically. *Raw* kidneys did not display any clear defects (data not shown). In contrast, *Bru* kidneys have proliferation and hypertrophy of the parietal epithelial lining of Bowman’s capsule (Fig. 2G). These defects are seen in both sexes. Affected glomeruli have irregular linings of parietal epithelium due to variations in cell size and nuclear density. In severe cases, multiple layers of parietal epithelium are present (data not shown). Changes in parietal epithelium are accompanied by increased numbers of fusiform cells consistent with fibroblasts in peri-glomerular interstitium.

To test whether the defects result in proteinuria, urine was collected from 5- to 6-month-old *Bru* and *Raw* mice; its protein content was quantified by Bradford assay and its composition was analysed by SDS–PAGE. No significant difference in protein content or composition was observed between the WT and mutants (data not shown).

**Mutations in Col4a1 result in variable tissue-specific BM defects**

Collagen IV is an abundant component of all BMs and is critical for the formation of stable BMs during embryonic development (13), but the specific expression of isoforms defines the characteristic properties of individual BMs. To further determine the causative defects of the observed phenotypes, the membranes of several tissues, including eye and kidney, were analysed in *Bru* and *Raw* mice by electron microscopy. In general mutants show variable changes in the thickness and appearance of BMs, and also local disruptions and detachment of matrix from cells can be observed.

The BM of the corneal epithelium is more severely affected in *Bru* animals, which display local interruptions, when compared with *Raw* in which the BM is continuous but less dense than in WT. (Fig. 3A). The lens capsule shows an irregular appearance in *Bru* when compared with the homogenous amorphous structure seen in *Raw* and WT lenses. In *Bru* mutants, a dilation of the vesicles from the endoplasmic reticulum (ER) of the lens epithelial cells can be observed (Fig. 3B). The BMs of the pigmented and non-pigmented epithelia of the ciliary body and iris in both *Raw* and *Bru* are locally disrupted or detached from the epithelial cells (Fig. 3C). It may be that these epithelial defects are responsible for the observed iridocorneal adhesion.

In general, the BM is more affected in *Bru* than in *Raw*, which correlates with the more severe histological changes. Typically, in epithelial BMs of the cornea in both *Raw* and *Bru*, almost normal BMs are found closely associated with hemidesmosomes. The interspace BMs of both mutants are less dense or even missing in *Bru*. This effect is also seen in the epithelium of the oesophagus (data not shown).

The vascular BMs surrounding the pericytes and endothelial cells of the retinal vessels are affected in both *Raw* and *Bru* mutants, displaying locally reduced thickness as well as focal interruptions (Fig. 3D). As no other histopathological phenotype was observed in *Raw* vessels, these changes may be linked with the observed arteriolar silversing seen by ophthalmoscope examination.

The inner limiting membrane (ILM) which covers the retina appears normal in most regions in *Raw* and *Bru*. Interestingly, local interruptions are observed in *Bru* (Fig. 3E), which can be accompanied by invasion of Mueller cell processes into the vitreous body. This may reflect the histological observation of fibro-vascular tufts in some *Bru* mice (10).

Analysis of the BMs of the kidney revealed no obvious defects in *Raw* mice. In *Bru*, the glomerular basal membrane (GBM) appears to be normal (Fig. 3F). However, in some areas, the endothelium underlying the GBM is locally degenerated, indicating a possible polarized pathological effect on these cells (data not shown). The tubular BMs of *Bru* mice were indistinguishable from normal BMs. However, Bowman’s capsule is severely affected. Rather than a continuous, dense linear structure, the BM shows variable thickness, folding and local splitting into multiple layers (Fig. 3G). These results indicate that changes in the BM of Bowman’s capsule may be the primary defect and determine the histopathological phenotype of *Bru* kidneys.

**Mutations in Col4a1 do not influence expression of other collagen IV isoforms**

During kidney organogenesis, a developmental switch reduces expression of α1(IV) and α2(IV) and activates the expression of α3(IV)–α5(IV), which are crucial for the mature GBM (14). In AS patients, the mutations result in a loss of a functional α3α4α5(IV) network and alter the expression of α1(IV) and α2(IV), whereby the expression of the α1α1α2(IV) network is maintained and partially compensates for the loss of the α3α4α5(IV) network (12).

Quantitative RT–PCR analysis on cDNA from adult eyes of *Bru* and *Raw* animals did not reveal any significant changes in the mRNA expression of the other collagen IV isoforms (data not shown). However, collagen type IV is subject to post- transcriptional modification and regulation, which could alter the protein levels of the different collagen IV isoforms (15). To investigate whether mutations in *Col4a1* lead to differences in protein expression, we performed immunohistochemistry on WT, *Raw* and *Bru* eye sections using collagen IV chain-specific monoclonal antibodies (16). No significant differences in distribution could be observed in the different mutants (data not shown). We conclude that no obvious changes in collagen IV mRNA and protein expression occur to compensate for the effects of mutant α1(IV).
DISCUSSION

BMs are complex aggregates of matrix components, and differences between composition and interactions underlie the diversity of individual membranes. Collagen type IV, the major structural component of BMs (17), is crucial for its normal functioning, although not for its initial formation during embryonic development (13). Six different alpha chains belong to the family of the type IV collagen molecules which can form three distinguishable networks (12). Mutations in the α3(IV), α4(IV) or α5(IV) chains cause AS (15,18–20), which is characterized by glomerulonephritis combined with deafness and ocular abnormalities (12). To date, no missense mutations have been described in vertebrates in Col4a1 or Col4a2. Heterozygous deficiency of Col4a1/Col4a2 in mouse results in no apparent phenotype but complete absence induces early embryonic lethality (13). Mutations in the homologous collagen IV genes in Caenorhabditis elegans are embryonic lethal (21,22).

Here, we describe an allelic series of Col4a1 point mutations that cause semi-dominant phenotypes in a number of tissues including eye and kidney. Homozygous Bru and Raw mutants are embryonic lethal; Raw homozygous mutants die by embryonic day 9.5. Mutant Col4a1 mRNAs are expressed in these mutants, and we suggest that in heterozygotes, the mutant proteins act as dominant negative molecules and affect synthesis, secretion or function of the α1.α1.α2(IV) network. This network is expressed in many BMs and defects are found in kidney, eyes and other tissues such as oesophagus and aorta. The BM defects are local and include variable thinning, partial disintegration, rupturing and detachment from underlying cells. These primary defects cause secondary effects on cells and tissues such as glomerulopathy and ocular phenotypes.

Collagen molecules contain large domains characterized by Gly-Xaa-Yaa repeats in which the glycine residue is most critical for stable triple helix formation (reviewed in 23). In our allelic series, we observed a striking genotype–phenotype correlation between individual mutations. The Raw mutation affects the Yaa residue and results in a very subtle phenotype with no apparent phenotype in WT. (A) The corneal epithelium of Raw is continuous but has areas in which the BM is less dense (arrow). In Bru, the BM can show interruptions (arrow). At hemidesmosomes (arrowhead), the BM appears irregular with a granular structure when compared with WT and Raw and a dilation of the ER can be observed (arrowhead). (C) The epithelial BM of the iris pigment epithelium has local disruptions and folds (black arrow) in both Raw and Bru. (D) The BM surrounding pericytes (PE) and endothelium (EN) of capillaries in the iris stroma (arrow) in Raw and Bru is fuzzier, less dense and has local interruptions. (E) In Bru, the ILM (arrowhead) covering the retina has local disruptions (arrow) which can lead to ectopia of Mueller cells (MC). The BM of the arterioles is thinner and has local disruptions (white arrow). The ILM in Raw is indistinguishable from WT. (F) The tubular BM (white arrow) and GBM (arrowhead) are mostly unaffected in Raw and Bru. Bowman’s capsule in Raw is unaffected (arrow) but shows a number of defects in Bru. (G) A different region of section 3F in Bru animals shows splitting and the formation of folds (arrow) in the BM of Bowman’s capsule in Bru. The epithelial cells have a hypertrophic appearance (arrowhead). Scale bar: (A and C–G) 1 μm; (B) 2 μm.
of the ER in the lens epithelium. If all collagen IV protomers containing a mutant α1(IV) chain were retained in the cell and/or degraded, there would simply be a reduction in the amount of protomer produced to 25% of WT.

Alternatively, some or all of the mutant protein may be secreted, may be incorporated in collagen IV networks and may alter the structure and interactive capacity of the collagen type IV network. This may compromise the stability and integrity of the BM or its formation and stable anchoring by disrupting interactions with other BM proteins or with matrix receptors, such as integrins or dystroglycan. This can be seen in local detachment of the matrix epithelial BMs of Raw and Bru (Fig. 3). The more pronounced disruption of the BM in the areas between hemidesmosomes shows the effect of mutant collagen IV on cell–matrix interactions, as different integrin receptors, such as α3β1 integrin, are found in these regions. The presence of specific receptors such as α6β4 integrin in hemidesmosomes (27) may stabilize the BM sufficiently at these sites. A direct blocking of interactions with α1β1 and α2β1 integrins can be ruled out as the mutations are not located within the major binding site (28).

The phenotypes of the Col4a1 mutants define a novel group of collagen IV defects unlike AS which is caused by defects in the α3.α4.α5(IV) network (12). Although both Bru mice and AS patients have kidney and eye pathologies, they are different. In AS, the GBM is mostly affected as mutations disrupt the α3.α4.α5(IV) network, which is crucial for the stability and function of the filtration barrier. Local degeneration of endothelial cells was observed in Bru, but no changes in podocytes are seen and the GBM appears to be unaffected. In contrast, Bowman’s capsule is severely affected inducing epithelial hypertrophy, which is probably secondary. Lenticonus is observed in 25% of AS patients (29) but has not been observed in Bru mice, although both AS and Bru have cataracts (29,30). A missense mutation has been described in COL4A4, which leads to autosomal dominant AS combined with cholesterolemia (31). Although the patients have typical GBM defects which are not observed in Raw and Bru, the arteriolar silverying in Raw could be explained by hypercholesterolemia. Indeed, Raw males have a mild reduction in the levels of HDL cholesterol. However there was no reduction in females and both sexes display arteriolar silverying. In addition, histopathology did not reveal any evidence indicating that the silvery appearance in the retinal arterioles is caused by fat deposition. Finally, in contrast to AS patients who can have sensorineural deafness, no deafness was observed in the Raw and Svc mutants, which were tested using the SHIRPA protocols (32) (Pat Nolan, personal communication), and Bru, which was tested using a click test. Overall, these differences reflect the individual molecular defects caused by mutations in the α1.α1.α2(IV) or α3.α4.α5(IV) network.

Bru eyes show a number of potentially glaucoma-related phenotypes including iris/corneal adhesions, buphthalmos and optic nerve cupping. They also display iris defects, corneal opacity and cataracts. The combination of these eye phenotypes is similar to AR anomaly which includes iridocorneal adhesions, iris hypoplasia, eccentric pupil, corneal opaqueness and glaucoma (2). Bru may be a model for a syndrome similar to AR’s anomaly, although so far no linkage has been reported of ASD to human chromosome 13q34, the location of COL4A1. However, it is worth noting that AR is genetically heterogeneous and a number of loci still remain to be identified (1,2).

The absence of abnormal histopathology underlines the subtlety of the Raw phenotype. However, ultrastructural analysis does reveal significant BM defects in some tissues, implying there is a threshold which has to be crossed before ultrastructural defects result in gross phenotypes and disrupt function. It is likely that the observed BM defects in retinal arterioles cause the arteriolar silverying, possibly by alterations in the reflective capacity of the vessel wall. The retinal arteriolar Raw phenotype, the BM defects in the retinal vessel wall and the bruising at birth in Bru and Svc animals strongly suggest that collagen type IV is important for vessel wall stability and vascular biology. During the revision of this manuscript, Gould et al. (33) showed that mutations in Col4a1 lead to porencephaly. We have not observed porencephaly in our mutants. However, Gould et al. (33) allude to ocular and renal defects in their animals, although the nature of these is not specified.

In conclusion, the diverse consequences of Col4a1 mutations described in this article define a spectrum of novel phenotypes, different from the loss of function mutation of Col4a1/4a2, which provides models for human disease pathologies.

MATERIALS AND METHODS

Animals

Studies were performed under guidance issued by the Medical Research Council and the UK Home Office.

Genome scan

A panel of 50 microsatellites (8) was amplified using DNA from 13 Bru mice. Primers were fluorescently labelled and analysed using ABI 310 Genetic Analyzer and ABI Genescan software (Applied Biosystems).

Identification of Col4a1 mutations

The Raw and Svc mutations were identified by sequencing of exons. For Bru, both the cDNA and exons of Col4a1 were sequenced. A list of primers is available upon request. RNA was isolated from Bru mice using Tri-Reagent (Sigma) and cDNA, generated using AMV Reverse-Transcriptase (Roche), was sequenced.

Phenotyping and histopathology

Bru eye phenotyping was performed using slit lamp examination as described earlier (http://www.eumorphia.org). Adult eyes and kidneys (3-month-old animals) were fixed overnight in Davidson’s solution (eyes) and 4% PFA (kidney), respectively, and embedded in paraffin wax. Sections were stained with haematoxylin–eosin using standard protocols.

Urine samples were collected from three animals (5–6 months old). Gel loading buffer containing DTT was added
directly to urine samples and proteins were separated using 10% SDS–PAGE. Gels were stained using silver staining (Biorad). Protein content was determined using standard Bradford assay.

Ultrasound analysis
Tissues were collected from three adult animals (3 months old) and fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 24 h at 4 °C. After post-fixation in 1% buffered osmium tetroxide and standard processing for embedding in epoxy resin (EPON 812), tissues were analysed using transmission electron microscopy (LEO 906E, Oberkochen, Germany).

Immunohistochemistry
Cryosections were fixed for 10 min in acetone followed by antigen retrieval using 0.1 M HCl/KCl for 10 min. After blocking in PBS containing 10% FCS, sections were incubated with chain-specific rat monoclonal collagen IV antibodies (16), followed by incubation with FITC-conjugated secondary antibodies (Jackson Laboratories). Images were collected using a fixed exposure time.

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