

Collagen IV is essential for basement membrane stability but dispensable for initiation of its assembly during early development

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Summary

Basement membranes are specialized extracellular matrices consisting of tissue-specific organizations of multiple matrix molecules and serve as structural barriers as well as substrates for cellular interactions. The network of collagen IV is thought to define the scaffold integrating other components such as, laminins, nidogens or perlecan, into highly organized supramolecular architectures. To analyze the functional roles of the major collagen IV isoform $\alpha1(IV)_2\alpha2(IV)$ for basement membrane assembly and embryonic development, we generated a null allele of the *Col4a1/2* locus in mice, thereby ablating both α -chains. Unexpectedly, embryos developed up to E9.5 at the expected Mendelian ratio and showed a variable degree of growth retardation. Basement membrane proteins were deposited and assembled at expected sites in mutant embryos, indicating that this isoform is dispensable for matrix deposition and assembly during early development.

However, lethality occurred between E10.5-E11.5, because of structural deficiencies in the basement membranes and finally by failure of the integrity of Reichert's membrane. These data demonstrate for the first time that collagen IV is fundamental for the maintenance of integrity and function of basement membranes under conditions of increasing mechanical demands, but dispensable for deposition and initial assembly of components. Taken together with other basement membrane protein knockouts, these data suggest that laminin is sufficient for basement membrane-like matrices during early development, but at later stages the specific composition of components including collagen IV defines integrity, stability and functionality.

Key words: Collagen IV, *Col4a1*, *Col4a2*, Knockout, Basement membrane, Development

Introduction

Basement membranes play fundamental roles in differentiation, proliferation, survival and migration of cells during embryonic development, but also serve as selective barriers and structural scaffolds (Schwarzbauer, 1999). Their similar ultrastructural appearance is in contrast to the variability of individual extracellular matrix proteins at specific sites, their mutual interactions and, accordingly, their supramolecular organization. The composition of basement membranes together with the repertoire of matrix receptors defines the specificity of cellular reactions.

Basement membranes comprise specialized matrix components from different protein families (Timpl, 1996). Laminins are a family of at least 15 distinct heterotrimeric molecules, which form gel-like aggregates (Colognato and Yurchenco, 2000). Nidogen-1 and -2 mediate the formation of ternary complexes between laminin and collagen IV in vitro (Fox et al., 1991; Kohfeldt et al., 1998), but their significance in vivo is not yet fully defined (Murshed et al., 2000; Schymeinsky et al., 2002). Heparan sulfate proteoglycan

perlecan is integrated into the networks of laminins and collagen IV and plays important roles in the maintenance of basement membrane integrity, its filtration functions and also the local storage of growth factors (Göhring et al., 1998). More complexity may be inferred from the identification of new isoforms of known molecules and discovery of additional specialized proteins (Erickson and Couchman, 2000).

So far, the analyses of human diseases and knockout animal models have defined distinct functions of the various basement membrane components for its overall integrity and function. For example, tissue-specific defects at various stages of development are caused by the lack of perlecan (Costell et al., 1999), by mutations within any of the genes coding for laminin-5 chains ($\alpha3$, $\beta2$, $\gamma2$) (Pulkkinen and Uitto, 1999), the laminin $\alpha2$ chain (Helbling-Leclerc et al., 1995) or by the disruption of the laminin $\alpha5$ gene (Miner et al., 1998). However, only the targeted inactivation of the laminin $\gamma1$ chain resulted in the lack of basement membranes due to the ablation of 10 out of the 14 currently known laminin isoforms, causing mutant embryos to die at E5.5 from early differentiation

defects (Smyth et al., 1999). The complexity of contributions of individual proteins to *in vivo* functions and their mutual interactions is further illustrated by the targeted deletion of the highly specific binding site of nidogen-1 on the laminin γ 1 chain causing local disintegration of basement membranes, which leads to renal agenesis and impaired lung development (Willem et al., 2002). In contrast, the corresponding ablation of nidogen-1 or -2 resulted in no obvious phenotypes (Murshed et al., 2000; Schymeinsky et al., 2002). Although the differential expression of components by neighboring cells (Ekblom et al., 1994; Thomas and Dziadek, 1993) and the central role of matrix receptors in coordinating the spatial and temporal aggregation of basement membranes (Henry and Campbell, 1998; Li et al., 2002) has shed new light on the molecular mechanisms of the supramolecular assembly of basement membranes, the contributions of individual proteins is not yet fully understood.

Members of the collagen IV family (Kühn, 1995) are found in all basement membranes and are characterized by their ability to form complex, covalently linked structural scaffolds, proposed to be required for the basement membrane assembly process (Timpl et al., 1981; Yurchenco and Furthmayr, 1984). Six individual chains have been identified, α 1(IV)- α 6(IV), which assemble into three distinct protomers, α 1. α 1. α 2(IV), α 3. α 4. α 5(IV) and α 5. α 5. α 6(IV) (Boutaud et al., 2000; Hudson et al., 2003). In addition to the ubiquitous α 1/ α 2 network, two independent networks, α 3. α 4. α 5(IV) and a combined aggregate of α 1. α 1. α 2/ α 5. α 5. α 6(IV) molecules, have been described (Borza et al., 2001). They are deposited later in development and replace the initial α 1. α 1. α 2(IV) network in a tissue-specific manner and define specialized basement membrane structures and functions. The α 3. α 4. α 5(IV) network is found in the glomerular basement membrane and some tubules of the kidney, in the lung, testis, cochlea and eye, whereas the α 5. α 5. α 6(IV) protomer is detected in the skin, esophagus, Bowman's capsule of the kidney and smooth muscle cells (Ninomiya et al., 1995). Therefore, mutations in any of the genes coding for the α 3(IV)- α 5(IV) chains cause tissue-specific phenotypes, associated with different forms of the Alport's syndrome (Hudson et al., 2003; Tryggvason, 1996) or related diseases (Badenas et al., 2002; Buzza et al., 2001). Yet, no genetic defects in either the α 1(IV) or α 2(IV) chains have been linked to human diseases so far, while mutations of collagen IV related genes in *Drosophila melanogaster* or *Caenorhabditis elegans* lead to embryonic lethal phenotypes (Borchiellini et al., 1996; Gupta et al., 1997). The α 1. α 1. α 2(IV) isoform is detected in the mouse embryo at the 32-64 cell stage (Dziadek and Timpl, 1985) and is therefore thought to be of crucial importance for the formation of the first sheet-like structured basement membrane in development. Together, these data support a fundamental role for the major collagen IV isoform during development.

We have defined for the first time the functional significance of the major collagen IV isoform α 1. α 1. α 2(IV) *in vivo* by the targeted inactivation of the *Col4a1/2* locus in mice. Surprisingly, deposition of basement membrane components was not dependent on the presence of collagen IV during early embryonic development and embryonic lethality occurred only at E10.5-11.5 because of impaired basement membrane stability. These data suggest therefore that collagen IV is not

critically important for the formation of basement membrane-like matrices, but is indispensable for structural integrity and functions of basement membranes at later stages.

Materials and methods

Generation of a *Col4a1/2* null allele

The murine *Col4a1/2* locus was isolated from a 129/Svj mouse genomic library (Stratagene, La Jolla) and subclones spanning 25 kb surrounding the shared promoter were generated by standard methods. The targeting vector (pNTK/HXEE-4) was combined from a 5 kb *EcoRI-XhoI* fragment from intron 1 of *Col4a1*, a 3 kb *HindIII-XhoI* fragment from intron 3 of *Col4a2* separated by a neomycin cassette under the control of the phosphoglycerokinase (pgk) promoter. R1 ES cells were transfected with the *NotI* linearized targeting vector as described previously (Mayer et al., 1997). G418 resistant clones were screened by Southern hybridization after *EcoRI* digestion with probe P1 (1.1 kb *XhoI-BamHI*), probe P2 or the neomycin gene.

Generation of a mouse strain with deficient *Col4a1/2* locus

Two independent clones (1D2, 11D3) with correct integration were injected into C57BL/6 blastocysts and transferred into pseudopregnant CD1 foster mothers. Highly chimeric males were obtained and crossed with C57BL/6 females to obtain heterozygous F₁ offspring. Genotyping was done as described above or by PCR using the primers *Col4a2-Ex2dw* (5'-GTTAGGAAGGGATCGAGC), *Hind+104up* (5'-ACCACCTCTGAGTTTCTGGA) and *Neodw* (5'-TCGTGCTTACGGTATCGCC), resulting in fragments of 597 and 650 bp for the wild-type and mutant alleles, respectively. Heterozygous mice were mated to obtain time-staged embryos.

Histological analysis and immunohistochemistry

E9.5-E11.5 embryos were dissected, fixed for 2-4 hours in 4% (w/v) paraformaldehyde in PBS and then embedded in paraffin wax, sectioned (5-10 μ m) and stained using Hematoxylin-Eosin or Periodic acid-Schiff reaction according to standard methods. For immunostaining, tissues were snap-frozen in OCT and cryosections (7-12 μ m) were transferred to silanized slides. Immunostaining was performed with polyclonal antibodies to nidogen-1, laminin-1 recognizing isoforms containing α 1, β 1 and γ 1 chains and collagen IV (Fox et al., 1991). Monoclonal antibodies (H12, H22 and H53) were generated against the individual collagen IV subunits α 1(IV), α 2(IV) and α 5(IV), respectively (Sado et al., 1995). Commercially available collagen IV antibodies (Chemicon Int.), PECAM-1 (PharMingen) and Cy2- or Cy3-conjugated secondary antibodies (Dianova) were used.

Ultrastructural analysis

Tissue specimens were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 24 hours at 4°C, postfixed in 1% buffered osmium tetroxide, and routinely processed for embedding in epoxy resin (EPON 812) and transmission electron microscopy (LEO 906E, Oberkochen; Germany).

Detection of collagen IV specific mRNA

Total RNA was isolated from E9.5 and E10.5 littermate embryos using Trizol (PEQ-Lab). Collagen IV-specific mRNAs were detected after reverse transcription using random primers (Roche) by PCR amplification using cDNA-specific primers (20 bases, Tm 58°-60°C) amplifying fragments according to database entries specific for α 1(IV) (a1N: MUSCOL4A1/J04694, pos. 272-613, 342 bp), α 2(IV) (a2N: MUSCOL4A2/J04695, pos. 92-260; 169 bp), α 5(IV) (a5N: AB041350; pos. 4858-5139; 282 bp) or annexin A5 (Anxa5: MUSANXV/D63423; pos. 51-242; 192 bp) as internal controls (Fig. 1). Quantitative PCR analyses (Fig. 4) were performed in a thermal cycler equipped with a real-time fluorescence detector (iCycler, Bio-

Rad) and the Absolute™ QPCR SYBR Green Mix (ABgene) was used according to the suppliers protocols. The primers (18-20 bases; Tm 58°C) were selected from published sequences (GenBank) to amplify cDNA fragments specific for $\alpha 1(\text{IV})$ and $\alpha 5(\text{IV})$ as given above, as well as for $\alpha 2(\text{IV})$ (MUSCOL4A2/J04695, pos. 92-200, 109 bp), $\alpha 3(\text{IV})$ (AF169387, pos. 4678-4832, 155 bp), $\alpha 4(\text{IV})$ (AF169388, pos. 4672-4881, 210 bp), $\alpha 6(\text{IV})$ (AB041351, pos. 4570-4876, 307 bp), laminin $\alpha 1$ chain (NM_008480, pos. 5011-5257, 247 bp), nidogen-1 (NM_010917, pos. 3889-4036, 148 bp) and GAPDH (NM_008084, pos. 581-1023, 443 bp). PCR-amplifications were performed in triplicates with 50 cycles at an annealing temperature of 58°C. The iCycler analysis software (BioRad) was used for data analysis and the amounts of products were calculated from standard curves generated in parallel from dilutions of each PCR products. Data were normalized to the levels of GAPDH-specific cDNA set as 100%. Calculation of mean values and standard deviations were based on triplicate assays, analyses were repeated three times and representative results are shown. Specificity of PCR products was tested by measurement of Tm-value and gel electrophoresis after 50 cycles.

Visualization of the vascular system by the use of the *Anxa5-lacZ* transgene

The *Anxa5-lacZ* fusion gene was generated by homologous recombination in ES cells and the derived mouse strain showed no obvious altered phenotype (Brachvogel et al., 2001; Brachvogel et al., 2003). By mating mice heterozygous for the *Col4a1/2* mutation with *Anxa5^{lacZ/lacZ}* mice, a strain was established with *Col4a1/2^{+/-}Anxa5^{lacZ/lacZ}* genotype and used for generating embryos with various *Col4a1/2* genotypes on a heterozygous *Anxa5^{lacZ/+}* background. Time-staged embryos were isolated, genotyped, stained for β -gal activity and used for vibratome sectioning as described (Brachvogel et al., 2001).

Results

Targeted inactivation of the *Col4a1/Col4a2* genes does not interfere with early embryonic development

The *Col4a1* and *Col4a2* genes are closely linked on mouse chromosome 8 in a unique head-to-head arrangement and separated by a short, shared promoter region similar to the human genes (Burbelo et al., 1988; Pöschl et al., 1988). The first exon of *Col4a1* and exons 1-3 of *Col4a2* encode the 5' untranslated regions together with the signal peptides, respectively. This unique arrangement enabled the simultaneous inactivation of both genes in ES cells by the deletion of a 1.5 kb region containing the 5' exons of each gene together with their shared promoter (Fig. 1A). After homologous recombination in ES cells, heterozygous clones with correct integration were used for the establishment of mouse lines as shown

by Southern blotting and PCR-analysis (Fig. 1B,C). The expression of correctly processed $\alpha 1(\text{IV})$ and $\alpha 2(\text{IV})$ mRNAs was ablated in mutant embryos, while they were present in E9.5 and E10.5 wild-type and heterozygous embryos as detected by RT-PCR (Fig. 1D).

Heterozygous mice appeared normal and did not display overt anatomical or behavioral abnormalities. However, no mice homozygous for the mutation could be identified after weaning, indicative of an embryonic lethal phenotype (Table 1). Surprisingly, genotypes of time-staged embryos showed no significant deviation from normal Mendelian ratio up to E9.5, but thereafter a steady decrease of homozygous mutants demonstrated embryonic lethality between E10.5-E11.5. In parallel an increase in disintegrated or resorbed embryos was seen. Occasionally, homozygous mutants were found at E11.5, but embryos never survived beyond E12. Collagen IV-deficient embryos showed no or only marginal changes in overall size and body shape at E9. However, a variable degree of growth retardation was observed at E9.5-E11 for many of the mutant embryos (Fig. 2). In most cases the smaller size was not linked to a significantly reduced number of somites. Occasionally, some mutant embryos were severely developmentally retarded and corresponded to stages 12-36 hours earlier, when compared to their normal littermates. Organ development was

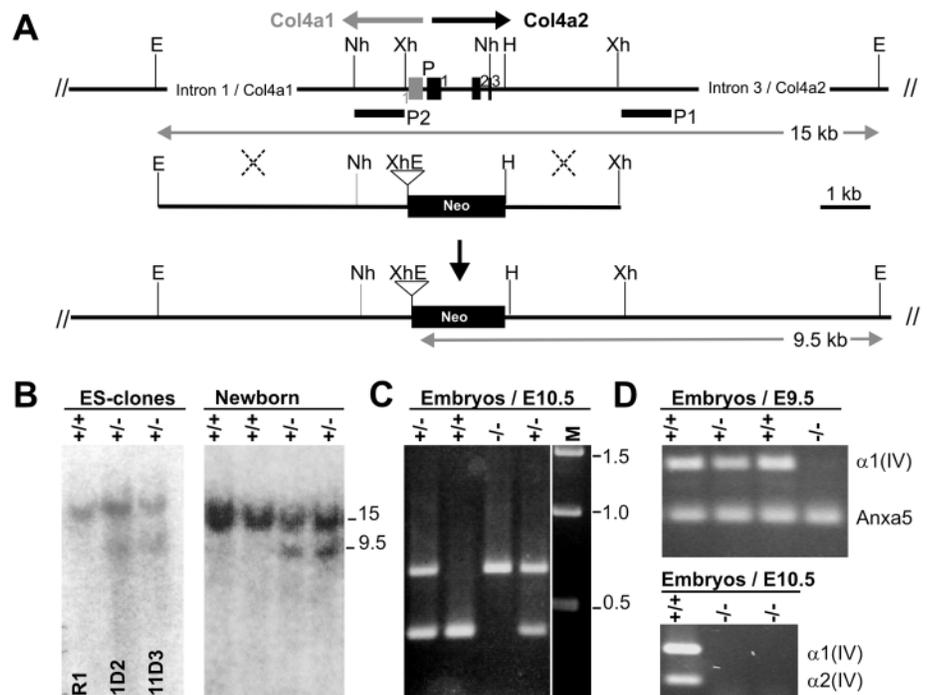


Fig. 1. Targeted inactivation of the *Col4a1/2* locus. (A) The murine *Col4a1/2* genes are arranged head-to-head with a common shared promoter element (P), exons (shaded squares) and relevant restriction sites (E, *EcoRI*; Xh, *XhoI*; Nh, *NheI*; H, *HindIII*) are indicated. The targeting construct containing the pgk/neomycin resistance cassette (Neo) and the mutant locus (bottom) are shown. Probes P1, P2 and the detected *EcoRI* fragments (arrows) are marked. (B) Genotyping of ES cells and newborns by Southern blotting. DNA was isolated, digested with *EcoRI* and hybridized with probe P1. The 15 kb and 9.5 kb bands are the wild-type and mutant alleles, respectively. (C) Genotyping of E10.5 embryos by PCR. The 0.7 and 0.4 kb fragments represent the mutant and wild-type alleles, respectively. Markers are indicated (kb). (D) Detection of $\alpha 1(\text{IV})$ and $\alpha 2(\text{IV})$ mRNAs in embryos at E9.5 and E10.5 by RT-PCR. Annexin A5 (*Anxa5*) was used as an internal control.

Table 1. Deficiency of collagen IV causes embryonic lethality at E10.5-E11.5

Stage	Litters	Resorbed	<i>n</i>	+/+	+/-	-/-	% expected (-/-)
E9.5	22	0	184	53	87	44	94
E10-10.5	23	3	189	58	96	35	68
E11-11.5	10	20	62	16	42	4	21
E12.5	9	19	68	19	49	0	0
Adult	18		119	43	76	0	0

Embryos at the indicated stages were isolated from intercrosses of heterozygous animals and the total number of intact (*n*) as well as disintegrated or resorbed embryos are indicated. Calculation of % expected (-/-): $n(-/-) \times 100 / 3n(+ / +) + n(+ / -)$.

similar to controls and all living collagen IV-deficient embryos had a beating heart. In addition, chorio-allantoic fusion had taken place and development of extra-embryonic structures, such as yolk sac and amnion showed no obvious structural defects (not shown). Together, these data demonstrate that early embryonic development and organogenesis occurs normally in the absence of collagen IV.

Basement membrane components are deposited in mutant embryos

The unexpected finding that collagen IV-deficient embryos developed up to E10.5 suggested that basement membrane-like structures might form in the absence of collagen IV and enable early development and differentiation. Therefore, we analyzed the deposition of well-characterized ubiquitously present basement membrane proteins by immunofluorescence (Fig. 3).

As expected, no $\alpha 1(\text{IV})/\alpha 2(\text{IV})$ -containing collagen could be detected in E9.5-E10.5 homozygous mutant embryos (Fig. 3). Surprisingly, however, the laminar deposition of laminins and nidogen-1 was still detected adjacent to epithelia, vascular endothelia, somites, notochord or the neuroectoderm, indicating that the absence of collagen IV did not perturb secretion and deposition of these components. However, the staining appeared weaker when compared with littermate controls (Fig. 3).

These findings suggested that the formation of basement membrane-like structures in collagen $\alpha 1/\alpha 2(\text{IV})$ -deficient embryos is either due to the compensatory expression of alternative collagen IV isoforms or that laminins are sufficient for the recruitment and overall organization of basement

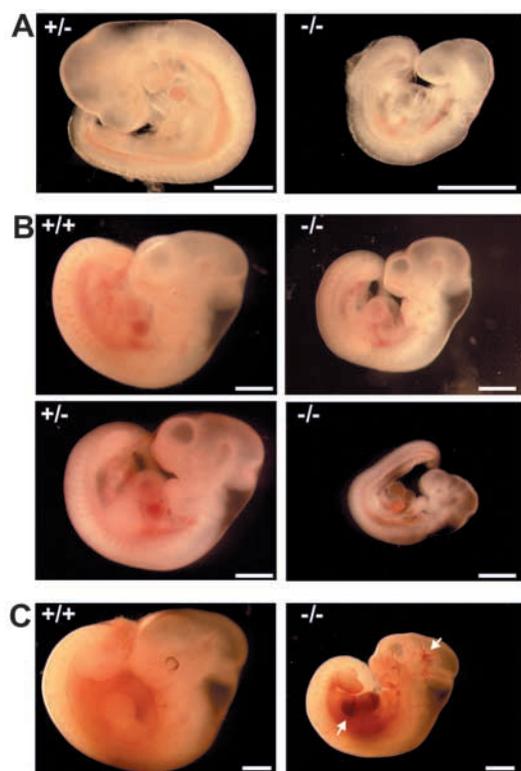


Fig. 2. Deficiency of collagen IV expression causes a variable degree of growth retardation in embryos at E9.5 (A), E10.5 (B) and E11.5 (C). The genotypes of representative littermates are indicated (+/+, +/-, -/-). Embryos at E11.5 show bleeding from the heart and dilation of blood vessels (arrows). Scale bars: 100 μm .

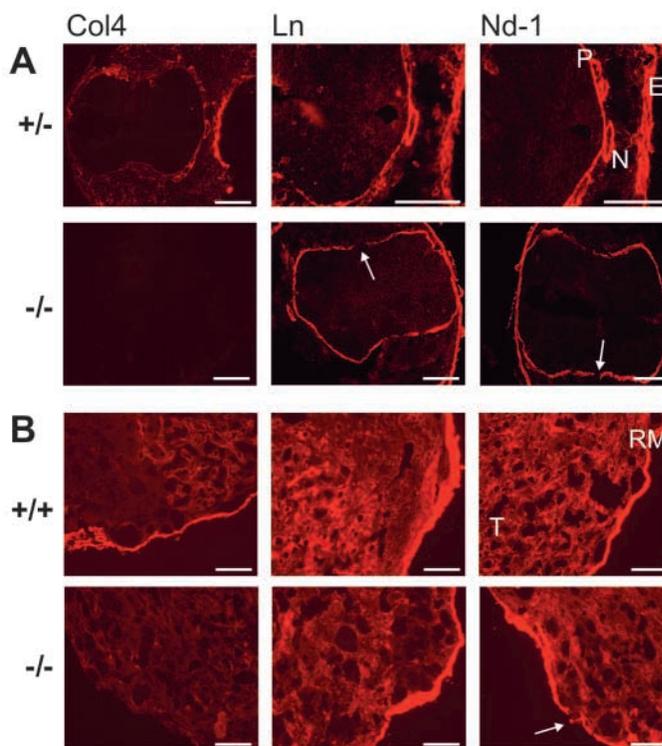


Fig. 3. Basement membrane-like structures are deposited in the absence of collagen IV. Collagen IV (Col4), nidogen-1 (Nd-1) and laminins containing $\alpha 1$, $\beta 1$ or $\gamma 1$ subunits (Ln) were detected by immunohistochemistry at E10.5 in embryos (A) or in Reichert's membrane (B) with the indicated genotypes. Basement membrane discontinuities are indicated by arrows. N, notochord; P, pia; E, epidermal basement membrane; RM, Reichert's membrane; T, trophoblast cells. Scale bars: 100 μm .

membrane protein aggregates. To distinguish between the two possibilities, we analyzed mRNA levels of $\alpha 1(\text{IV})$ - $\alpha 6(\text{IV})$ chains in embryos at E9.5 and E10.5 by quantitative RT-PCR (Fig. 4A). In wild-type embryos only mRNA specific for the $\alpha 5(\text{IV})$ chain was expressed in addition to the $\alpha 1(\text{IV})$ and $\alpha 2(\text{IV})$ chains, while $\alpha 3(\text{IV})$, $\alpha 4(\text{IV})$ and $\alpha 6(\text{IV})$ mRNAs were not present in detectable amounts. Similarly, in $\alpha 1/\alpha 2(\text{IV})$ -deficient embryos only $\alpha 5(\text{IV})$ -specific mRNA was expressed, whereas other collagen IV chains were only detected in trace amounts. The mRNA levels of laminin $\alpha 1$ chain and nidogen-1 were also not significantly altered in mutant embryos.

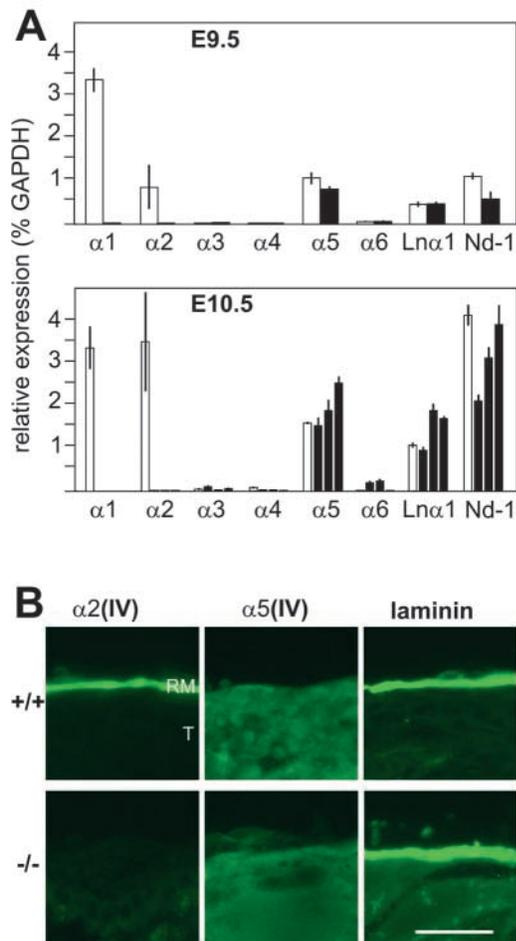


Fig. 4. Deficiency of $\alpha 1(\text{IV})/\alpha 2(\text{IV})$ collagen is not compensated by the increased expression of other collagen IV chains. (A) Relative mRNA levels of collagen IV isoforms ($\alpha 3$ - $\alpha 6$), laminin $\alpha 1$ chain (Ln- $\alpha 1$) and nidogen-1 (Nd-1) were determined by quantitative RT-PCR in RNA isolated from individual wild-type (white bars) and $\alpha 1(\text{IV})/\alpha 2(\text{IV})$ -deficient embryos (black bars) from E9.5 and E10.5 littermates. The $\alpha 3(\text{IV})$, $\alpha 4(\text{IV})$ and $\alpha 6(\text{IV})$ mRNAs are not detectable or are present at only very low levels in any genotype. In mutant embryos no compensatory upregulation of $\alpha 5(\text{IV})$, laminin $\alpha 1$ chain and nidogen-1 is observed. Relative mRNA levels are expressed as a percentage of GAPDH expression. (B) No deposition of $\alpha 5(\text{IV})$ protein is observed by immunofluorescence in the Reichert's membrane of normal (+/+) or mutant embryos (-/-) at E10.5. A faint intracellular staining in trophoblast cells indicates the expression of low levels of non-secreted $\alpha 5(\text{IV})$ protein. Antibodies detecting laminins containing the $\alpha 1$, $\beta 1$ or $\gamma 1$ chains and $\alpha 2(\text{IV})$ collagen were used as positive controls. Scale bar: 10 μm .

Additionally, we could not detect any collagen IV chains in embryos (not shown) or the Reichert's membrane of mutant embryos by immunohistochemistry using subunit-specific antibodies (Fig. 4B). Only low levels of intracellular staining were seen for $\alpha 5(\text{IV})$, but the protein could not be detected in deposited matrices. Taken together, these data demonstrate that no significant amounts of any collagen IV isoform could be secreted in $\alpha 1/\alpha 2(\text{IV})$ -deficient embryos and further suggest that basement membrane components can be deposited in the absence of any collagen IV protomer during early embryogenesis.

Deficiency of collagen IV results in altered basement membrane structures

Although immunofluorescence analysis clearly demonstrated the presence of basement membrane-like structures in mutant embryos, the staining looked more patchy and appeared weaker when compared to littermate controls (Fig. 3). In addition, discontinuities or ruptures of basement membranes were apparent both in mutant embryos (Fig. 3A) and in extraembryonic tissues (Fig. 3B). The most obvious changes were visible in the specialized Reichert's membrane underlying the parietal endoderm cells. It is normally formed

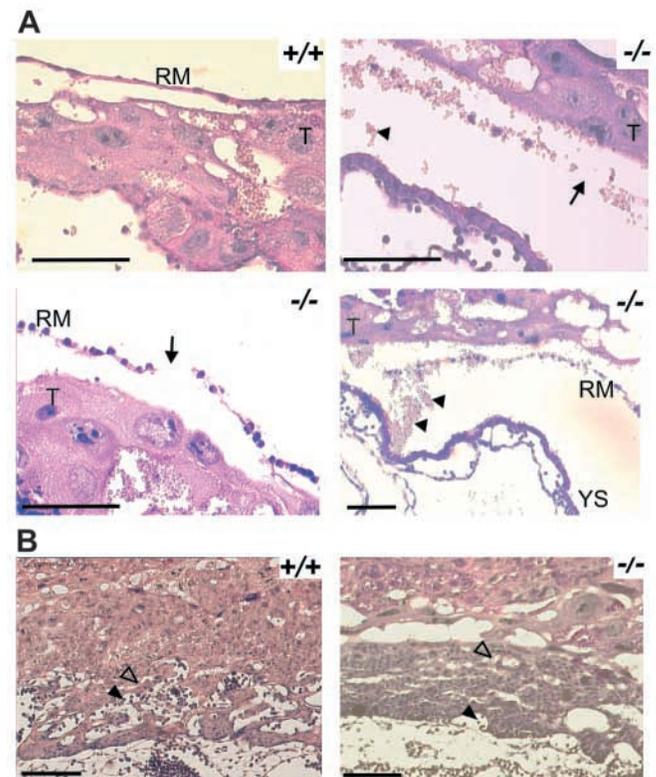
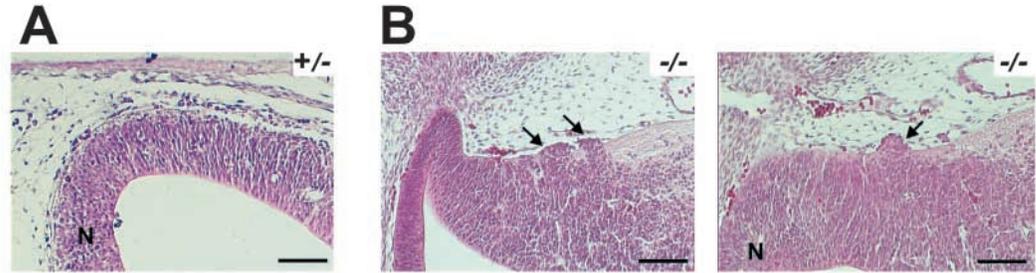


Fig. 5. Defects of placental structures. Hematoxylin-Eosin staining of paraffin wax sections of deciduas (E11.5) from collagen IV-deficient (-/-) embryos and normal littermates (+/+). (A) The Reichert's membrane (RM) of collagen IV-deficient embryos appears thinner, and ruptures (arrows) are causing severe bleeding of maternal blood (arrowhead) into the yolk sac cavity. YS, yolk sac. (B) The development of the labyrinth layer of the placenta is retarded in mutant embryos (-/-) as compared to wild-type embryos (wt). The contact between maternal (open arrowheads) and embryonic blood (closed arrowheads) is reduced. Scale bar: 100 μm .

Fig. 6. (A) Wild-type control. (B) Neuronal ectopias (arrows) are present in E11.5 mutant embryos because of the migration of neuronal cells through the pial basement membrane into the surrounding mesenchymal layers (arrows). N, neuroectoderm. Scale bars: 100 μ m.



from typical basement membrane matrix proteins such as collagen IV, laminins, nidogens and perlecan, and defines a stable barrier between parietal endoderm cells and the trophoblast layer (Fig. 5A). In the mutants, however, it appeared fragile, thin or disorganized and breaches in the basement membrane were visible in many places. As a consequence, excessive amounts of maternal blood were found in the yolk sac cavity (Fig. 5A), which may be one of the major reasons leading to death and resorption of mutant embryos.

The overall organization of the placenta was retained in mutant embryos, but an impaired development of the labyrinth layer was observed, as seen by its reduced thickness in collagen IV-deficient embryos (Fig. 5B). As a consequence, the maternal and the embryonic blood systems, recognized by their typical blood cells, are separated by cells and deposited matrix when compared with the intimate contact seen in wild-type littermates. This may limit the efficiency of the exchange of nutrients, which becomes increasingly important at later stages of development. We therefore conclude that the impaired placental development contributes to the observed phenotype of growth retardation of collagen IV-deficient embryos.

No gross abnormalities in organ development or tissue structures were observed in collagen IV-deficient embryos, but indications of an increased instability of basement membranes was detected in various tissues. In the brain, neuronal ectopias were apparent in embryos at E11.5 (Fig. 6), characterized by cells protruding into the surrounding mesenchyme. Local disruption of the pial basement membrane (see Fig. 8) is most probably the reason for the aberrant migration of neural cells.

The observation of bleeding into the pericardium and the presence of dilated blood vessels at E10.5–11.5 (Fig. 2C) also suggested that stability and internal organization of embryonic vascular basement membranes is perturbed. To test whether collagen IV deficiency also interferes with vascular development, we used the recently described *Anxa5-lacZ* transgene, which specifically detects perivascular cells, to visualize most blood vessels and capillaries in collagen IV mutant embryos (Brachvogel et al., 2001). β -gal staining demonstrated that the overall formation of the vasculature was indistinguishable between collagen IV mutant and wild-type embryos, as major vascular elements and the capillary networks in the embryo (Fig. 7A) and the yolk sac (not shown) were present and looked normal. This proves that the recruitment of perivascular cells (Carmeliet, 2000), detected by expression of the transgene, was not perturbed. However, subtle changes in the spatial organization were observed in the perineural vascular plexus surrounding the neuroectoderm, reflected by a reduced density of capillaries, a less regular arrangement of blood vessels and irregular protrusions of capillaries into the neural layer (Fig. 7B). These data indicate

that vascular development is independent of collagen IV, while the local organization of capillary networks may depend on the contact with collagen IV-containing basement membranes.

Ultrastructural analysis of basement membranes in collagen IV-deficient embryos

Ultrastructural analysis of basement membranes in E10.5 embryos (Fig. 8) further supported the changes we observed by histological analysis. The epidermal (Fig. 8A) and pial basement membranes (Fig. 8B) of collagen IV-deficient embryos were variable in thickness when compared to littermate controls. In some regions the matrix was amorphously deposited or completely absent. In addition, the

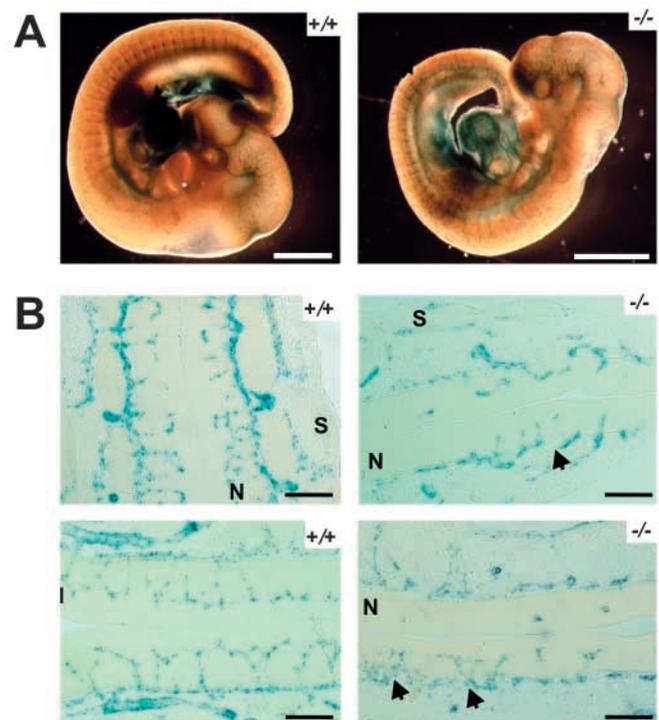
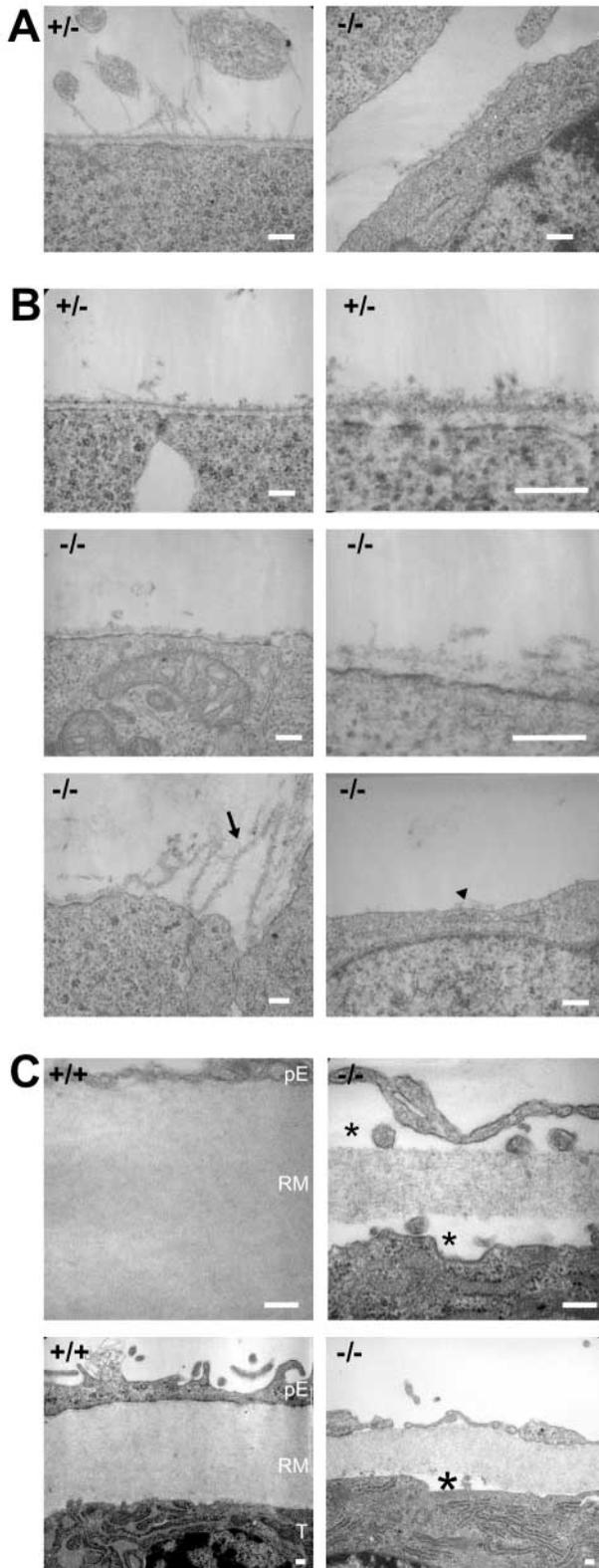


Fig. 7. Deficiency of collagen IV does not affect the development of the vascular system, but causes aberrant organization of capillary structures. (A) The vascular system was detected by staining for β -gal in E10.5 wild-type (+/+) and collagen IV-deficient (-/-) embryos on a *Anxa5⁺/lacZ* background. No gross differences are seen in whole-mount stainings. Scale bar: 1 mm. (B) The capillary plexus forming close to the pial basement membrane is less dense in mutant (-/-) embryos and capillaries entering the neuroectoderm are irregularly arranged (arrows) and detected in lower numbers in collagen IV-deficient embryos than in littermate controls (+/+). Scale bars: 100 μ m.

appearance of deposited basement membrane-like matrices was more irregular and less electron-dense. The contact to underlying cells was lost at some sites and coincided with detached and irregularly folded basement membranes (Fig. 8B), indicating that the intrinsic cohesion of remaining matrix

components is still sufficient to stabilize its laminar structure. Together, these data suggest a reduced interaction of cells with collagen IV-deficient basement membranes and it is likely that signaling, differentiation, growth and survival of cells is affected.

Reduced thickness and an aberrant internal structure of basement membranes was most obvious in the Reichert's membrane (Fig. 8C). While this thick basement membrane had a homogenous appearance in normal embryos, electron-dense string-like structures in a loose and irregular arrangement were evident in the mutants. We speculate that these structures reflect a modified supramolecular organization of components in the absence of the stabilizing collagen IV network that becomes visible after fixation of specimens. At many sites also the contact with the trophoctoderm layer and parietal endoderm cells was lost and again suggests defects in cell-matrix interactions.



Discussion

The collagen IV network has been suggested to provide the basic scaffold into which the laminin networks and perlecan oligomers are integrated by the cross-linking nidogens to finally assemble the sheet-like basement membrane complex (Fox et al., 1991; Timpl and Brown, 1996). We show that deposition of basement membrane matrices is not critically dependent on the presence of collagen IV, and embryos develop up to E10-E11, indicating that collagen IV is dispensable during early development, but essential for the structural integrity of these matrices at later stages.

The presence of basement membrane-like matrices in the absence of the major collagen IV isoform $\alpha1.\alpha1.\alpha2(IV)$ shows that the secretion and deposition of other basement membrane proteins is sufficient to provide the essential functions of basement membranes during early development. The ability of laminin to form gel-like aggregates in vitro in the presence of calcium ions (Yurchenco and Cheng, 1993) suggests that minimal matrices, containing laminins as major structural components, are sufficiently stable to form laminar structures and enable differentiation, proliferation and survival of cells. The hypothesis that only laminins are crucial during early development is supported by the fact that the deletion of the laminin $\gamma1$ chain caused fatal consequences during the peri-implantation period (Smyth et al., 1999), but not the ablation of any other basement membrane component analyzed so far (Costell et al., 1999; Murshed et al., 2000; Schymeinsky et al., 2002). However, there is currently no ultimate model available to describe the molecular organization of basement membrane components in early embryos. It remains therefore open at present whether laminins alone are sufficient or whether any

Fig. 8. Ultrastructural abnormalities in basement membranes of collagen IV-deficient embryos. Epidermal (A) and pial basement (B) membranes in E10.5 mutant embryos (-/-) are amorphyously deposited or absent when compared to normal controls. In some areas the deposited material detaches from the surface of cells and forms irregular folds (arrow) or is almost completely absent (arrowhead). (C) Reichert's membrane of mutant embryos (E10) is significantly thinner than in wild-type in comparable areas and shows an altered texture. Additionally, detachment from trophoctoderm (T) and parietal endoderm cells (pE) is observed (*). Scale bars: 0.2 μm.

other basement membrane component is required for its stabilization. The absence of single components, like nidogens, perlecan or even collagen IV may be tolerated to form minimal basement membrane-like matrices that support at least some basic functions.

The major basement membrane components are correctly deposited, suggesting that the absence of collagen IV does not negatively regulate secretion or deposition of laminins, nidogens or perlecan. Occasionally, we observed a weaker staining intensity in mutants, which may be due to a decreased synthesis. We favor, however, the idea that this effect is based on a reduced retention of basement membrane components into a complex supramolecular aggregate in the absence of the covalently stabilized collagen IV network. A similar finding has been observed in mice lacking the nidogen-binding module of the laminin $\gamma 1$ chain, in which nidogen was not retained in basement membranes despite normal protein amounts (Willem et al., 2002).

The development of embryos up to E10-E11 demonstrates that the collagen $\alpha 1. \alpha 1. \alpha 2(\text{IV})$ network is not crucial for blastocyst formation, maturation, implantation and initial morphogenetic events. The constraints on the intrinsic stability of basement membranes to maintain its barrier functions increase gradually in the embryo during progressive proliferation of cells and the differentiation and expansion of cell layers. Therefore, the observed local disintegration of basement membranes in collagen IV-deficient embryos later in development is probably due to reduced stability of matrix aggregates. One example is the vascular bleeding in the heart and arteries as the cell-cell contacts are not sufficient to stabilize the tissue architecture. A second example is the fracture of the Reichert's membrane and accordingly, insufficient barrier between maternal and embryonic environments causes excessive bleeding into the yolk sac cavity and resorption of embryos. The loss of barrier function is also reflected in the development of neuronal ectopias in E11.5 mutant embryos because of the local disintegration of the basement membrane separating the embryonal neuroectoderm from the surrounding mesenchyme. Similar cortical defects have been described for mice lacking perlecan, mutations inhibiting the laminin-nidogen interaction or upon inactivation of cell surface receptors (Costell et al., 1999; Georges-Labouesse et al., 1998; Graus-Porta et al., 2001; Halfter et al., 2002; Moore et al., 2002). These defects cause fragmented pial basement membrane between E10.5-12.5 (Halfter et al., 2002) through which Cajal Retzius cells and other neuronal cells migrate into the surrounding mesenchyme. Together, these phenotypes support the notion that collagen IV has an essential role to maintain the structural integrity of basement membranes at stages associated with increasing mechanical demands. Yet, we cannot exclude, at the moment, the possibility that impaired cell-matrix interactions cause the detachment of basement membranes from underlying cell layers and contributes to the observed phenotypes. Loss of cell-matrix contact, as seen at many sites, could also be an explanation for the gradual increase of growth retardation preceding the lethality around E10-E11 and it may influence proliferation and differentiation of cells. Alternatively, impaired placental development and the observed local distortion of the capillary networks may decrease nutrition of the embryos.

Although basement membrane-like deposits formed in collagen IV-deficient embryos, they showed distinct differences to those of controls in ultrastructural analysis. Reduced thickness, discontinuities, amorphous deposits or loss of any matrix deposits were seen. In contrast to the homogenous appearance of matrix in normal embryos, electron-dense, irregular aggregates were detected in the absence of collagen IV. A similar ultrastructure was seen before in a cell line known to secrete a collagen IV-free basement membrane-like matrix (Brauer and Keller, 1989). Therefore, we propose that the altered supramolecular architecture of basement membranes in the absence of collagen IV results in the formation of aberrant aggregates. Mutations of collagen IV-related genes cause embryonic lethality in invertebrates. Reduced expression of the collagen IV-related gene *Dcg* in *Drosophila* (Borchiellini et al., 1996) and null mutations in collagen IV chain homologues *emb-9* and *let-2* in *Caenorhabditis* resulted in failure of basement membrane structures and finally caused embryonic lethality because of the lack of functional muscle attachment sites (Gupta et al., 1997). Although it was surprising that corresponding mutants in mouse embryos are able to develop up to E10-E11, the residual functionality of collagen IV-deficient basement membranes seems to be sufficient for basic functions and enable early embryonic development.

In contrast to invertebrates, mammals contain multiple collagen IV isoforms and the consequences of mutations in collagen IV variants reflect the pattern of expression and specific basement membrane functions. Mutations of the *COL4A3-COL4A6* genes in humans are associated with various forms of Alport's related syndromes (Gunwar et al., 1998; Hudson et al., 2003; Kashtan, 2000; Tryggvason, 1996). During development the ubiquitous collagen IV network, $\alpha 1. \alpha 1. \alpha 2(\text{IV})$, is replaced in a tissue- and stage-specific manner by the minor isoforms and it is believed that the local expression of these chains restricts the impairment of basement membrane function to organs expressing these chains, such as kidney or lung. The detection of basement membrane-like structures in the absence of the major collagen IV isoform $\alpha 1. \alpha 1. \alpha 2(\text{IV})$ may be compensated for by the presence of $\alpha 3(\text{IV})$ - $\alpha 6(\text{IV})$ chains, which may assemble into two distinct protomers, $\alpha 3. \alpha 4. \alpha 5(\text{IV})$ or $\alpha 5. \alpha 5. \alpha 6(\text{IV})$ (Boutaud et al., 2000). However, immunostaining for distinct collagen IV chains did not reveal significant amounts of $\alpha 3(\text{IV})$ - $\alpha 6(\text{IV})$ chains in wild-type embryonic basement membranes or in mutant embryos. By quantitative RT-PCR we could only detect trace amounts of $\alpha 3(\text{IV})$, $\alpha 4(\text{IV})$ and $\alpha 6(\text{IV})$ mRNAs in normal and mutant embryos. Therefore, no significant amounts of any heterotrimeric collagen IV protomer can be assembled and secreted in mutants and monomeric $\alpha 5(\text{IV})$ chains are only found intracellularly. These data suggest that collagen IV aggregates are dispensable for the deposition of basement membrane-like structures during early embryonic development. However, it remains to be analyzed how the $\alpha 1. \alpha 1. \alpha 2(\text{IV})$ deficiency influences the tissue-restricted formation of the mixed $\alpha 1. \alpha 1. \alpha 2 / \alpha 5. \alpha 5. \alpha 6(\text{IV})$ network later in development.

Interestingly, the phenotype of collagen IV-deficiency shows some similarity to the targeted inactivation of the collagen-specific chaperone Hsp47 (Nagai et al., 2000). These mutant mice died before E11.5, the secretion of processed collagen IV,

as well as collagen I, was reduced and defective basement membrane structures were also observed. As inactivation of only collagen I causes embryonic lethality at E13.5 because of ruptures of major blood vessels and mesenchymal necrosis (Harbers et al., 1984; Lohler et al., 1984), we speculate that the phenotype of Hsp47-deficient mice mimics in some aspects that of collagen IV-deficient embryos described here. Additional phenotypes in Hsp47-deficient embryos, such as formation of aberrant epithelia and the disruption of blood vessels, may be explained by the chaperone functions of Hsp47 on the formation of stable protomers of other members of the collagen family.

Based on the data presented, we propose that deficiency of collagen IV has no, or only a limited, influence on the deposition and function of basement membrane-like structures during early development. Recently, it was proposed that the secretion of laminins and their recruitment by specific cellular receptors like β 1-integrins or dystroglycan, represents the first crucial step for basement membrane formation (Li et al., 2002). The local concentration of deposited laminins would thus enable their efficient assembly into gel-like aggregates in the presence of calcium ions (Yurchenco and Cheng, 1993; Yurchenco and Cheng, 1994). Such laminin-based minimal matrices could provide the flexibility and variability that is necessary for the differentiation and proliferation of cells when mechanical demands on the integrity of basement membranes are limited. However, the presence of specific collagen IV networks is essential for the intrinsic cohesiveness of basement membranes under conditions of increasing mechanical demands and the formation of stable and functional basement membranes.

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