Histol Histopathol (2007) 22: 1077-1084

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

The absence of one or both nidogens does not alter basement membrane composition in adult murine kidney

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Summary. Nidogen-1 and nidogen-2 are major components of all basement membranes and are considered to function as link molecules between laminin and collagen type IV networks. Surprisingly, the knockout of one or both nidogens does not cause defects in all tissues or in all basement membranes. In this study, we have elucidated the appearance of the major basement membrane components in adult murine kidney lacking nidogen-1, nidogen-2, or both nidogens. To this end, we localized laminin-111, perlecan, and collagen type IV in knockout mice, heterozygous (+/-) or homozygous (-/-) for the nidogen-1 gene, the nidogen-2 gene, or both nidogen genes with the help of light microscopic immunostaining. We also performed immunogold histochemistry to determine the occurrence of these molecules in the murine kidney at the ultrastructural level. The renal basement membranes of single knockout mice contained a similar distribution of laminin-111, perlecan, and collagen type IV compared to heterozygous mice. In nidogen double-knockout animals, the basement membrane underlying the tubular epithelium was sometimes altered, giving a diffuse and thickened pattern, or was totally absent. The normal or thickened basement membrane of double-knockout mice also showed a similar distribution of laminin-111, perlecan, and collagen type IV. The results indicate that the lack of nidogen-1, nidogen-2, or both nidogens, plays no crucial role in the occurrence and localization of laminin-111, collagen type IV, and perlecan in murine tubular renal basement membranes.

Key words: Nidogen-1, Nidogen-2, Mouse kidney, Laminin-111, Perlecan, Collagen type IV

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Introduction

Basement membranes are specialized structures of the extracellular matrix with multiple functions. They provide structural support and regulate cell behaviour (Timpl, 1996; Timpl and Brown, 1996). As thin condensed matrices, they divide the cells of the parenchymal tissues from the interstitial matrix. The main basement membrane components, collagen type IV, laminin-isoforms, nidogen-1, nidogen-2 and perlecan are involved in numerous cell biological functions (Kleinmann et al., 1989; Timpl, 1989, 1996; Timpl and Brown, 1996; Aumailley et al., 2005). They play a central role in tissue compartmentalization and are important in maintenance of cell phenotypes as well as supplying morphogenetic stimuli for development and tissue remodelling (Ekblom, 1993; Kleinman et al., 1993; Ekblom et al., 1996; Streuli, 1996). Laminin-nidogen complexes are involved in epithelial-mesenchymal interactions (Dziadek, 1995).

Nidogen-1, also referred to as entactin-1, is a small rod–shaped molecule (150 kDa) consisting of three globular domains [G1, G2, G3]. These domains are connected by a flexible link and a rod (Fox et al., 1991; Mayer and Timpl, 1994). Nidogen-1 is known to play an important role in basement membrane assembly during mouse development (Miosge et al., 1999a; Miosge et al., 2000a). Together with other basement membrane components, nidogen-1 shows a high in vitro binding activity for laminin-111 (Fox et al., 1991) with a binding site localized on the laminin γ1 III3-5 chain (Mayer et al., 1993). Nidogen-1 also binds to collagen type IV (Aumailley et al., 1989) and is considered to build ternary complexes between the laminin-111 and collagen type IV networks (Fox et al., 1991; Mayer et al., 1995). This is the reason why nidogen-1 is thought to be a crucial link protein, stabilizing network formation in basement membranes (Timpl and Brown, 1996).

Nidogen-1 knockout mice develop normally and

show intact basement membranes with an increased amount of nidogen-2 in muscle and a similar amount in kidney basement membrane, as investigated at the ultrastructural level (Murshed et al., 2000). A lack of nidogen-1 does not result in any alteration in the distribution of collagen type IV and laminin, whereas the anionic charges across the glomerular wall are significantly altered (Lebel et al., 2003). Furthermore, the density of alpha(v)-integrin is found to be increased and the filtration properties of the glomerular wall altered, suggesting changes in permselectivity properties.

Nidogen-2 (200 kDa), also referred to as entactin-2, is known as an isoform of nidogen-1, with 46% sequence identity with nidogen-1 (Kohfeldt et al., 1998). Both nidogens show similar binding activities towards collagen type IV and perlecan. Moreover, it has previously been shown, that both nidogens bind with similar affinity to laminin-111 (Miosge et al., 2002). Deficiency of nidogen-2, however, does not result in any overt basement membrane abnormality and nidogen-1 showed indistinguishable staining patterns and intensities in the control and mutant animals (Schymeinsky et al., 2002).

The lack of both nidogen isoforms does not prevent embryonic development and is compatible with survival to birth (Bader et al., 2005). Furthermore, nidogen-1 and nidogen-2 do not appear to be crucial in establishing tissue architecture during organ development. Electron microscopy of the kidney reveals a fully formed dual basement membrane characteristic of the glomerulus with normal podocytes and slit membranes. In contrast, the basement membranes underlying the tubular epithelium show various patterns. They are sometimes absent or appear to be thickened and diffuse (Bader et al., 2005).

In order to investigate the role of nidogen-1 and nidogen-2 in basement membrane architecture, we localized laminin-111, perlecan, and collagen type IV in nidogen-1 knockout, nidogen-2 knockout, as well as in nidogen double-knockout mice and compared them with heterozygous mice at the light and electron microscopic level. Heterozygous mice served as controls because nidogen-1 and nidogen-2 were still present in the basement membrane and the ultrastructure of their basement membrane was identical with wild type animals (Murshed et al., 2000; Schymeinsky et al., 2002; Bader et al., 2005). The results show that neither the knockout of nidogen-1 or nidogen-2 alone, nor of both nidogens lead to variations in the distribution of laminin-111, perlecan, and collagen type IV in adult murine kidney.

Materials and methods

Tissue processing

Homozygous and heterozygous nidogen-1 mutant mice (Murshed et al., 2000), homozygous and heterozygous nidogen-2 mutant mice (Schymeinsky et al., 2002), as well as homozygous and heterozygous nidogen double-mutant mice (Bader et al., 2005) have all been described previously. For light microscopy, mouse kidneys (n=3 for each group) were fixed in 3.7% paraformaldehyde in phosphate buffer, pH 7.4, at 4°C and the tissues were then embedded in paraffin according to standard protocols (Miosge et al., 2000a,b; Gersdorff et al., 2005a). Serial sections of 5 µm thickness were cut with a Reichert's microtome. For immunogold histochemistry, renal cortex pieces (n=3 for each group), 1mm^3 in size, were fixed in 4% paraformaldehyde and 0.5% glutaraldehyde for 15min, dehydrated in a graded series of ethanol up to 70%, and embedded in the acrylic resin LR-Gold® (London Resin Company, Reading, UK). Semithin (1 µm) and ultrathin (90nm) sections were cut according to procedures previously described in detail (Miosge et al., 2000a,b; Gersdorff et al., 2005b).

Sources of antibodies

An affinity-purified polyclonal antibody (1.53 mg/ml) against perlecan (Schulze et al., 1995) was applied. A monoclonal rat anti-mouse antibody was prepared against murine laminin-111 (1.62 mg/ml), which reacted exclusively with the laminin a1 chain in immunoblots and was suitable for light and electron microscopic immunohistochemistry of laminin-111 (Miosge et al., 1995, 1999b). A polyclonal antibody against murine collagen type IV (1 mg/ml) (Andujar et al., 1985) was purchased from Quartett, Berlin, Germany. The secondary antibody used for the light microscopic immunostaining was a goat-anti-rabbit or goat-anti-rat IgG (Dakopats, Hamburg, Germany).

Light microscopic immunohistochemistry

Sections were deparaffinized, rehydrated and the endogenous peroxidase was blocked. They were then treated with 10 µg/ml protease XXIV (Sigma, Deisenhofen, Germany) for 5min. The anti-perlecan antibody was used at a dilution of 1:20 in TBS (tris buffered saline, pH 7.4) for 24hr at 37°C. The anti-laminin-111 antibody was used at a dilution of 1:100 in TBS and was incubated for 1hr at RT. The anti-collagen type IV antibody was used at a dilution of 1:20 in TBS for 1hr at RT. The secondary antibody was applied at a dilution of 1:50 in 1% BSA/TBS, followed by the PAP complex (diluted 1:150 in 1% BSA/TBS) each for 30min at RT. As negative controls, each immunoreaction was accompanied by a reaction omitting the primary antibody. No immunostaining was then observed.

Electron microscopic immunogold histochemistry

Gold particles with a diameter of 16 nm were prepared by boiling 49.5ml distilled water and 0.5 ml tetrachloroauric acid (1%) with 1.2 ml sodium citrate

(1%). The gold particles were used to label the antibodies (Herken and Miosge, 1991; Gersdorff et al., 2005b). Tissue sections on formvar® coated nickel grids were incubated for 15min at RT with 1% BSA/TBS and then incubated with anti-perlecan (diluted 1:50 in TBS, 24hr), anti-laminin-111 (diluted 1:100 in TBS, 1hr) or anti-collagen type IV (diluted 1:100 in TBS, 1hr) antibodies. The sections were rinsed with water and stained with uranyl acetate and lead citrate, each for 10 min. The stained sections were examined with a Leo 906E electron microscope. To exclude non-specific binding, we incubated control sections with pure gold solution or without any antibody. All controls proved negative.

Results

Light microscopic immunohistochemistry for nidogen-1 knockout renal tissue

Immunoperoxidase staining for laminin-111 showed reactions in all basement membrane zones of adult murine kidney, including tubular and glomerular basement membrane and the collecting tubule in heterozygous (Fig. 1A) as well as homozygous (Fig. 1B) kidney tissues. Furthermore, we detected staining for perlecan in heterozygous (Fig. 1C) and homozygous tissues (Fig. 1D) in all basement membrane zones of murine kidney and in basement membranes of blood vessels at a comparable level. Immunoperoxidase staining for collagen type IV showed similar reactions for the collecting tubule and the tubular and glomerular basement membrane of heterozygous tissue (Fig. 1E) as well as nidogen-1 knockout tissue (Fig. 1F). The cytoplasm or the nuclei of adjacent cells were not stained. Controls were all negative (data not shown).

Light microscopic immunohistochemistry for nidogen-2 knockout renal tissue

Laminin-111 was found in all basement membrane zones of murine kidney in nidogen-2 knockout and heterozygous mice. In addition, staining for perlecan was detected in the collecting tubules as well as in the tubular and glomerular basement membranes in both heterozygous and homozygous animals. Collagen type IV was localized in the tubular as well as in the glomerular basement membranes in knockout and heterozygous mice at a comparable level. Furthermore, the collecting tubule was similarly stained (data not shown).

Light microscopic immunohistochemistry for nidogen double-knockout renal tissue

In most mice lacking both nidogen isoforms normal kidneys developed. Only a small percentage of double-null animals showed either unilateral (6%) or bilateral (8%) renal aplasia (Bader et al., 2005). At the light microscopic level, no alterations of the tubular basement membranes were distinguishable. In double knockout and in heterozygous mice, the light microscopic immunostaining showed a similar staining for laminin-111 in the collecting tubule as well as in the tubular and glomerular basement membranes. The same was true for perlecan. Furthermore, we detected staining for collagen type IV in both, heterozygous and homozygous animals, in all basement membrane zones of murine kidney and in basement membranes of blood vessels at a comparable level (data not shown).

Electron microscopic immunogold histochemistry for nidogen-1 knockout and nidogen-2 knockout renal tissue

For the ultrastructural localization of laminin-111, perlecan and collagen type IV in renal basement membranes the immunogold method was chosen to allow a clear identification of laminin-111, perlecan, and collagen type IV deposits in different extracellular matrix compartments and cell types.

It has been shown at the ultrastructural level, that nidogen-1 knockout murine kidney tissues show equal amounts of nidogen-2 in renal basement membranes (Murshed et al., 2000). Nidogen-2 knockout murine kidney tissue shows no obvious alterations in the localization of nidogen-1 (Schymeinsky et al., 2002).

With the help of immunogold histochemistry, we showed staining for laminin-111 in renal basement membranes in heterozygous (Fig. 2A) and in nidogen-1 knockout (Fig. 2B) murine tissues with similar gold particle amounts and distributions. The same was true for laminin-111 in nidogen-2 knockout murine kidney (Fig. 2C).

In addition, immunogold labelling for perlecan was seen in all basement membranes of murine kidneys in heterozygous tissue (Fig. 2D), in nidogen-1 knockout (Fig. 2E), as well as nidogen-2 knockout (Fig. 2F) renal tissues without any differences in its distribution.

Collagen type IV was detected in the basement membrane of the proximal tubule with no ultrastructural changes in heterozygous tissues (Fig. 2G) and none in nidogen-1 knockout tissues (Fig. 2H). Furthermore, nidogen-2 knockout murine kidneys showed similar gold particle amounts (Fig. 2I) without any differences in the distribution.

Electron microscopic immunogold histochemistry for nidogen double-null renal tissue

Most mice, lacking both nidogen isoforms, develop kidneys with all parts of the nephron and collecting system (Bader et al., 2005). Moreover, the numbers of glomeruli were similar in control and in nidogen double-knockout renal tissue. Even so, the tubular basement membrane was sometimes altered in the same mouse. It partially appeared thickened and diffuse or was totally absent. In this study, we examined all three types of basement membranes.

In normal appearing basement membranes of double-knockout tissue, the localization of laminin-111 (Fig. 3A) showed gold particle amounts and distribution with a weak labelling. This was also true for perlecan and collagen type IV.

The ultrastructural localization of laminin-111 in thickened and diffuse basement membranes of kidneys, lacking both nidogen isoforms (Fig. 3B), showed no

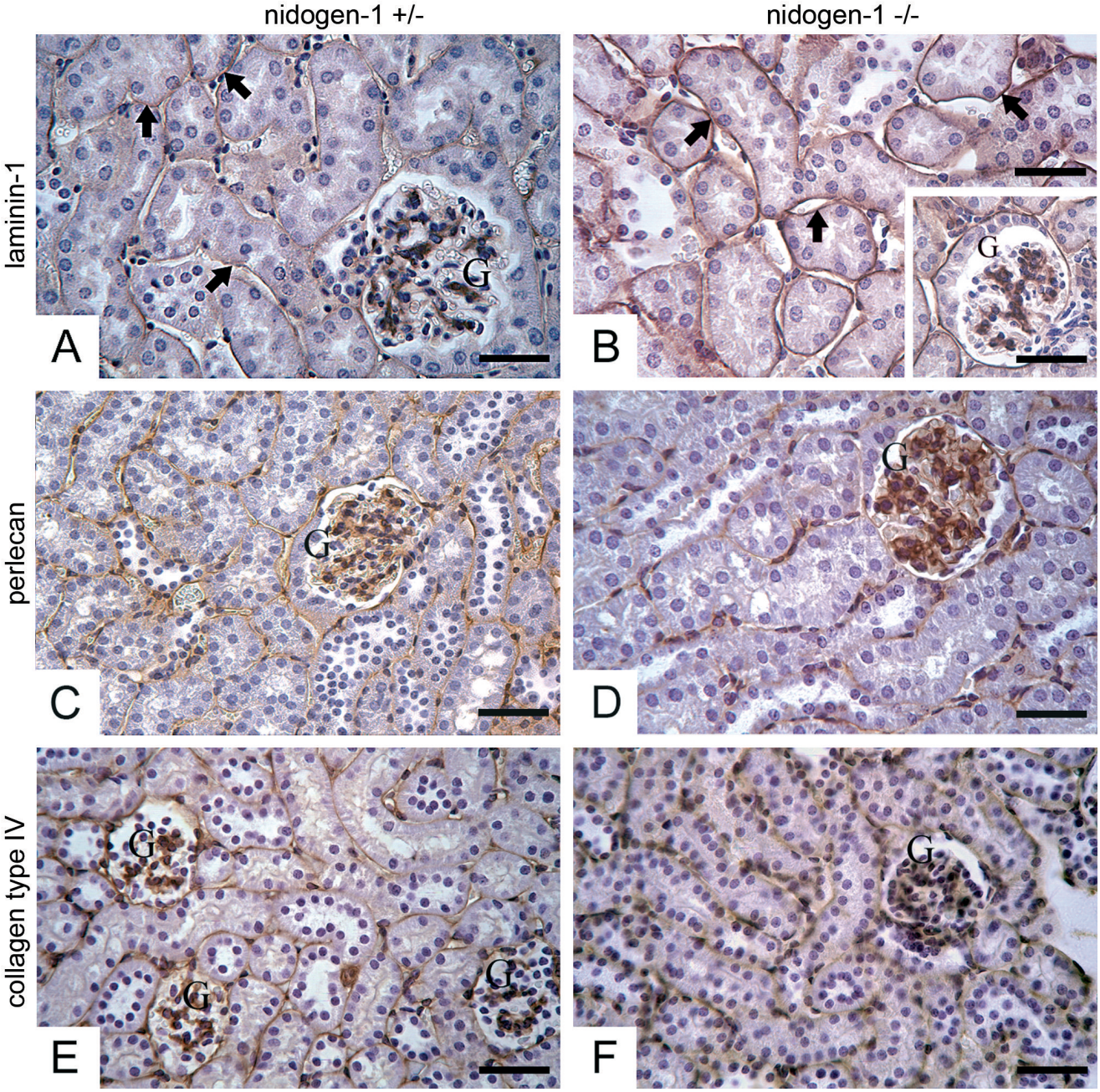

Fig. 1. Light microscopic immunostaining in kidney tissue of control mice (**A, C, E**) and nidogen-1 knockout mice (**B, D, F**). **A.** Labelling for laminin-111 is seen in tubular basement membranes (black arrow) and in glomerular basement membranes (G) in heterozygous tissue. **B.** Staining for laminin-111 is seen in tubular basement membranes (black arrow) and the glomerular basement membranes (inset, G) in nidogen-1 knockout mice. **C.** Labelling for perlecan is seen in tubular basement membranes and the glomerular basement membranes (G) in heterozygous tissue. **D.** Staining for perlecan is found in tubular basement membranes (black arrow) and the glomerular basement membranes (G) in nidogen-1 knockout tissue. **E.** Staining for collagen type IV in tubular basement membranes and in glomerular basement membranes in heterozygous tissue. **F.** Labelling for collagen type IV in nidogen-1 knockout mice. Bars: 50 µm in A, B, D; 60 µm in B-inset, C, E, F.

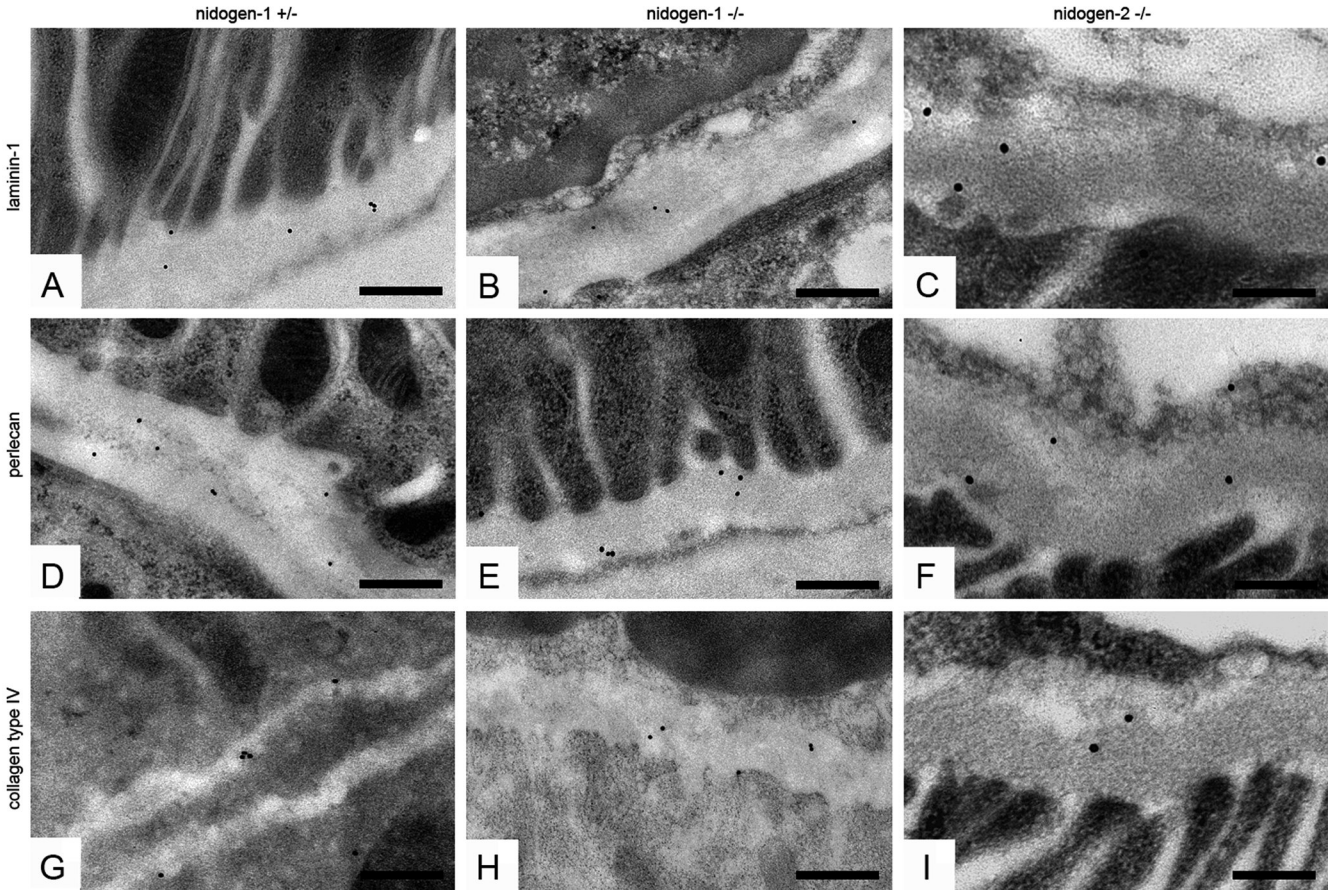

Fig. 2. Immunogold histochemistry in renal tissue of heterozygous (**A, D, G**), nidogen-1 knockout (**B, E, H**) and nidogen-2 knockout tissue (**C, F, I**). Staining for laminin-111 in heterozygous (**A**), in nidogen-1 (**B**), and in nidogen-2 knockout proximal tubular basement membranes (**C**). Labelling for perlecan in heterozygous renal tissue (**D**), in nidogen-1 (**E**) as well as in nidogen-2 knockout (**F**) in proximal tubular basement membranes. Staining for collagen type IV in heterozygous (**G**) as well as in nidogen-1 (**H**) and nidogen-2 (**I**) single knockout basement membranes. Bars: 0.28 µm in A, B, D, E, G, H; 0.23 µm in C, F; 0.21 µm in I.

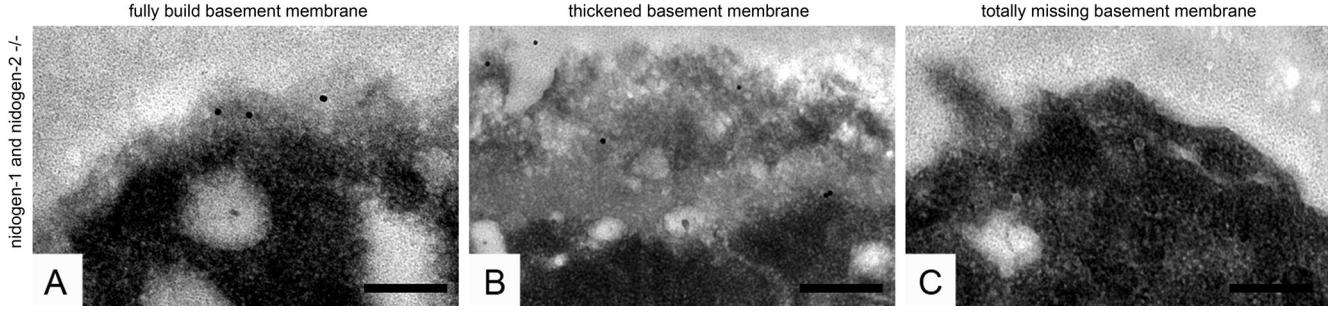

Fig. 3. Immunogold histochemistry of nidogen double-knockout in fetal kidney basement membranes (**A, B, C**). **A.** Localization of laminin-111 in a normal appearing proximal tubular basement membrane lacking both nidogens. **B.** Staining for laminin-111 in a thickened and diffuse renal basement membrane. **C**. Labelling of laminin-111 in regions of double-knockout renal tissue totally lacking a basement membrane. Bars: 0.23 µm in A; 0.28 µm in B, C.

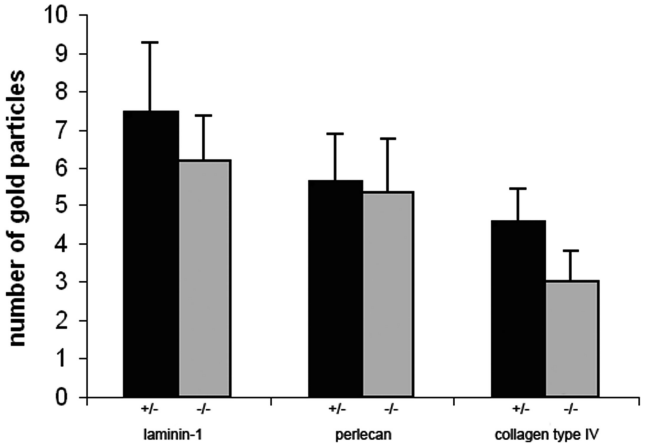

Fig. 4. Statistical analysis of ultrastructural immunogold staining of the proximal tubule. Analysis was performed for heterozygous mice (black bars) and for nidogen-1 knockout mice (gray bars). Bar 1 shows the mean values of the number of gold particles for the laminin-111 reactions, bar 2 the mean values for the perlecan reactions. Bar 3 shows the mean values of the number of gold particles for collagen type IV. The difference was statistically significant with p values < 0.001. T on bars represent the standard error of the mean.

changes in gold particle amounts and in its distribution compared to heterozygous kidney. In addition, the amounts of perlecan and collagen type IV showed no alterations.

In regions of double-knockout renal tissue totally lacking a basement membrane, neither laminin-111 (Fig. 3C), perlecan, nor collagen type IV were localized in the supposed basement membrane area as well as in the adjacent extracellular matrix.

Statistical analysis

The number of gold particles was counted for proximal tubules on eleven randomly chosen micrographs of identical size (2 μm^2) and magnification. The mean values for the ultrastructural staining for either heterozygous or nidogen-1 knockout mice are shown in Fig. 4. The mean values for nidogen-2 knockout and nidogen double-knockout mice show similar values (data not shown). To indicate the mean values of laminin-111, collagen type IV, and perlecan, we performed the Wilcoxon-Mann-Whitney test. The differences were statistically significant with p values < 0.001.

Discussion

Nidogens are small rod-shaped molecules with three globular domains [G1, G2 and G3] connected by a flexible link and a rod (Fox et al., 1991; Mayer and Timpl, 1994). With their high in vitro binding activity to a single laminin-type epidermal growth factor-like module γ1 III4 on the laminin γ1 chain (Mayer et al., 1993) and their binding activity to collagen type IV (Aumailley et al., 1989), nidogens are considered to hold a key position as a link molecule between the independent laminin and collagen type IV networks, forming ternary complexes (Fox et al., 1991; Timpl and Brown, 1996).

Surprisingly, nidogen-1 as well as nidogen-2 single knockout mice show only mild phenotypes. Nidogen-1 knockout animals show no overt abnormalities. They are fertile and their basement membrane structures appear normal (Murshed et al., 2000). Lebel et al. (2003) showed no alteration in the distribution of collagen type IV and laminin-111 in nidogen-1 murine knockout kidney, whereas the anionic charges across the glomerular wall were significantly altered. The density of alpha(v)-integrin was increased and the lack of nidogen-1 appears to modify the molecular organization, leading to alterations in functional properties of the glomerular wall (Lebel et al., 2003).

In adult kidney, nidogen-2 staining is found in all basement membranes and shows no alterations upon loss of nidogen-1. Nevertheless, nidogen-2 staining is intensified in certain basement membranes, such as endothelial basement membranes of skeletal muscle and basement membranes surrounding cardiocytes (Murshed et al., 2000; Miosge et al., 2002).

Nidogen-2 knockout mice also show no overt abnormalities and are also fertile (Schymeinsky et al., 2002). Again, the mice show no overt ultrastructural alterations in basement membranes.

Nidogen-1 and nidogen-2 show similar binding activities to the laminin γ1 fragment (Miosge et al., 2002). The lack of both nidogen isoforms does not prevent embryonic development and is compatible with survival to birth (Bader et al., 2005). However, nidogen double-knockout mice have basement membrane defects and developmental abnormalities in certain organs. This strongly argues for tissue-specific roles of nidogens in basement membrane assembly and function (Böse et al. 2006).

We localized laminin-111, perlecan and collagen type IV at the light and electron microscopic level in murine kidney of control mice, nidogen-1 knockout, nidogen-2 knockout as well as nidogen double-knockout mice, to examine amounts of the major basement membrane components.

The ultrastructure of basement membranes appears different depending on the method of fixation. Conventional fixation visualizes a layered basement membrane (Inoue et al., 1983). In contrast, with rapid freeze-substitution, a homogeneous basement membrane is seen (Goldberg and Escaig-Haye, 1986). The mild fixation carried out in this study also results in a homogeneous basement membrane.

Our results showed, that neither perlecan, laminin-111 nor collagen type IV were accumulated in any of the renal basement membranes, as seen in the statistical analysis (Fig. 4). Furthermore, no changes in the amounts of the investigated molecules were found in nidogen knockout kidney tissues compared with control mice. An ultrastructural colocalization of nidogen-1 and nidogen-2 with laminin-111 has been shown and the

binding affinity of laminin γ1 III4 for nidogen-1 and nidogen-2 is similar (Miosge et al., 2000b, 2002). The occurrence of both nidogens in the same renal basement membranes suggests that they may have related functions and could substitute for one another (Miosge et al., 2000b). Although embryonic basement membranes do not have a fully developed ultrastructural architecture until nidogen-1 becomes present (Miosge et al., 2000a), our data also suggest, that one knocked out nidogen alone is not essential for renal basement membrane formation or maintenance (Murshed et al., 2000; Schymeinsky et al., 2002). Nevertheless, in nidogen-double knockout murine kidney, the tubular basement membranes partially appeared thickened and diffuse or were totally absent in one and the same mouse.

In early embryonic development, laminin-111 plays a key role (Sasaki et al., 2004) because all other major basement membrane components are dispensable in early developmental stages. Mice lacking the laminin γ1 chain do not develop a basement membrane and die at a very early embryonic stage (Smyth et al., 1999). The deletion of the nidogen-binding site in the laminin γ1 chain results in developmental defects (Willem et al., 2002). The mice die immediately after birth and show renal agenesis and impaired lung development. Ultrastructural analysis shows locally restricted ruptures in the basement membrane of the elongating Wolffian duct and of alveolar sacculi. These data indicate that the nidogen-binding site is required for early kidney and lung morphogenesis (Willem et al., 2002).

Our studies and those of Murshed et al. (2000), Schymeinsky et al. (2002), and Bader et al. (2005) suggest, that nidogen-1 compensates for the absence of nidogen-2 and vice versa. However, nidogen double-knockout mice show alterations in kidney basement membrane ultrastructure, but do not show differences in the number and distribution of the major basement membrane components.

Moreover, in the light of previous in vivo studies of our group and combined with the results of many others it seems to be allowed to suggest a modification of the model of nidogens as major link molecules for basement membrane assembly to state that nidogens only serve as link molecules in certain organs and developmental processes.

Acknowledgements. We would like to thank Christina Zelent for technical assistance and Cyrilla Maelicke, B.Sc. for editing the manuscript. Parts were taken from the doctoral thesis of Petra Burckhard.

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Accepted March 30, 2007