Molecular Pathogenesis of Genetic and Inherited Diseases

Laminin α 1 Chain Corrects Male Infertility Caused by Absence of Laminin α 2 Chain

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Laminins are important for basement membrane structure and function. The laminin α 2 chain is a major component of muscle basement membranes, and mutations in the laminin α 2 gene lead to congenital muscular dystrophy in humans and mice. Although the laminin α 2 chain is prominently expressed in testicular basement membranes, its role in testis has remained unclear. Here, we show that laminin α 1, α 2, β 1, β 2, γ 1, and γ 3 chains are the major laminin chains in basement membranes of seminiferous tubules. In laminin α 2 chain-deficient dy^{3K}/dy^{3K} mice, lack of laminin α 2 chain led to concurrent reduction of laminin γ 3 chain and abnormal testicular basement membranes. Seminiferous tubules of laminin α 2 chain-deficient dy^{3K}/dy^{3K} mice displayed a defect in the timing of lumen formation, resulting in production of fewer spermatides. We also demonstrate that overexpression of laminin α 1 chain in testis of dy^{3K}/dy^{3K} mice compensated for laminin α 2 chain deficiency and significantly reversed the appearance of the histopathological features. We thus provide genetic data that laminin α chains are essential for normal testicular function *in vivo*. (*Am J Pathol* 2005, 167:823–833)

Basement membranes are thin sheets of extracellular matrix that surround epithelia, endothelia, muscle, fat, and Schwann cells. All basement membranes contain laminins, collagen type IV, nidogens, and heparan sulfate proteoglycans.¹ Laminins are heterotrimers of α -, β -, and γ -chains. The five α -, three β -, and three γ -chains give rise to at least 15 different protein isoforms that differ in their tissue distribution.² Laminin α 1 and α 5 chains are crucial for early embryonic development and organogenesis, whereas laminin α 2, α 3, and α 4 chains are essential

for postnatal development.³ Laminin α 2 chain is deposited in basement membranes of muscle, and laminin α 2 chain deficiency leads to congenital muscular dystrophy MDC1A.^{4–6} Two knock out models (dy^{W}/dy^{W} and dy^{3K}/dy^{3K}) and three spontaneous mouse strains (dy/dy , dy^{2J}/dy^{2J} , and dy^{Pas}/dy^{Pas}) representing animal models for congenital muscular dystrophy with laminin α 2 chain deficiency have been reported.^{7–12} All strains develop severe clinical signs of muscular dystrophy. The laminin α 2 chain is also expressed in other tissues such as the central and peripheral nervous systems, thymus, and testis; and non-muscle defects are associated with laminin α 2 chain deficiency.^{13–18} Interestingly, dy/dy mice expressing reduced levels of laminin α 2 chain do not reproduce¹⁹ (www.jax.org). This may not only be due to muscle weakness, but could also be due to testicular defects. Furthermore, abnormal basement membrane structures are detected in testicular biopsies from men with impaired fertility,²⁰ and *in vitro* studies point toward a role for collagen IV and laminins in spermatogenesis.^{21,22} Thus, basement membranes appear significant for spermatogenesis. However, genetic evidence for a role of basement membrane components for spermatogenesis is lacking.

Normal spermatogenesis is an intricate process that takes place in the seminiferous epithelium of the mammalian testis. The seminiferous tubules are lined by small cells called spermatogonia. Alternating with the spermatogonia are the highly polarized epithelial cells, the Sertoli cells, which act as nursery units for the developing sperm. The Sertoli cells are attached to each other, to the spermatogonia, and to the basement membrane of the seminiferous tubule to form the blood-testis barrier. During spermatogenesis, spermatogonia differentiate into spermatocytes that will cross through the blood-testis

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Table 1. Relative Amounts of Human Laminin $\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 4$, and $\alpha 5$ mRNA in Testis

Laminin $\alpha 1$	10.0 $\pm$ 0.076
Laminin $\alpha 2$	9.6 $\pm$ 0.13
Laminin $\alpha 3$	2.1 $\pm$ 0.076
Laminin $\alpha 4$	3.0 E-4 $\pm$ 0.21
Laminin $\alpha 5$	3.2 $\pm$ 0.31

Quantitative real-time PCR with sequence-specific hybridization probes was performed. Laminin levels were normalized to GAPDH and expressed as relative mRNA amounts and are shown as means $\pm$ SD ($n = 3$).

barrier as they mature and traverse the tubular lumen.^{23,24}

In the present study, we provide genetic evidence that laminin α chains are vital for spermatogenesis. We first examined the expression pattern of laminin α , β , and γ chains in wild-type and laminin $\alpha 2$ chain-deficient dy^{3K}/dy^{3K} testes. We demonstrate that lack of laminin $\alpha 2$ chain leads to concomitant loss of the laminin $\gamma 3$ chain and an abnormal basement membrane. Seminiferous tubules of laminin $\alpha 2$ chain-deficient dy^{3K}/dy^{3K} mice displayed a defect in the timing of lumen formation. Furthermore, significantly fewer spermatides were produced in testes lacking laminin $\alpha 2$ chain. Finally, we show that overexpression of laminin $\alpha 1$ chain in testis of dy^{3K}/dy^{3K} mice compensates for laminin $\alpha 2$ chain deficiency and partially reverses the appearance of the histopathological features.

Materials and Methods

Transgenic Mice

Laminin $\alpha 2$ chain-deficient dy^{3K}/dy^{3K} mice were previously described.⁷ Transgenic mice deficient in laminin $\alpha 2$ chain but overexpressing laminin $\alpha 1$ chain in various tissues (dy^{3K} LN $\alpha 1$ TG mice) were recently described.²⁵

Quantitative Real-Time Polymerase Chain Reaction (PCR)

Total RNA from human testis was obtained from Becton-Dickinson, Franklin Lakes, NJ. Total RNA from mouse

Figure 1. ELISA titration of affinity-purified antibodies against laminin $\gamma 1$ and $\gamma 3$ chains. **A:** The laminin $\gamma 1$ chain antibody was used against $\gamma 1$ LN/LEa ($\circ$) and $\gamma 3$ LN/LEa ($\bullet$). **B:** The laminin $\gamma 3$ chain antibody was used against $\gamma 3$ LN/LEa ($\bullet$) and $\gamma 1$ LN/LEa ($\circ$). Although the laminin $\gamma 3$ chain antibody showed some binding to the laminin $\gamma 1$ fragment, no staining was seen in dy^{3K}/dy^{3K} testis, which is a rich source of laminin $\gamma 1$ chain (Figure 3).

testes was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) according to manufacturer's specifications. First-strand cDNA synthesis from total RNA was performed using Superscript II RT (Invitrogen). Quantitative real-time PCR was performed on a LIGHTCYCLER (Roche Diagnostics, Mannheim, Germany), with quantitation of GAPDH mRNA synthesized by the cells as con-

Figure 2. Immunostaining of laminin α and β chains. Cross-sections of testes from wild-type and dy^{3K}/dy^{3K} mice were stained with antibodies against laminin $\alpha 1$ to $\alpha 5$ and $\beta 1$ chains. Laminin $\alpha 2$ chain was absent from dy^{3K}/dy^{3K} testis, whereas other laminin α chains and laminin $\beta 1$ chain appeared normally expressed. **Inset** in LM $\alpha 4$ panel shows double staining with laminin $\alpha 2$ chain in red and laminin $\alpha 4$ chain in green. Bar = 80 μ m.

Figure 3. Immunostaining of laminin β and γ chains, perlecan, and collagen IV. Cross-sections of testes from wild-type and dy^{3K}/dy^{3K} mice were stained with antibodies against laminin $\beta 2$ and $\gamma 1$ to $\gamma 3$ chains, perlecan, and collagen IV. Laminin $\gamma 3$ chain was absent from dy^{3K}/dy^{3K} testis whereas other γ chains, laminin $\beta 2$ chain, perlecan, and collagen IV appeared normally expressed. Bar = 80 μ m.

trol.²⁶ Primers and probes were designed by TIB Molbiol (Berlin). Primer pairs were as follows: for human $\alpha 1$, Fw-ACTTC-CGGCTACCGTGA and Rev-GTTTCGCTGGAAG-GCAAT; for human $\alpha 2$, Fw-GTACCCCAACATCTCCA-CTGTC and Rev-AAGTTGTTTCCAGAAGACGAAGT; for human $\alpha 3$, Fw-TACCACCTACTGACCACCTCC and Rev-AAGCCGTAGTCCAGAGTTGTC; for human $\alpha 4$, Fw-AA-

GTCCGTCCCAGAAGCA and Rev-CCAAGCGTGGTGT-CAGTAG; for human $\alpha 5$, Fw-CAGTCCTGAGGACTG-AAGTG and Rev-CCACTGAAGTTGTAAATGGTGC; for human GAPDH, Fw-AACAGCGACACCCACTCCTC and Rev-GGAGGGGAGATTCACTGTGGT; for mouse $\gamma 3$, Fw-GCTGGAAGAGCCTTGAGAA and Rev-GTAGAGGA-GACCTCAGCTTCATT; for mouse $\alpha 1$, Fw-AGAGATTGTA-GATGGCAAGGTC and Rev-TGTTTGATGTGGGCAGGA-TAG; and for mouse GAPDH, Fw-TTGTCAAGCAATGCA-TCCTGC and Rev-CCGTTCACTCTGGGATGAC. Probes for detection were as follows: for human $\alpha 1$, fluorescein-CTGGGTTTCATACGGGCACAAAAGACT and LIGHTCYCLER red (LCR) 640-TTTATCCATCGAGCTGTTTCGTGG; for human $\alpha 2$, fluorescein-GTGAGCTCCACACTCATGAAAT-CTCTCA and LCR 640-GTCTCGTGTGGCAAGATACAT-CAGAAGA; for human $\alpha 3$, fluorescein-GCAGGTCACTC-TGGAAGATGGTTACA and LCR 640-TGAATTGAGCAC-CAGCGATAGC; for human $\alpha 4$, fluorescein-TACTGAGG-TGGAAAATCTCTGATGCC and LCR 640-TTATTGAC-TTTCACATGACCTGTCCATTT; for human $\alpha 5$, fluorescein-GTCATCGACATACAGCCAGACTCCC and LCR 640-TG-GCATTGCTGTAGAAGGCGAC; for human GAPDH, fluo-rescein-CATGGCCTCCAAGGAGTAAGACCCCT and LCR 640-ACCACCAGCCCCAGCAAGAGCA; for mouse $\gamma 3$, fluo-rescein-GTCACCTTGGCAGCTTGCACA and LCR 640-CT-GAGGAAGCCCGTGTGAAGGC; for mouse $\alpha 1$, fluo-rescein-CTGAAAGCCCCACACCCATTCCA and LCR 640-TC-GGCAGACACCAACGATCCCATTTA; and for mouse GAPDH, fluorescein-CACCCAGAAGACTGTGGATGGC-CCCT and LCR 640-TGGAAAGCTGTGGCGTGATGGC-CG. Standard curves were obtained for each set with serial dilutions of RNA. Amplification curves were generated by LIGHTCYCLER software. The number of cycles (n) corresponding to the start of the logarithmic phase of the fluo-rescence signals was used to calculate the amount of mRNA by the formula $2^{(\Delta n)}$, where $\Delta n = n_{GAPDH} - n_{laminin}$. Each increase in Δn by 1 equals a twofold increase of specifically amplified mRNA. Statistical significance was examined by using Student's t -test

Immunofluorescence

Tissues were immersed in Tissue Tek and frozen in liquid nitrogen. Cryosections (8 μ m) were either stained with hematoxylin and eosin (H&E) or analyzed by immunofluorescence. Primary antibodies (Abs) were rat monoclonal Abs 200,²⁵ 4H8-2 (Alexis Biochemicals, Lausanne, Switzerland), and 1928 (Chemicon, Hampshire, UK) against laminin $\alpha 1$, laminin $\alpha 2$, and laminin $\beta 1$ chain, respectively, and rat monoclonal Ab N6 against GATA-1 (Santa Cruz, Santa Cruz, CA). The rabbit polyclonal Abs against

Table 2. Relative Amounts of Laminin $\gamma 3$ mRNA in Wild-Type and dy^{3K}/dy^{3K} Testes

Wild type	0.38 $\pm$ 0.05
dy^{3K}/dy^{3K}	0.47 $\pm$ 0.16

Quantitative real-time PCR with murine laminin $\gamma 3$ sequence-specific hybridization probes was performed. Laminin expression was normalized to GAPDH and expressed as relative mRNA amounts and is shown as means $\pm$ SD ($n = 3$).

laminin $\alpha 3$,²⁷ $\alpha 4$,²⁸ $\alpha 5$,²⁹ $\beta 2$,³⁰ $\beta 3$,²⁷ and $\gamma 2$ ²⁷ have been described previously. Additional rabbit Abs against laminin $\gamma 1$ and $\gamma 3$ chains were raised against their recombinant LN+LEa fragments following the procedure used for laminin $\alpha 1$ LN+LEa fragment.^{31,32} Enzyme-linked immunosorbent assay (ELISA) assays followed standard protocols. Rabbit polyclonal Abs against perlecan³³ and collagen type IV (Chemicon) and goat polyclonal Ab against protamine-2 (Santa Cruz) were used. Protamine-2 staining was quantified using the Volocity imaging system (Improvision, Inc., Lexington, MA). Statistical significance was examined by using Student's *t*-test.

Transmission Electron Microscopy

Transmission electron microscopy was performed as described previously.²⁵

Laminin Extraction and Immunoblotting

Proteins were isolated from 100 mg of wild-type and LN α 1TG testes by brief sonication in 1 mmol/L EDTA in 1 $\times$ Tris-buffered saline (TBS) with 1:25 dilution of protease inhibitors (Roche Diagnostics). Samples were incubated at 4°C for 1 hour and spun down at 13,000 rpm at 4°C. The supernatants were collected, and the protein concentration was determined using BCA assay (Pierce, Rockford, IL). One hundred micrograms of each sample was boiled for 5 minutes and separated on 5% polyacrylamide-sodium dodecyl sulfate gels for 90 minutes at 100 V. Proteins were transferred to Hybond-P polyvinylidene difluoride membranes (Amersham, Buckinghamshire, UK). Membranes were blocked for 1 hour in 5% nonfat dry milk in 1 $\times$ TBS with 0.01% Tween-20 and incubated overnight at 4°C with a rabbit polyclonal antibody detecting laminin $\alpha 1$, $\beta 1$, and $\gamma 1$ chains (Sigma, St. Louis, MO) (1:500). Blots were washed in 1 $\times$ TBS with 0.01% Tween-20, and subsequently horseradish peroxidase-linked anti-rabbit secondary antibody (Santa Cruz) was applied onto the membranes (1-hour incubation). Detection was performed with ECL kit (Amersham), and the immunoreactive protein bands were visualized on ECL films (Amersham). Equal loading was confirmed by Coomassie blue staining of sodium dodecyl sulfate-polyacrylamide gels.

Results

Expression of Basement Membrane Components in Human and Mouse Testes

To examine the expression of laminin α chain mRNAs in human testis, we performed quantitative real-time PCR.

This demonstrated high levels of laminin $\alpha 1$ and $\alpha 2$ chain mRNAs, moderate levels of laminin $\alpha 3$ and $\alpha 5$ chain mRNAs, and low levels of laminin $\alpha 4$ chain mRNA (Table 1). Thus, the main laminin α chain mRNAs in testis are laminin $\alpha 1$ and $\alpha 2$. The limitations of testis biopsies of patients with known mutations in the gene encoding laminin $\alpha 2$ chain prompted our use of the dy^{3K}/dy^{3K} mouse, which is deficient in laminin $\alpha 2$ chain.⁷ A panel of antibodies was used to study the expression of laminin α , β , and γ chains in mouse testis. All antibodies except the laminin $\gamma 1$ chain antibody have previously been characterized.^{25,27–30,32} The specificity of the antibodies against laminin $\gamma 1$ and $\gamma 3$ chains was verified by ELISA (Figure 1). As previously reported, laminin $\alpha 1$ chain was expressed in the basement membrane of wild-type mice.¹⁸ In dy^{3K}/dy^{3K} mice, the expression of laminin $\alpha 1$ chain did not appear to be altered based on immunofluorescence (Figure 2). As expected, laminin $\alpha 2$ chain was expressed in the basement membrane of wild-type testis¹⁸ and completely absent in dy^{3K}/dy^{3K} mice (Figure 2). Laminin $\alpha 3$ chain was confined to blood vessels in testes of both wild-type and dy^{3K}/dy^{3K} mice (Figure 2). Double staining with laminin $\alpha 2$ chain revealed that laminin $\alpha 4$ chain was expressed outside the epithelium, in smooth muscle and in blood vessels (Figure 2). In human testis, it has also been demonstrated that laminin $\alpha 4$ chain is expressed in smooth muscle rather than in the basement membrane.³⁴ In testes of both wild-type and dy^{3K}/dy^{3K} mice, weak immunoreactivity for laminin $\alpha 5$ chain was found in the basement membrane and in blood vessels (Figure 2).

Both laminin $\beta 1$ and $\beta 2$ chains were expressed in the basement membrane and in blood vessels of testes from wild-type and dy^{3K}/dy^{3K} mice (Figures 2 and 3), whereas laminin $\beta 3$ chain could not be detected (data not shown). Laminin $\gamma 1$ chain was expressed in the basement membrane and in blood vessels of testes from wild-type and dy^{3K}/dy^{3K} mice (Figure 3). Similarly, laminin $\gamma 2$ chain was expressed in the same locations albeit at lower levels (Figure 3). Previously, it has been shown that laminin $\gamma 3$ chain immunoreactivity is localized to the apical surface of the seminiferous tubules.³⁵ However, using a similar assay but different antibodies, we found laminin $\gamma 3$ chain expression in the basement membrane of wild-type testis (Figure 3). Also, in human testis, laminin $\gamma 3$ chain expression was confined to the basement membrane region rather than the apical surface of epithelial cells (data not shown). Noticeably, the expression of laminin $\gamma 3$ chain was absent in dy^{3K}/dy^{3K} mice (Figure 3). This reduction appeared to be at the translational level because laminin $\gamma 3$ chain mRNA was expressed at normal levels in testis of dy^{3K}/dy^{3K} mice (Table 2). Thus, laminin $\gamma 3$ chain is associated with laminin $\alpha 2$ chain in the basement mem-

Figure 4. Analyses of testes from wild-type and dy^{3K}/dy^{3K} mice. **A:** Cryosections of testes from wild-type and dy^{3K}/dy^{3K} mice were stained with H&E. Seminiferous tubules in wild-type testis had well-developed lumens, whereas lumen formation seemed to be delayed in dy^{3K}/dy^{3K} mice. Bar = 80 μ m. High-magnification photomicrographs of the seminiferous tubules of wild-type animals showed tubules containing Sertoli cells (**blue arrowhead**), spermatogonia (**black arrowhead**), primary spermatocytes (**blue arrow**), and early and late spermatides (**black arrows**). The number of spermatides in dy^{3K}/dy^{3K} mice were considerably reduced. Bar = 25 μ m. **B:** Transmission electron microscopy of testes from wild-type and dy^{3K}/dy^{3K} mice. **Asterisks** denote the transverse sections of spermatid flagella, which were seen in wild-type but not dy^{3K}/dy^{3K} mice. Bar = 1 μ m. **C:** Transmission electron microscopy of testes from wild-type and dy^{3K}/dy^{3K} mice. The basement membrane, clearly present along the basal surface of the seminiferous tubule of wild-type mice, was sometimes thinner and disrupted in dy^{3K}/dy^{3K} mice. **Black arrows** denote a normal basement membrane, **white arrows** denote a thinner basement membrane, and **asterisks** denote a disrupted basement membrane. Bar = 0.5 μ m.

brane of seminiferous tubules and forms a trimer with either laminin $\beta 1$ or $\beta 2$ chains. Other basement membrane components such as perlecan and collagen type IV appeared normally expressed in basement membranes and blood vessels in testes of both wild-type and dy^{3K}/dy^{3K} mice (Figure 3).

Histopathological Features of Laminin $\alpha 2$ Chain-Deficient Testis

Dy^{3K}/dy^{3K} mice die at 4 to 5 weeks of age before reaching the reproductive age of 6 weeks. However, already at the age of 3 weeks, germ cells appear coinciding with the appearance of lumen within seminiferous tubules.³⁶ At 3 weeks of age, dy^{3K}/dy^{3K} mice are significantly smaller than wild-type littermates.⁷ Commensurate with the overall reduction in body size, there was general reduction in the size of various organs including testis. Organs such as kidney, heart, bladder, and eye showed normal morphology (data not shown), suggesting that development in general is not significantly impaired. However, testis showed abnormal morphology. Examination of sections of testes from 26- to 30-day-old dy^{3K}/dy^{3K} mice revealed that the laminin $\alpha 2$ chain-deficient males appear to have a defect in the timing of lumen formation. In wild-type testis, lumen had formed in the majority of seminiferous tubules. In contrast, seminiferous tubules of dy^{3K}/dy^{3K} mice contained either small lumens or no lumens (Figure 4A). Also, a reduced amount of round and elongated spermatides were detected in laminin $\alpha 2$ chain-deficient testis (Figures 4A and 7D). The ultrastructure of testes from wild-type and dy^{3K}/dy^{3K} mice at the age of 23 days was examined by electron microscopy. This revealed the presence of spermatid flagella in the wild-type testis but not in dy^{3K}/dy^{3K} testis (Figure 4B). Previous studies have shown that laminin $\alpha 2$ chain deficiency results in defective muscle and sciatic nerve basement membranes.^{10,16,37} Also, the basement membrane underlying the Sertoli cells in dy^{3K}/dy^{3K} testis was sometimes thinner and disrupted (Figure 4C).

To further show that the number of spermatides is reduced in dy^{3K}/dy^{3K} testis, we stained for the presence of protamines, which are the major DNA-binding proteins in the sperm nucleus.³⁸ Significantly less protamine-2 was expressed in testis of dy^{3K}/dy^{3K} mice (Figure 5, A and B). Histologically, spermatogonia seemed normal in dy^{3K}/dy^{3K} mice (Figure 4A). Alternating with the spermatogonia are the Sertoli cells that are identified by their large nuclei near the basement membrane. By histological means, the Sertoli cells also appeared normal in dy^{3K}/dy^{3K} mice (Figure 4A). In addition, GATA-1 immunostainings confirmed that Sertoli cells were readily detected in dy^{3K}/dy^{3K} testes. In both wild-type and dy^{3K}/dy^{3K} testes, nuclear immunoexpression of GATA-1³⁹ was detected close to the basement membrane (Figure 5A). Ectoplasmic specializations are actin filament-endoplasmic reticulum complexes that occur in Sertoli cells at sites of intercellular attachment. To study the ectoplasmic specializations, we determined the distribution of actin filament using rhodamine-phalloidin in 26-day-old testis. In

Figure 5. Expression pattern of protamine-2, GATA-1, and actin in wild-type and dy^{3K}/dy^{3K} testes. **A:** Cross-sections of testes from wild-type and dy^{3K}/dy^{3K} mice were stained with antibodies against protamine-2, GATA-1 (red), and laminin $\gamma 1$ (green) and with rhodamine-phalloidin. Protamine-2 staining was markedly reduced in dy^{3K}/dy^{3K} testis. GATA-1 and laminin $\gamma 1$ chain double staining demonstrated that the nuclei of Sertoli cells were present near the basement membrane. Actin staining revealed that apical ectoplasmic specializations are missing in dy^{3K}/dy^{3K} testis. Bar in **top panel** = 250 μm and in **middle and bottom panels** = 80 μm . **B:** Quantification of protamine-2 and actin staining in wild-type and dy^{3K}/dy^{3K} testes. Significantly fewer protamine-2 ($P < 0.0008$) and actin ($P < 0.0004$) stainings were observed in dy^{3K}/dy^{3K} testes.

agreement with previous findings,^{40,41} actin was concentrated at the basal Sertoli-Sertoli junctions and at the apical Sertoli-spermatid junctions in wild-type testis (Figure 5A). Although actin staining was normal at the basal part of the seminiferous tubules, significantly reduced staining was observed at the apical side of dy^{3K}/dy^{3K} seminiferous tubules (Figure 5, A and B). Only focal patches were seen, and these data suggest that apical junctions are not present in the dy^{3K}/dy^{3K} testis.

Laminin $\alpha 1$ Chain Transgene Partially Compensates for Laminin $\alpha 2$ Chain Deficiency in Testis

Laminin $\alpha 1$ chain is also prominently expressed in testis, but whether it is important for normal testicular function

Table 3. Relative Amounts of Laminin $\alpha 1$ mRNA in Wild-Type, dy^{3K}/dy^{3K} , $LN\alpha 1TG$, and $dy^{3K}LN\alpha 1TG$ Testes

WT	12.4 $\pm$ 2.3
dy^{3K}/dy^{3K}	29.2 $\pm$ 10.5
$LN\alpha 1TG$	47.4 $\pm$ 13.8
$dy^{3K}LN\alpha 1TG$	51.1 $\pm$ 8.4

Quantitative real-time PCR with murine laminin $\alpha 1$ sequence-specific hybridization probes was performed. Laminin expression was normalized to GAPDH and expressed as relative mRNA amounts and is shown as means $\pm$ SD ($n = 3-8$). Significantly more laminin $\alpha 1$ chain mRNA was seen in dy^{3K}/dy^{3K} testis compared with wild-type testis ($P < 0.0026$), whereas significantly less laminin $\alpha 1$ chain mRNA was seen in dy^{3K}/dy^{3K} testis compared with $LN\alpha 1TG$ testis ($P < 0.0417$) or $dy^{3K}LN\alpha 1TG$ testis ($P < 0.0049$).

cannot be easily tested because mice deficient in laminin $\alpha 1$ chain die during early embryonic development.^{42,43} Interestingly, laminin $\alpha 1$ chain mRNA is up-regulated ~ 2.4 -fold in dy^{3K}/dy^{3K} testis, compared with wild-type testis. (Table 3). However, it seems that this up-regulation is inadequate to compensate for laminin $\alpha 2$ chain deficiency. We therefore tested whether an additional up-regulation of laminin $\alpha 1$ chain in testis could compensate for laminin $\alpha 2$ chain absence. Recently, we generated and characterized mice overexpressing laminin $\alpha 1$ chain in various tissues including skeletal muscle.²⁵ In testes of these mice (derived from line 12) an approximately four-fold overexpression of laminin $\alpha 1$ mRNA (Table 3) and a twofold overexpression of the protein was noted, compared with wild-type testis (Figure 6). The expression of laminin $\beta 1$ and laminin $\gamma 1$ chains was also up-regulated

Figure 6. Immunoblotting of laminin $\alpha 1$, $\beta 1$, and $\gamma 1$ polypeptides. Immunoblotting of protein extracts from testes of wild-type and transgenic mice overexpressing laminin $\alpha 1$ chain ($LN\alpha 1TG$) was performed. The polyclonal antiserum detects all three chains of laminin-111 (laminin $\alpha 1$ chain at 400 kD and laminin $\beta 1$ and laminin $\gamma 1$ chains at 200 kD). Both $\alpha 1$ and $\beta 1/\gamma 1$ chains were up-regulated about twofold. The migration distances of 400- and 200-kD proteins are shown to the **left**. Coomassie blue-stained loading control is shown in the **bottom panel**.

Figure 7. Immunostaining of laminin $\alpha 1$ chain in testes of different genotypes. Cross-sections of testes from wild-type, dy^{3K}/dy^{3K} , $LN\alpha 1TG$, and $dy^{3K}LN\alpha 1TG$ mice were stained with antibodies against laminin $\alpha 1$ chain. Bar = 80 μm .

about twofold in transgenic testis (Figure 6). The transgenic mice were previously mated with $dy^{3K}/+$ mice to generate $dy^{3K}LN\alpha 1TG$ mice. In these mice, laminin $\alpha 1$ chain can compensate for laminin $\alpha 2$ chain deficiency in muscle and reverses the appearance of histopathological features of muscular dystrophy.²⁵ Next, we analyzed testis from 25-day-old $dy^{3K}LN\alpha 1TG$ mice lacking laminin $\alpha 2$ chain in testis but overexpressing laminin $\alpha 1$ chain. In testes of these mice, laminin $\alpha 1$ chain mRNA was up-regulated ~ 4 -fold (Table 3). To determine whether transgenically expressed laminin $\alpha 1$ chain is different from endogenous expression, we compared laminin $\alpha 1$ chain expression in testes of wild-type, dy^{3K}/dy^{3K} , $LN\alpha 1TG$, and $dy^{3K}LN\alpha 1TG$ mice by immunofluorescence. Laminin $\alpha 1$ chain was expressed in the basement membrane of seminiferous tubules in wild-type, dy^{3K}/dy^{3K} , $LN\alpha 1TG$, and $dy^{3K}LN\alpha 1TG$ mice (Figure 7). This was further verified with double stainings against laminin $\alpha 4$ chain, which is mainly expressed in smooth muscle outside the epithelium (data not shown). Thus, transgenically expressed laminin $\alpha 1$ chain appears to be expressed in a similar manner as endogenous laminin $\alpha 1$ chain.

Hematoxylin and eosin-stained sections showed that lumen had formed in the majority of seminiferous tubules of $dy^{3K}LN\alpha 1TG$ mice (Figure 8A). Round and elongated spermatides were readily detected in $dy^{3K}LN\alpha 1TG$ testis (Figure 8, A and D). Immunofluorescence analysis revealed that there was almost as much protamine-2 in $dy^{3K}LN\alpha 1TG$ testis as in wild-type testis (Figure 8, A and C). We next analyzed testes from 5- to 9-month-old mice to see whether the effect of laminin $\alpha 1$ chain overexpression on testicular morphology was sustained. No difference in the amounts of protamine-2 between control and $dy^{3K}LN\alpha 1TG$ testes was seen, suggesting that spermatogenesis proceeds normally in mature $dy^{3K}LN\alpha 1TG$ testis (Figure 8, B and C). Also, the basement membrane was restored in testis of $dy^{3K}LN\alpha 1TG$ mice (Figure 9).

Figure 8. Overexpression of laminin $\alpha1$ chain in testis partially compensates for laminin $\alpha2$ chain deficiency. Cryosections of testes from 25-day-old (**A**) and 9-month-old (**B**) control and $dy^{3K}LN\alpha1TG$ mice were stained with H&E and antibodies against protamine-2. Histologically, seminiferous tubules of $dy^{3K}LN\alpha1TG$ mice appeared very similar to those of wild-type mice. **C:** Quantification of protamine-2 and staining in control and $dy^{3K}LN\alpha1TG$ testes. Slightly less protamine-2 was detected in 25-day-old ($P < 0.2690$) but not in 9-month-old ($P < 0.8788$) $dy^{3K}LN\alpha1TG$ testes. Bar = 80 μm . **D:** Quantification of the number of elongated spermatids per seminiferous tubule in 25-day-old wild-type, dy^{3K}/dy^{3K} , and $dy^{3K}LN\alpha1TG$ testes. The number of elongated spermatids in dy^{3K}/dy^{3K} testis was significantly smaller than in wild-type testis ($P < 0.0001$), whereas the number of elongated spermatids in $dy^{3K}LN\alpha1TG$ testis was not significantly different from wild-type testis ($P < 0.6359$).

Figure 9. Restoration of the basement membrane in $dy^{3K}LN\alpha1TG$ testis. Transmission electron microscopy of testis from 5-month-old wild-type and $dy^{3K}LN\alpha1TG$ mice. The basement membrane is clearly present along the basal surface of the seminiferous tubule of both wild-type and $dy^{3K}LN\alpha1TG$ mice. **Black arrows** denote the basement membrane. Bar = 0.25 μm .

Moreover, as previously indicated, male $dy^{3K}LN\alpha1TG$ mice are able to produce offspring.²⁵

To test whether rescue of the testis phenotype is due to a laminin isoform containing the $\alpha1$ and $\gamma3$ chains, we analyzed the expression of laminin $\gamma3$ chain in $dy^{3K}LN\alpha1TG$ testis. Notably, the expression of laminin $\gamma3$ was not restored in the basement membrane of the seminiferous tubules (Figure 10). These data show that loss of laminin $\alpha2$ and $\gamma3$ chains are compensated for by laminin $\alpha1$ chain (which appears not to be associated with laminin $\gamma3$ chain) and the $\beta1/\gamma1$ chains because these chains are up-regulated on overexpression of laminin $\alpha1$ chain (Figure 6).

Discussion

Here, we show that the presence of laminin $\alpha2$ chain in the basement membrane of seminiferous tubules is important for spermatogenesis. In wild-type mice, the first wave of spermatogenesis begins shortly after birth; the first meiotic divisions occur at about postnatal day 20, and at day 35 the first spermatozoa are produced.⁴⁴ Although laminin $\alpha2$ chain-deficient dy^{3K}/dy^{3K} mice die of unknown causes at about 35 days of age,⁷ the first wave of spermatogenesis can still be studied. Lack of laminin $\alpha2$ chain affected the structure of the basement membrane, which was thinner

Figure 10. Immunostaining of laminin $\gamma3$ chain in $dy^{3K}LN\alpha1TG$ testis. Cross-sections of testes from wild-type and $dy^{3K}LN\alpha1TG$ mice were stained with antibodies against laminin $\gamma3$ chain. Expression of laminin $\gamma3$ chain was not restored in $dy^{3K}LN\alpha1TG$ testis. Bar = 80 μm .

and disrupted to a variable degree in dy^{3K}/dy^{3K} testis. Seminiferous tubules of laminin $\alpha 2$ chain-deficient dy^{3K}/dy^{3K} testis displayed a defect in the timing of lumen formation and reduced amounts of round and elongated spermatozoa. These data show that testis maturation is impaired in laminin $\alpha 2$ chain-deficient mice.

Lack of laminin $\alpha 2$ chain in the basement membrane of testis resulted in reduced expression of laminin $\gamma 3$ chain and disruption of the basement membrane as defined by electron microscopy. Other basement membrane components (such as other laminin chains, collagen type IV, and perlecan) were present despite irregular basement membranes. However, the presence of basement membrane components does not necessarily reflect the presence of a basement membrane defined by electron microscopy. Also, it has been shown that the basement membrane in laminin $\alpha 2$ chain-deficient muscle is disrupted despite the presence of other basement membrane components.^{25,37} Previously, it has been demonstrated that laminin $\gamma 3$ chain is the only laminin chain that is not associated with basement membranes.³⁵ In testis, laminin $\gamma 3$ chain was shown to be expressed on the luminal side of the seminiferous tubule.³⁵ However, using novel antibodies specific for laminin $\gamma 3$ chain,³² we showed here that laminin $\gamma 3$ chain is expressed exclusively in the basement membranes of testis and also in other organs (data not shown). Furthermore, because no other laminin chains have been shown to be expressed on apical surfaces of testis epithelium, it is likely that the antibody used by Koch et al³⁵ cross reacts with an unrelated apical antigen. Thus, we suggest that spermatogenesis may be dependent on laminin-213 or laminin-223.⁴⁵

Besides the $\alpha 2$ chain, the $\alpha 1$ chain is the other major laminin α chain in testis. Dy^{3K}/dy^{3K} testis has elevated levels of laminin $\alpha 1$ chain. This led us to question whether further increasing laminin $\alpha 1$ chain levels could rescue the abnormal testicular phenotype. An additional twofold increase in laminin $\alpha 1$ chain expression did rescue the testicular phenotype. Very likely, overexpression of laminin $\alpha 1$ chain in testis is the major cause of rescue, but we cannot rule out that the increased expression of laminin $\alpha 1$ chain in other tissues also is beneficial.

It is noteworthy that overexpression of laminin $\alpha 1$ chain did not normalize laminin $\gamma 3$ chain expression in testis, taking into account that laminin $\gamma 3$ chain mRNA is made in dy^{3K}/dy^{3K} testis. Also in peripheral nerve, loss of laminin $\alpha 2$ chain leads to a dramatic reduction of laminin $\gamma 3$ chain expression, and no restoration is seen on forced expression of laminin $\alpha 1$ chain (K. Gawlik and M. Durbeek, unpublished data). Hence, it appears that laminin $\alpha 1$ chain does not form a trimer with laminin $\gamma 3$ chain, at least not in testis or peripheral nerve. Laminin $\alpha 1$ chain-mediated rescue of the testis defect most likely involves laminin $\gamma 1$ chain because this chain is up-regulated on overexpression of laminin $\alpha 1$ chain. Laminin $\gamma 2$ chain was also shown to be expressed in the basement membrane of the seminiferous tubules, but so far, the laminin $\alpha 3$ chain is the only α chain known to associate with laminin $\gamma 2$ chain.⁴⁶ Therefore, it is peculiar that laminin $\alpha 3$ chain expression in testis was confined to blood vessels rather than the epithelial basement membrane.

Further studies are needed to identify the α chains associated with the $\gamma 2$ chain in the basement membranes of seminiferous tubules.

The detailed mechanisms by which lack of laminin $\alpha 2$ chain deficiency leads to impaired spermatogenesis remain to be clarified. Basement membranes provide structural stability of organs and send signals to cells through cell surface receptors.⁴⁷ Laminin $\alpha 2$ chain receptors include members of the integrin family and dystroglycan.⁴⁸ Integrins have been identified as laminin binding proteins in Sertoli cells,⁴⁹ and integrin $\alpha 6\beta 1$ and dystroglycan are both confined to the basal compartment of the seminiferous epithelium.^{18,50,51} Mice deficient in integrin $\alpha 6$ subunit die at birth,⁵² and a targeted disruption of dystroglycan results in early embryonic lethality.⁵³ Thus, tissue-specific ablation of the genes could be used to directly study the involvement of these two genes in spermatogenesis. The expression patterns of these two receptors are not altered in dy^{3K}/dy^{3K} testis (data not shown). Nevertheless, signal transduction cascades through laminin $\alpha 2$ chain and integrins or dystroglycan might be perturbed in dy^{3K}/dy^{3K} testis. In muscle, laminin $\alpha 2$ chain appears to promote myotube stability by preventing apoptosis,⁵⁴ and in dystrophic muscle, increased apoptotic cell death might be involved in the process of muscle fiber degeneration.^{7,55} Yet, in laminin $\alpha 2$ chain-deficient testis, we did not notice increased apoptotic cell death (data not shown).

Finally, to our knowledge, there is no published literature that deals with the issue of MDC1A and fertility, but our data indicate that male patients with laminin $\alpha 2$ chain-deficient muscular dystrophy may be infertile. Recently, we demonstrated that laminin $\alpha 1$ chain distinctly reduces muscular dystrophy in laminin $\alpha 2$ chain-deficient mice.²⁵ Here, we showed that laminin $\alpha 1$ chain can compensate for laminin $\alpha 2$ chain absence in testis. Lentiviral vectors have been shown to be efficient in transducing both muscle and Sertoli cells of testis.^{56,57} Thus, delivery of laminin $\alpha 1$ chain into muscle and testis using lentiviral vectors will be tested and could constitute promising therapeutic strategies for treatment of muscular dystrophy with associated infertility.

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