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Transgenic isolation of skeletal muscle and kidney defects in laminin beta2 mutant mice: Implications for Pierson syndrome

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Summary

Pierson syndrome is a recently defined disease usually lethal within the first postnatal months and caused by mutations in the gene encoding laminin β2 (*LAMB2*). The hallmarks of Pierson syndrome are congenital nephrotic syndrome accompanied by ocular abnormalities, including microcoria (small pupils), with muscular and neurological developmental defects also present. *Lamb2*−/− mice are a model for Pierson syndrome; they exhibit defects in the kidney glomerular barrier, in the development and organization of the neuromuscular junction, and in the retina. *Lamb2*−/− mice fail to thrive and die very small at 3 weeks of age, but to what extent the kidney and neuromuscular defects each contribute to this severe phenotype has been obscure, though highly relevant to understanding Pierson syndrome. To investigate this, we generated transgenic mouse lines expressing rat laminin β2 either in muscle or in glomerular epithelial cells (podocytes) and crossed them onto the *Lamb2*−/− background. Rat β2 was confined in skeletal muscle to synapses and myotendinous junctions, and in kidney to the glomerular basement membrane. In transgenic *Lamb2*−/− mice, β2 deposition only in glomeruli prevented proteinuria but did not ameliorate the severe phenotype. In contrast, β2 expression only in muscle restored synaptic architecture and led to greatly improved health, but the mice died from kidney disease at 1 month. Rescue of both glomeruli and synapses was associated with normal weight gain, fertility, and lifespan. We conclude that muscle defects in *Lamb2*−/− mice are responsible for the severe failure to thrive phenotype, and that renal replacement therapy alone will be an inadequate treatment for Pierson syndrome.

Introduction

Basement membranes (BMs) are thin sheets of specialized extracellular matrix that underlie all epithelial and endothelial cells, surround all muscle fibers, fat cells, and Schwann cell/axon units in peripheral nerves, and encase the entire central nervous system. BMs have been shown to influence cell proliferation, differentiation, and migration. They are also involved in filtration and tissue compartmentalization and serve as barriers to cancer cells.

All BMs contain members of four protein families: type IV collagen, nidogen/entactin, sulfated proteoglycans, and laminin (reviewed in Sasaki et al., 2004). The particular protein isoforms present in a given BM are presumed to impart unique functional properties important for behavior of the adjacent cells and for organ development and function. Perhaps the best evidence for this is the fact that mutations in genes encoding BM proteins cause human disease,

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despite substitution by related isoforms in some cases. For example, Alport syndrome is a type IV collagen disease of kidney and inner ear (Kashtan, 2004); Knobloch syndrome is a collagen XVIII (heparan sulfate proteoglycan) disease affecting the eye (Suzuki et al., 2002); and Herlitz's junctional epidermolysis bullosa and congenital muscular dystrophy type 1A are laminin diseases of the skin and neuromuscular system, respectively (Burgeson and Christiano, 1997; Patton, 2000).

Laminins exist in BMs as α-β-γ heterotrimers. In mammals there are five α, four β, and three γ chains that assemble nonrandomly to form at least fifteen different heterotrimers (see Aumailley et al., 2005 for a new laminin nomenclature; reviewed in Miner and Yurchenco, 2004). Evidence from zebrafish labelports the existence of a fourth β chain (Parsons et al., 2002). Mutations in ten of the eleven known mouse laminin genes have been reported. These studies have shown that laminin is absolutely required for BM formation (Miner et al., 2004; Smyth et al., 1999) and that laminins play roles in diverse developmental and physiological processes (reviewed in Miner and Yurchenco, 2004; Yurchenco et al., 2004).

Recently, mutations in the laminin β2 gene (*LAMB2*) were reported in a rare, lethal human disease called Pierson syndrome (Zenker et al., 2004a; Zenker et al., 2005). Pierson syndrome, also called microcoria-congenital nephrosis syndrome (OMIM 609049), is an autosomal recessive disease with congenital nephrotic syndrome/diffuse mesangial sclerosis, distinct ocular abnormalities including microcoria (small pupil), and impairment of vision and neurodevelopment (Zenker et al., 2004b). These features are consistent with the phenotype of mice lacking laminin β2, which stop gaining weight at one week of age and die at approximately three weeks of age. *Lamb2*−/− mice exhibit heavy proteinuria/congenital nephrotic syndrome with podocyte foot process effacement (Noakes et al., 1995b), aberrantly formed and functionally impaired neuromuscular junctions (Knight et al., 2003; Nishimune et al., 2004; Noakes et al., 1995a; Patton et al., 1998), and both structural and functional abnormalities in the retina (Libby et al., 1999). These phenotypes reflect the fact that laminin β2 has been reported to be concentrated in the kidney glomerulus and at the skeletal neuromuscular junction (Hunter et al., 1989) and to be deposited in the retina (Libby et al., 2000; Libby et al., 1997).

While the eye abnormalities would not be expected to impair the growth and longevity of *Lamb2*−/− mice, the glomerular and neuromuscular defects likely contribute to the severity of the failure to thrive phenotype. However, it has been difficult to determine the extent to which each of these is involved. Moreover, laminin β2 is expressed at other sites quite widely throughout the body (Sasaki et al., 2002), so there may exist defects in other tissues that are being masked by the severity of the kidney and muscle defects. To investigate these issues, which have relevance in terms of understanding both basic laminin biology and Pierson syndrome, we have used tissue-specific laminin β2 transgenes to isolate the neuromuscular and kidney defects, rescuing each one individually and both together by mating the transgenes onto the *Lamb2*−/− background. A similar approach was previously used to isolate skeletal muscle defects from those in peripheral nerve in *Lama2* mutant mice (Kuang et al., 1998). Our results suggest that the neuromuscular defect, rather than the kidney defect, is responsible for the severe overt phenotype. Furthermore, a requirement for laminin β2 in tissues other than muscle and kidney to maintain normal viability could not be demonstrated.

Materials and methods

Construction of rat laminin β2 transgenes

To construct the MCK-B2 transgene, the full length rat laminin β2 cDNA (Green et al., 1992) (gift from Joshua R. Sanes, Harvard University, Cambridge, MA) was inserted between the 3.3 kb mouse muscle creatine kinase (MCK) enhancer/promoter and the SV40 large T-antigen transcription termination and polyadenylylation signal sequences (Jaynes et al.,

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1988) (gift from Jean Buskin and Stephen Hauschka, University of Washington, Seattle, WA). To construct the NEPH-B2 transgene, the rat β2 cDNA plus SV40 3′ processing signals from MCK-B2 were flanked with loxP sites and inserted downstream of the 4.2 kb mouse nephrin promoter (Eremina et al., 2002) (gift from Susan Quaggin, University of Toronto, Toronto, Canada).

Production of laminin β2 knockout and transgenic mice

Production of mice carrying the targeted *Lamb2* mutation (gift from Joshua R. Sanes) has been previously described (Noakes et al., 1995a). Briefly, the MC1*neo* cassette was inserted into the *Age*I site in the second exon of mouse *Lamb2*. Antibodies to epitopes encoded by mRNA sequences 3′ of this insertion did not stain homozygous mutants (Noakes et al., 1995b; Sasaki et al., 2002), indicating that the insertion generates a null mutation. Mice were genotyped by PCR of DNA extracted from tail clips using the following primer pairs: for the mutant allele (MC1*neo*-specific), 5′-CGAATTCGAACACGCAGATGCAG-3′ and 5′-CCGGGCGCCCCTGCGCTGACAGC-3′; for the wild-type allele, 5′-TGACCCACTGTCCTCAGTGCTG-3′ and 5′-GAGTGTAGGATAGGTACCTTAG-3′.

To produce transgenic mice, the transgenes were digested and gel-purified away from plasmid vector sequences and then microinjected individually into the pronuclei of single-celled B6CBAF2/J embryos by the Washington University Mouse Genetics Core. Founders and their transgenic offspring were identified by PCR of DNA extracted from tail clips using the following primers: for MCK-B2, 5′-CTGGCTAGTCACACCCTGTAGG-3′ (MCK forward) and 5′-CTGGATAGCAGCTTCCTCGAG-3′ (β2 reverse); for NEPH-B2, 5′-GAAGCAGCAGAATGAGTTCACAC-3′ (nephrin forward) and 5′-ATACGAAGTTATTCGAAGTCGAG-3′ (vector/loxP sequences between the nephrin promoter and the β2 cDNA).

Antibodies, immunostaining, and histology

Mouse mAbs D5 and D7 that recognize the rat laminin β2 COOH-terminal coiled-coil domain (Hunter et al., 1989; Sanes et al., 1990) were gifts from Joshua R. Sanes and were also obtained from the Developmental Studies Hybridoma Bank (Iowa City, IA). Polyclonal antisera to the mouse laminin β2 LF domain (formerly called domain IV) (Sasaki et al., 2002), which cross react with rat β2, were a gift from Takako Sasaki and the late Rupert Timpl (Max Planck Institute for Biochemistry, Martinsried, Germany). Rat mAb 5A2 to the mouse laminin β1 LN domain (formerly VI) (Abrahamson et al., 1989; St John et al., 2001) was a gift from Dale Abrahamson (University of Kansas Medical Center, Kansas City, KS). Rabbit antiserum 8948 to the mouse laminin α5 LEb and L4b domains (formerly IIIb and IVa) has been described (Miner et al., 1997). Alexa 488- and Cy3-conjugated secondary antibodies were obtained from Molecular Probes (Eugene, OR) and Chemicon (Temecula, CA), respectively.

For immunostaining, fresh tissues were immersed in OCT and quick-frozen in 2- methylbutane cooled in a dry ice-ethanol bath. Seven μm frozen sections were cut in a cryostat and air dried on gelatin-coated slides. Antibodies were diluted in PBS with 1% BSA, applied to sections for 1 hour, and rinsed in PBS. Secondary antibodies were then applied in a similar fashion. TRITC-conjugated α-bungarotoxin (Molecular Probes) was included to localize synaptic sites in skeletal muscle. After rinsing the sections were mounted in 90% glycerol containing 0.1X PBS and 1 mg/ml *p*-phenylenediamine and viewed under epifluorescence with a Nikon Eclipse 800 compound microscope. Images were captured with a Spot 2 cooled color digital camera (Diagnostic Instruments, Sterling Heights, MI).

For conventional light microscopy, tissues were fixed in 10% buffered formalin, dehydrated through graded ethanols, embedded in paraffin, sectioned at 4 μm, and stained with periodic-

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acid-Schiff (PAS). For electron microscopy, tissues were fixed, embedded in plastic, sectioned, and stained as previously described (Kikkawa et al., 2003; Noakes et al., 1995b).

Evans blue analysis

To assay the integrity of skeletal muscle fibers, Evans blue dye (Sigma, St. Louis, MO) at 10 mg/ml in 0.9% NaCl was injected intraperitoneally at 10 μl/g body weight. After 6 hrs mice were perfused under anesthesia with 10 ml PBS and then 40 ml of 2% paraformaldehyde in PBS. The diaphragm with rib insertions was dissected out and post-fixed overnight with 3% paraformaldehyde and 1% glutaraldehyde in PBS at 4°C. The diaphragm was then detached from the ribs and photographed with a QImaging Micropublisher digital camera (QImaging, Burnaby, BC, Canada) attached to a stereomicroscope.

Urinalysis

Urine was collected from mice at various ages either by manual restraint, by caging on raised wire floors (Ancare, Bellmore, NY) for several hours, or by bladder puncture under anesthesia at the time of sacrifice. One μL of urine was run on precast 4 to 20% SDS polyacrylamide gels (Invitrogen/Novex, Carlsbad, CA), and gels were stained with Coomassie blue and destained by standard methods. For quantitation, urinary protein and creatinine concentrations were measured with a Cobas Mira Plus analyzer (Roche, Somerville, NJ).

Results

Generation and characterization of laminin β2 transgenic mice

We wished to isolate the muscle and kidney defects observed in *Lamb2*−/− mice so that they could be studied independently of one another. The failure to thrive phenotype of *Lamb2*−/− mice is so severe that indirect effects of the kidney and neuromuscular diseases on each other, and perhaps on other tissues, could not be ruled out. One approach to accomplish this would be to make cell type-specific knockouts using Cre/loxP technology. However, we chose to attempt to selectively rescue each defect individually using cell type-specific laminin β2 transgenes expressed on the *Lamb2*−/− background.

Muscle-specific β2 transgene.—The reported developmental defects in *Lamb2*−/− muscles are restricted to the synapse. Consistent with this, laminin β2 is concentrated in the synaptic portion of the myofiber BM; it also accumulates at myotendinous junctions (MTJs) (Patton et al., 1997) and can be detected extrasynaptically with some antibodies (Hunter et al., 1989; Sasaki et al., 2002; Wewer et al., 1997). Previous studies have shown that β2 is expressed by skeletal muscle in vivo (Moscoso et al., 1995) and is concentrated at synapse-like sites that form on cultured myotubes in vitro even in the absence of neurons (Martin et al., 1995). Together, these data suggest that skeletal muscle is capable of concentrating laminin β2 at synaptic sites independent of motor neuron expression of β2, should there be any. We therefore constructed a β2 transgene (MCK-B2; Fig. 1A) that should be expressed at high levels in skeletal muscle, using the well-characterized mouse MCK enhancer/promoter. This element has also been shown to drive expression in cardiac muscle with ~100 fold less activity, but, importantly, it is not significantly active in kidney (Johnson et al., 1989). We chose to use the rat β2 cDNA because of the availability of mouse mAbs that recognize rat but not mouse β2 (Sanes et al., 1990). These mAbs facilitated specific detection of transgene-derived β2.

Seven MCK-B2 transgenic founders were generated. Because mouse tail contains multiple tissue types, including some skeletal muscle, transgene expression was assayed by staining frozen sections of a tail biopsy from each founder with anti-rat β2 monoclonal antibodies. Muscle synaptic sites were identified by co-staining with TRITC-conjugated α-bungarotoxin, which binds to acetylcholine receptors. Two of the seven founders expressed rat β2, and it was

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concentrated at synapses. These two were mated to *Lamb2* +/− mice to generate *Lamb2* +/−; MCK-B2 lines.

Analysis of offspring showed that only one of the two lines deposited the transgene-derived β2 consistently at all synapses, and this line was used for subsequent studies. An immunohistochemical survey of tissues was performed to determine whether expression was muscle-specific. Rat β2 was detected at skeletal muscle synapses (Fig. 2C,D), but not in extrasynaptic regions of the myofiber BM. This is in contrast to the endogenous mouse β2, which is found in both synaptic and extrasynaptic regions (Fig. 2 and Sasaki et al., 2002). Expression was also observed in cardiac muscle and in some visceral smooth muscle, but not in nerve, kidney, lung parenchyma, skin, liver, retina, intestinal mucosa, or brain (Fig. 2E-H and data not shown). Mosaic expression was observed in the vascular smooth muscle of arteries (data not shown). Based on these results, we conclude that the MCK-B2 transgene behaves in an appropriate tissue-specific fashion and that the presumed expression of the transgene throughout the skeletal muscle fiber nevertheless leads to concentration of β2 at synapses, as previously shown in vitro (Martin et al., 1995).

Podocyte-specific β2 transgene.—The known defects in *Lamb2*−/− kidney are restricted to the glomerular filter, consistent with the fact that β2 is highly concentrated in the glomerular BM (GBM) (Hunter et al., 1989). The GBM is synthesized by two cells: the podocytes that are present in the urinary space and lie on the outer aspect of the GBM, and the endothelial cells that line the glomerular capillaries on the inner aspect of the GBM (St John and Abrahamson, 2001). In order to restrict rat β2 transgene expression to the glomerulus, we chose to use a podocytespecific promoter element isolated from the *Nphs1* gene (Eremina et al., 2002), which encodes nephrin (Kestila et al., 1998), to make the NEPH-B2 transgene (Fig. 1B). For added flexibility in future experiments, the rat β2 cDNA and the adjacent SV40 sequences were flanked by loxP sites so that transgene expression could be halted by Cre-mediated recombination.

Three NEPH-B2 transgenic founders were obtained, and each was mated to a *Lamb2* +/− mouse to generate three independent lines. Transgene expression was assayed in offspring by immunostaining kidney sections for rat β2, which was never detected in non-transgenic controls (Fig. 3A). All three transgenes were expressed, and rat β2 was only detected in the GBM (Fig. 3B). However, deposition was segmental and weak in two of the three lines, so only the third was used for subsequent experiments. Rat β2 deposition was not detected in any other tissues examined, including skeletal muscle, heart, intestine, and lung (Fig. 3C,D and data not shown).

Tissue-specific transgenic rescue of *Lamb2*−/− defects

Skeletal muscle rescue.—To determine whether muscle-derived laminin β2 was sufficient to rescue the *Lamb2*−/− neuromuscular junction differentiation defects, and to attempt to isolate the glomerular filtration defect, *Lamb2* +/− mice were crossed with *Lamb2* +/−; MCK-B2 mice to generate *Lamb2*−/−; MCK-B2 mice. *Lamb2*−/−; MCK-B2 mice were significantly more healthy than *Lamb2*−/− mice and were usually indistinguishable from littermate controls with respect to overall appearance and body weight during the pre-weaning period (Fig. 4A). However, they routinely died at one month of age, only ~10 days later than *Lamb2*−/− mice. SDS-PAGE analysis of urine showed massive proteinuria before and after weaning, with albumin being the major urinary protein (Fig. 4B and data not shown). Quantitation of urinary protein to creatinine ratios showed that proteinuria in *Lamb2*−/−; MCK-B2 mice was comparable to that observed in *Lamb2*−/− mice. Consistent with the heavy proteinuria, *Lamb2* −/−; MCK-B2 mice were edematous near the time of death.

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Immunohistochemical analyses showed that in skeletal muscle, rat β2 was concentrated at synaptic sites in *Lamb2*−/−; MCK-B2 mice (Fig. 5). Interestingly, as discussed in detail in the Discussion, even with the more sensitive polyclonal antibody, little β2 was detectable along the extrasynaptic portions of the myofiber. (One exception was the MTJ, as shown below.) Expression was also detected in cardiac muscle but not in kidney or in other non-muscle tissues, consistent with the data in Fig. 2.

We previously showed that loss of β2 from synapses in *Lamb2*−/− muscle leads to both loss of laminin α5, β2's partner in the synaptic laminin α5β2γ1 (laminin-11 or Lm-521), and increased synaptic β1 (Patton et al., 1997), which presumably compensates for the absence of β2. Here, restoration of β2 to the synaptic cleft in *Lamb2*−/−; MCK-B2 mice led to the reappearance of α5 and the disappearance of β1 (Fig. 5C-F). Not only was the molecular composition of the synaptic BM normalized, but the endplate also acquired the normal pretzel-like shape (Fig. 5B) that *Lamb2*−/− synapses lack (Noakes et al., 1995a). Ultrastructural analyses further confirmed the rescue of synaptic organization: the muscle endplate established junctional folds, and Schwann cell processes did not aberrantly extend into the synaptic cleft (Fig. 6A-C). We conclude that muscle-derived β2 is deposited into the synaptic cleft BM and is sufficient to rescue synaptic structural and compositional defects in the *Lamb2* mutant. The overall improvement in health of the animal suggests that synaptic function is also improved.

As mentioned previously, laminin β2 is also normally concentrated at MTJs, but developmental defects in *Lamb2*−/− MTJs have not previously been reported. Injection of Evans blue, a dye that accumulates only in damaged and regenerating skeletal and cardiac muscle fibers (Straub et al., 1997), into *Lamb2*−/− mice resulted in labeling of muscle fibers in the diaphragm, but primarily at the ends, near MTJs (Fig. 7A,B). (This pattern contrasts with the labeling observed in typical dystrophic muscle, which occurs along the entire length of muscle fibers.) Furthermore, transmission electron microscopy showed that the BM associated with the MTJ was somewhat discontinuous and less compact compared to control, and the folding at the end of mutant muscle fibers exhibited reduced complexity (Fig. 6E,F). Together, these data suggest that in the absence of laminin β2, MTJs are structurally and functionally defective.

In *Lamb2*−/−; MCK-B2 mice, β2 was detectable at most if not all MTJs (Fig. 7F), and this correlated with improved ultrastructure in some fibers (Fig. 6G). But surprisingly, muscle fibers in *Lamb2*−/−; MCK-B2 mice were still labeled by Evans blue near MTJs (Fig. 7C). A possible explanation stems from our analysis of *Lamb2*+/−; MCK-B2 MTJs, which should contain both endogenous mouse and transgene-derived rat β2. However, double staining for total and for transgene-derived rat β2 revealed that many MTJs do not contain rat β2, though they contain the endogenous mouse β2 (Fig. 7G,H). This shows that transgene-derived protein accumulates at MTJs inefficiently compared to endogenous protein. It then follows that transgene-derived β2 should also accumulate inefficiently at MTJs in *Lamb2*−/−; MCK-B2 mice (though it does accumulate, as shown in Fig. 7F), which could lead to a mild injury to the developing muscle and the observed Evans blue uptake in the highly active diaphragm.

Kidney rescue.—To determine whether podocyte-derived laminin β2 is sufficient to restore the glomerular filtration barrier, and to attempt to isolate the neuromuscular defects, *Lamb2* +/−; NEPH-B2 mice were crossed with *Lamb2* +/− mice to generate *Lamb2*−/−; NEPH-B2 mice. These mice were overtly indistinguishable from and had growth curves similar to *Lamb2* −/− mice (Fig. 4A), and they died at 3 to 4 weeks of age, suggesting little improvement in overall health. However, immunohistological analysis revealed efficient deposition of rat β2 into the GBM (Fig. 8), and urinalysis revealed that there was no proteinuria in *Lamb2*−/−; NEPH-B2 mice (data not shown). The abrogation of proteinuria led to no obvious improvement in synaptic architecture in the absence of synaptic β2 (Fig. 6D), demonstrating for the first time that synaptic defects in *Lamb2*−/− mice are not secondary to ill health caused by proteinuria.

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Based on these results, we conclude that the severe phenotype in *Lamb2*−/− mice stems primarily from the neuromuscular junction defects.

We previously showed that there is a laminin β1 to β2 developmental transition in the forming GBM (Miner and Sanes, 1994). In *Lamb2*−/− mice, β1 remains in the GBM rather than being eliminated, thus serving a structural role to maintain GBM integrity, but nevertheless failing to maintain the glomerular barrier to protein (Noakes et al., 1995b). Here, restoration of podocyte-derived β2 to the GBM was accompanied by proper elimination of β1 as glomeruli matured, though it was still detectable in the mesangial matrix (Fig. 8). Ultrastructural analysis showed normal podocyte foot process architecture in *Lamb2*−/−; NEPH-B2 glomeruli at all stages, even at a time near death (Fig. 6H,K and data not shown). In contrast, analysis of *Lamb2* −/− and *Lamb2*−/−; MCK-B2 glomeruli revealed widespread foot process effacement (Fig. 6H-J), consistent with the heavy proteinuria (Fig. 4B).

Combined transgenic rescue of muscle and kidney defects

We next generated *Lamb2*−/−; MCK-B2; NEPH-B2 mice through appropriate crosses. Except for occasional mild proteinuria, these mice have manifested no obvious defects and can live for well over a year, some to eighteen months. Both males and females have exhibited normal fertility and are overtly indistinguishable from control littermates. Light microscopic analysis of PAS-stained paraffin sections revealed no significant pathology in either skeletal muscle or kidney in older mice (Fig. 9), and ultrastructural analysis of glomeruli and neuromuscular junctions showed no significant abnormalities (data not shown). Interestingly, Evans blue only occasionally labeled the ends of muscle fibers in the diaphragms of older doubly rescued mice (Fig. 7D and data not shown), and all MTJs contained β2 (Fig. 7I-L), suggesting that subsequent to any initial injury at developmental stages, as in Fig. 7C, MTJs are apparently repaired. Furthermore, no histopathology was observed in other tissues reported to be rich in β2, including lung, retina, pancreas, or gut (data not shown). Based on these results, a requirement for β2 in BMs other than those associated with muscle and the kidney glomerulus can not be demonstrated, at least in the context of the controlled environment of a mouse cage.

Discussion

Accumulating evidence shows that laminins play crucial roles in the development and function of many organs, as well as in such fundamental developmental processes as gastrulation, notochord formation, and neural tube closure (Miner and Yurchenco, 2004). The growth of the laminin family during the evolution of multicellular organisms has allowed a segregation of laminins into two distinct groups in vertebrates: one group containing laminins absolutely required during embryogenesis for basic developmental processes, and the second containing laminins with more restricted, specialized functions in postnatal animals.

Here we have focused on the laminin β2 chain, which falls squarely into the latter group. While laminin β2 has no obvious function during development in utero, and *Lamb2*−/− pups are indistinguishable from control littermates during the first postnatal week, homozygotes die at ~3 weeks of age with severe defects in both the glomerular filter and the neuromuscular junction (Noakes et al., 1995a; Noakes et al., 1995b). Whether there might be defects in other tissues (besides retina; Libby et al., 1999), and how the kidney and muscle defects each contribute to the lethal failure to thrive phenotype, have been important unanswered questions. The recent finding of mutations in *LAMB2* associated with Pierson syndrome (Zenker et al., 2004a; Zenker et al., 2005) underscores the importance of these issues with regard to human development and disease.

To investigate whether the kidney or the synapse defect is most detrimental to the animals' health, we used tissue-specific laminin β2 transgenes to rescue each defect individually. We

have clearly shown that the defects in synaptic development and maturation are much more devastating: rescuing the GBM defects with the podocyte-specific β2 transgene (NEPH-B2) did not improve the overt phenotype of the *Lamb2* mutant, whereas rescuing the structure and presumably the function of the neuromuscular junction with the muscle-specific β2 transgene (MCK-B2) greatly improved weight gain and extended lifespan by ~50%. The latter mice must have died from nephrotic syndrome, because preventing it through simple addition of the NEPH-B2 transgene allowed for a very long life (well over 1 year) and normal fertility in *Lamb2*−/−; MCK-B2; NEPH-B2 mice. Furthermore, we can conclude from this result that for normal longevity of caged mice, laminin β2 is only required in muscle and glomerular BMs, the only sites where we could detect β2 in *Lamb2*−/−; MCK-B2; NEPH-B2 mice. Such an important conclusion would not have been possible had we chosen to use the more conventional Cre/loxP conditional knockout approach to specifically mutate *Lamb2* in skeletal muscle or podocytes, because all tissues not expressing Cre would contain their normal complement of β2.

Because the MCK regulatory element is active in both skeletal and cardiac muscle and, to a much lesser extent, in smooth muscle (Jaynes et al., 1988; Johnson et al., 1989), we cannot be absolutely certain that the basis for improvement in the health of *Lamb2*−/−; MCK-B2 mice is solely due to restored synaptic architecture and function. But the fact that *Lamb2*−/− hearts exhibit no obvious morphological or histopathological defects, such as ventricular dilation or cardiomyocyte necrosis or fibrosis (J. H. Miner, B. L. Patton, and G. Jarad, unpublished), suggests that the improved health of *Lamb2*−/−; MCK-B2 mice is not due to transgene expression in the heart. Moreover, that cardiac defects are not generally associated with Pierson syndrome (Zenker et al., 2004b) is also consistent with the lack of a critical role for laminin β2 in the heart.

So why do defects in synaptic structure and function kill *Lamb2*−/− mice? The mice usually die at ~21 days of age, which is also the time of weaning. Together with the highly significant 50% decrease in mean quantal content at mutant nerve terminals (Knight et al., 2003), the most likely explanation for death is that the mice are simply unable to properly use their muscles to obtain nutrition. Before weaning this involves suckling for many hours each day, in addition to competing with littermates for access to teats; after weaning it involves obtaining and chewing solid food and reaching for water. Consistent with this explanation, we have found that hand feeding *Lamb2*−/− mice can extend their life to up to 40 days of age, though they remain very small and weak. A lack of proper nutrition is fully consistent with the observed failure to thrive phenotype.

Although laminin β2 is concentrated at skeletal muscle synapses, it can normally also be detected extrasynaptically all along the muscle fiber with certain antibodies (Sasaki et al., 2002; Wewer et al., 1997), suggesting that the muscle deposits β2 along its entire length. Here, however, little MCK-B2 transgene-derived β2 was detected in the muscle fiber BM, other than at synapses and MTJs. Because there is no reason to suspect that the MCK regulatory element is not active in nuclei throughout the entire muscle fiber, these data suggest that skeletal muscle fibers are programmed to deposit β2 only in synaptic and MTJ BMs. This was predicted in part by in vitro data demonstrating that the laminin β2 protein contains an inherent signal that targets it to synapse-like sites on cultured myotubes (Martin et al., 1995). Another cell type—perhaps the interstitial fibroblast—is likely to be responsible for deposition of laminin β2 along the extra-junctional portions of the muscle fiber.

During kidney glomerular development, distinct BMs synthesized by the epithelial podocytes and the invading endothelium fuse to form the immature GBM (Abrahamson and Perry, 1986). The laminin component of the maturing GBM continues to be synthesized by both the podocytes and the endothelial cells (St. John and Abrahamson, 2001). Our approach to restore

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laminin β2 to the mutant GBM by expressing it exclusively in podocytes via the nephrin promoter was successful in several respects, including localization to the GBM, restoration of the glomerular barrier to protein, and restriction of laminin β1 to the mesangium. This indicates that though GBM laminin β2 is normally synthesized by both podocytes and endothelial cells, expression is podocytes is sufficient for normal GBM structure and function.

Our results have important implications for Pierson syndrome, which is caused by mutations in human *LAMB2* (Zenker et al., 2004a; Zenker et al., 2005). Individuals with this disease present with congenital nephrotic syndrome, diffuse mesangial sclerosis, and microcoria (fixed narrowing of the pupils) associated with absent dilator pupillae muscles in the iris. Other eye abnormalities include an almost absent ciliary body, an enlarged cornea, an abnormal lens shape, maldevelopment of the choroid, and arrested retinal development. No obvious structural abnormalities of the brain have been found, but muscular hypotonia and abnormal movements have been reported (Zenker et al., 2004b). Affected individuals usually die within weeks after birth from kidney failure, but some have lived for over two years. The kidney and retinal defects seem to correlate quite well with defects in the *Lamb2* mutant mouse, and the muscular hypotonia may stem from similar defects in development and function of the neuromuscular junction. However, cornea, lens, and pupillary defects have not been found in the mouse.

Because of the availability of renal replacement therapy, through either dialysis or transplantation, the congenital nephrosis is the most treatable aspect of Pierson syndrome. However, the results of our transgenic rescue studies show that ameliorating the nephrotic syndrome on its own is not significantly beneficial in extending the life of the *Lamb2*−/− mouse. This calls into question whether renal replacement therapy alone is warranted in Pierson syndrome patients. Indeed, one patient who received chronic dialysis treatment and lived for 1.8 years exhibited significant neurological and developmental deficits including severe hypotonia, psychomotor delay, and blindness (Zenker et al., 2004a). Recently, a missense mutation in *LAMB2* was reported in a patient exhibiting congenital nephrotic syndrome but not the other features of Pierson syndrome (Hasselbacher et al., 2005), demonstrating that there is heterogeneity in the severity of disease resulting from *LAMB2* mutations in humans. As more patients with *LAMB2* mutations are discovered and genotype-phenotype correlations are discerned, a better understanding of the neurological and muscular defects will hopefully emerge and lead to the development of extrarenal treatment paradigms.

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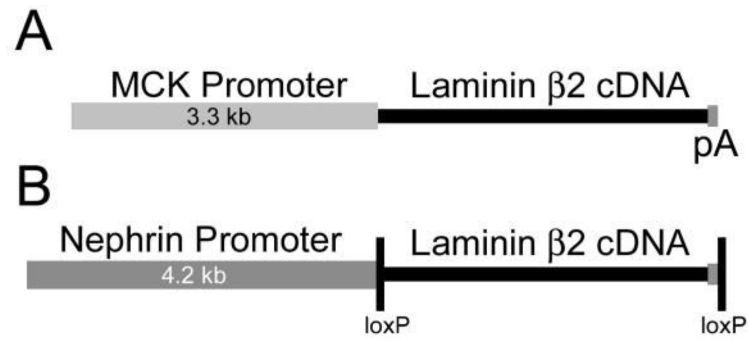

Fig. 1.
Structure of the transgenes. (A) The MCK-B2 transgene drives rat laminin β2 expression from the mouse muscle creatine kinase (MCK) promoter, and transcription termination and polyadenylylation signal sequences from SV40 (pA) ensure processing to mRNA. (B) The NEPH-B2 transgene drives β2 expression from the mouse nephrin promoter. The β2 cDNA and SV40 sequences were flanked by loxP sites for future manipulations.

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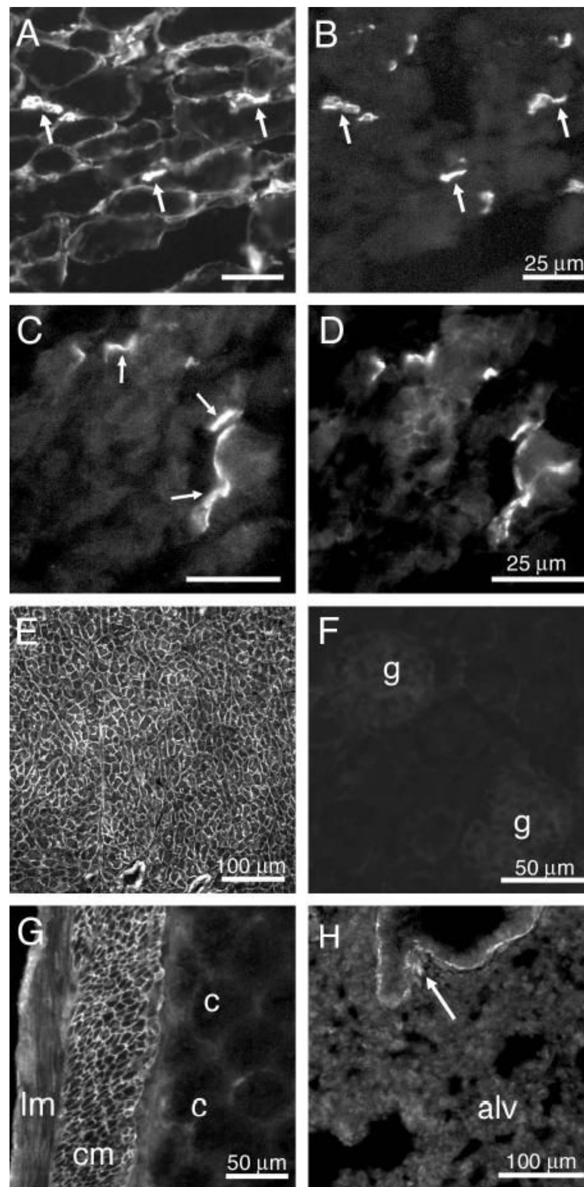

Fig. 2.
Localization of endogenous and MCK-B2 transgene-derived laminin β2. (A,B) In control skeletal muscle, endogenous mouse laminin β2 (A) is concentrated at synapses (arrows) doubly labeled by α-bungarotoxin (B); β2 is also found in extrasynaptic regions of muscle fibers (A). (C,D) In MCK-B2 transgenics, antibody specific for transgene-derived rat β2 (C) only labels synapses in skeletal muscle (arrows), identified by α-bungarotoxin (D). (E-H) Transgene-derived rat laminin β2 is also found in cardiac muscle BMs (E), in circular (cm) but not longitudinal smooth muscle (lm) or crypt (c) epithelial BMs of intestine (G), and weakly in large airway smooth muscle of lung (arrow in H) but not in alveolar (alv) BMs. No rat β2 was detected in glomeruli (g) in kidney (F). Scale bars in A-D, 25 μm; in E and H, 100 μm; in F and G, 50 μm.

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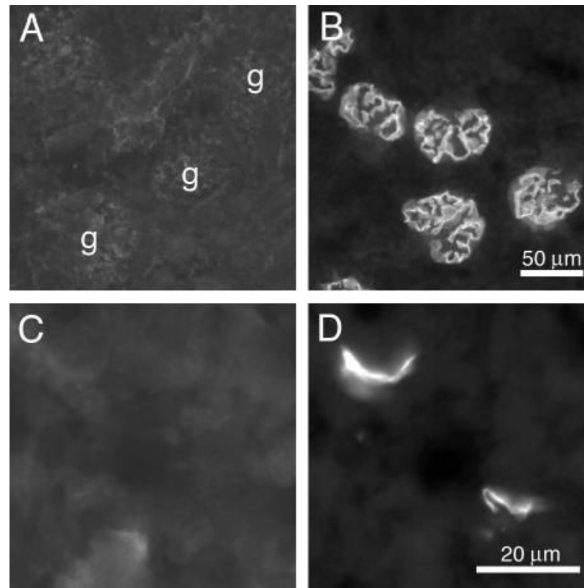

Fig. 3.
NEPH-B2 transgene-derived laminin β2 accumulates specifically in the GBM. (A,B) Antibody specific for transgene-derived rat β2 does not stain kidney glomeruli (g) from a control mouse (A) but stains GBM in kidney from NEPH-B2 transgenic mice (B). (C,D) NEPH-B2 transgene-derived β2 is not deposited at skeletal muscle synapses (C) identified by double staining with α-bungarotoxin (D). Scale bar in B, 50 µm; in D, 20 µm.

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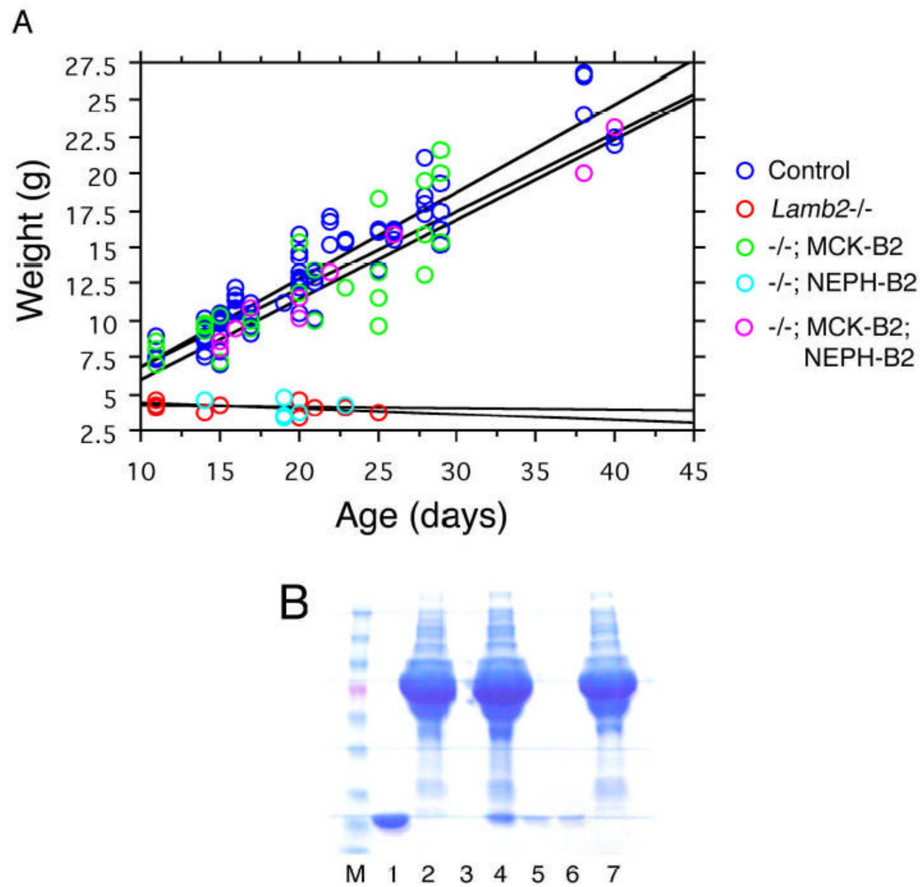

Fig. 4.

(A) Growth curves (weight vs. age) of control mice, *Lamb2*−/− mice, and *Lamb2*−/− mice carrying transgenes. *Lamb2*−/− and *Lamb2*−/−; NEPH-B2 mice fail to surpass a weight of ~4 g and die at 3 to 4 weeks of age. In contrast, *Lamb2*−/−; MCK-B2 mice grow at a rate similar to controls but die at 1 month of age. *Lamb2*−/−; MCK-B2; NEPH-B2 doubly transgenic mice exhibit normal weight gain and a long life. (B) Proteinuria in *Lamb2*−/−; MCK-B2 mice. One μL of urine was analyzed by SDS-PAGE and stained with Coomassie blue. M, markers; the pink band is albumin. Lanes 1, 5, and 6: urine from *Lamb2*+/−; MCK-B2 mice. Lanes 2, 4, and 7: urine from *Lamb2*−/−; MCK-B2 mice. Lane 3 was empty.

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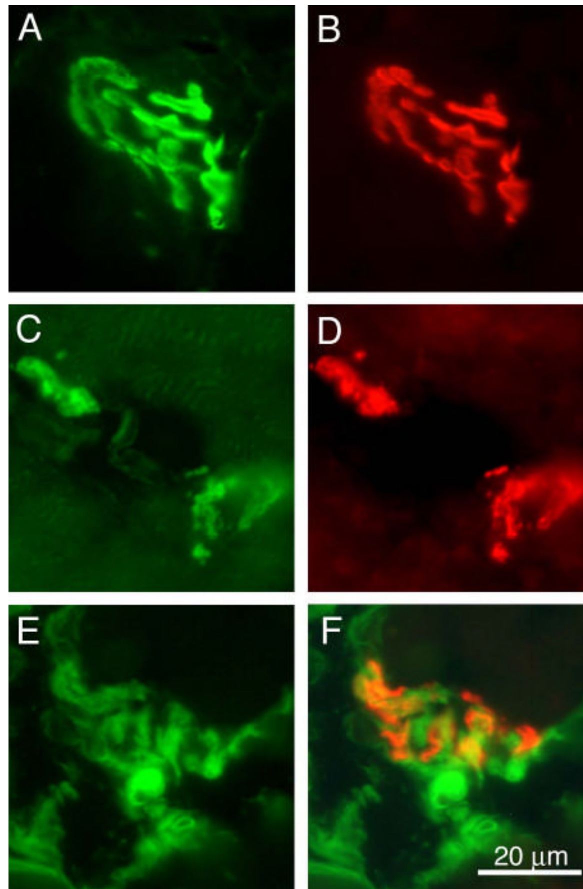

Fig. 5.

Restoration of proper synaptic architecture and BM composition in skeletal muscle from *Lamb2*−/−; MCK-B2 mice. (A,B) Colocalization of rat laminin β2 (A) with α-bungarotoxin (B) in a highly ramified skeletal muscle synapse. (C,D) Restoration of laminin α5 to synaptic BMs (C) identified by α-bungarotoxin (D). (E,F) Laminin β1 (E, green in F) is not associated with the α-bungarotoxin-positive synaptic clefts (red in the merged image in F). Scale bar in F, 20 µm for A-F.

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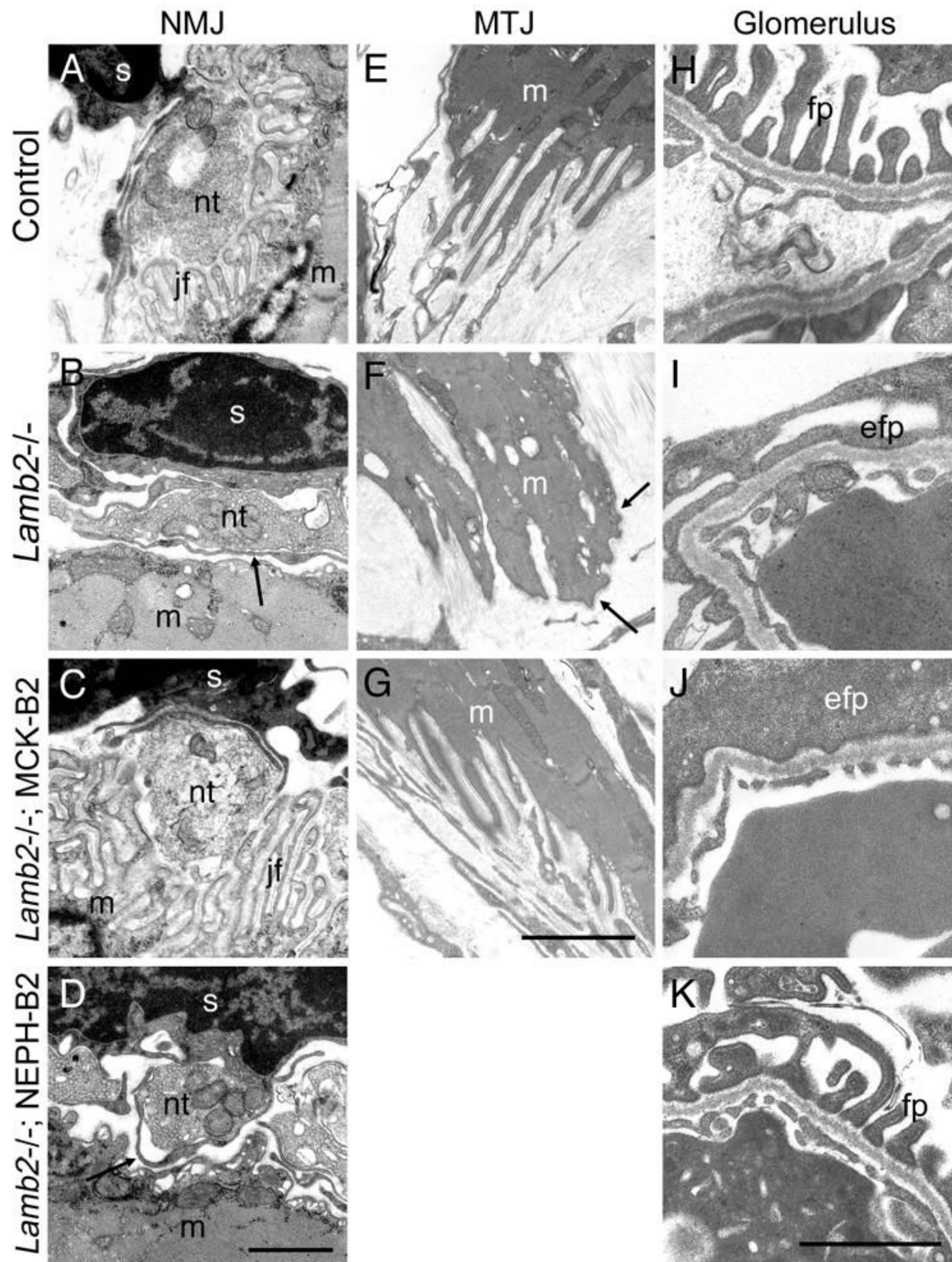

Fig. 6.

Ultrastructural analysis of neuromuscular junctions (NMJ), myotendinous junctions (MTJ), and glomerular filtration barriers. (A-D) A control synapse (A) shows a Schwann cell (s) capping the vesicle-rich nerve terminal (nt) adjacent to the muscle (m) endplate containing numerous junctional folds (jf). In the *Lamb2*−/− synapse (B), junctional folds are absent and the Schwann cell extends processes (arrow) between the nerve terminal and the muscle. Synaptic deposition of laminin β2 in *Lamb2*−/−; MCK-B2 mice restores synaptic architecture to normal (C). In *Lamb2*−/−; NEPH-B2 mice, glomerular deposition of β2 and prevention of proteinuria has no restorative effect on the synapse (D). (E-G) MTJ from a control (E) exhibits numerous infoldings of the muscle fiber (m) with continuous BMs. In the *Lamb2*−/− MTJ (F), infoldings are less complex, and the BMs (arrows) appear fuzzy. MCK-B2 transgene-derived β2 restores much of the normal MTJ architecture (G). (H-K) Glomerular capillary segment

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from a control (H) shows the interdigitated podocyte foot processes (fp) adjacent to the GBM. Effaced foot processes (efp) are evident in the *Lamb2*−/− (I) and *Lamb2*−/−; MCK-B2 (J) mice, which are proteinuric. Deposition of rat β2 into the GBM in *Lamb2*−/−; NEPH-B2 mice prevents proteinuria and foot process effacement (K). Scale bars in D and K are 1 μm for A-D and H-K, respectively; scale bar in G is 2 μm for E-G.

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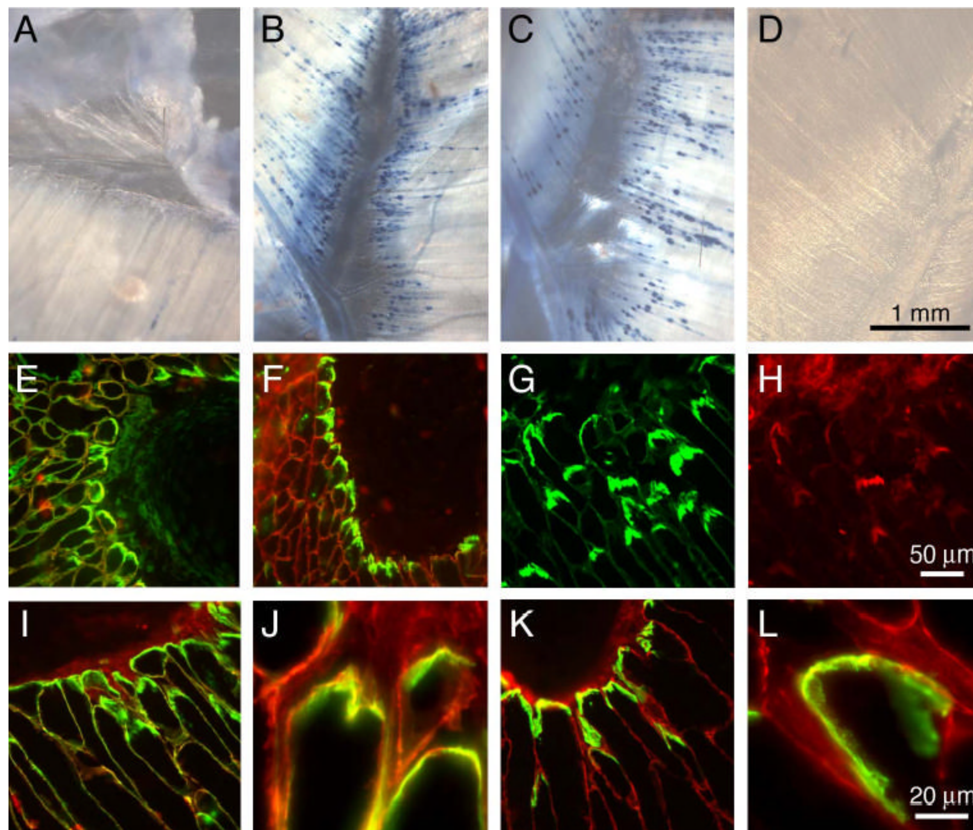

Fig. 7.

Functional and compositional analyses of MTJs. (A-D) Evans blue analysis reveals normal MTJ integrity in a control mouse diaphragm (A), but evidence of damage is observed in *Lamb2* −/− (B) and *Lamb2*−/−; MCK-B2 (C) mice at 3 weeks of age. A diaphragm from an older adult *Lamb2*−/−; MCK-B2; NEPH-B2 mouse shows no damage (D), suggesting eventual repair of MTJs. (E,F) Double staining for laminin β2 (green) and laminin α2 (red) in intercostal muscles reveals concentration of β2 at MTJs in both control (E) and *Lamb2*−/−; MCK-B2 (F) mice. (G,H) Double staining for mouse plus rat β2 (G) and rat β2 only (H) in a Lamb2+/−; MCK-B2 mouse shows that many MTJs contain little if any transgene-derived protein. (I-L) Double staining for total laminin β2 (green) and α2 (red) in intercostal muscles from aged control (I,J) and *Lamb2*−/−; MCK-B2; NEPH-B2 (K,L) mice reveals efficient accumulation of β2 at MTJs. Note the lack of transgene-derived β2 in extra-junctional BMs in rescued mutant muscle fibers (F,K). Scale bar in D, 1 mm for A-D; in H, 50 µm for A-I and K; in L, 20 µm for J and L.

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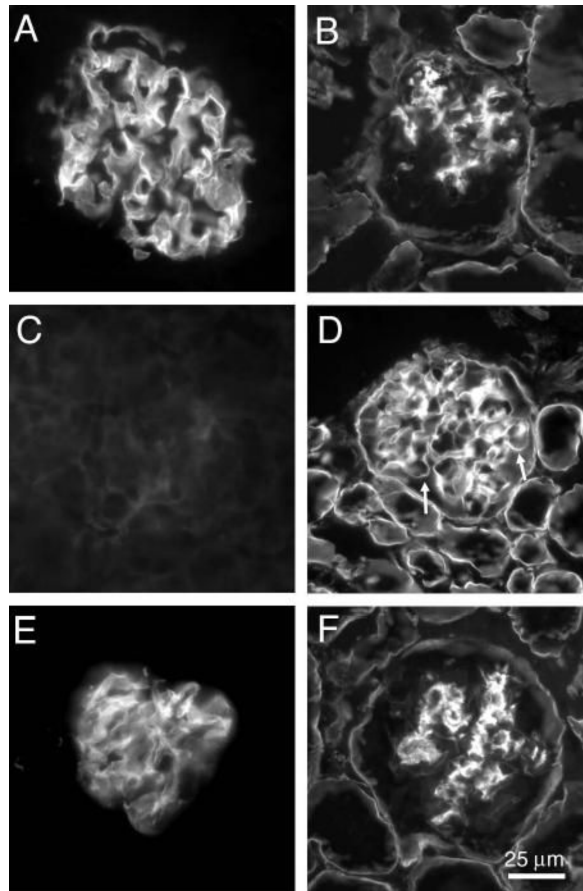

Fig. 8.

Analysis of laminin β chain composition in glomeruli. (A,B) In *Lamb2*+/−; NEPH-B2 mice, the GBM contains rat laminin β2 (A), and laminin β1 is restricted to the mesangial matrix (B). (C,D) In *Lamb2*−/− mice, no β2 is present in the GBM (C), and β1 substitutes for it in the GBM (arrows in D). (E,F) In *Lamb2*−/−; NEPH-B2 mice, rat β2 is deposited into the GBM (E), and β1 is restricted to the mesangial matrix (F) as in controls. Scale bar in F, 25 μm for A-F.

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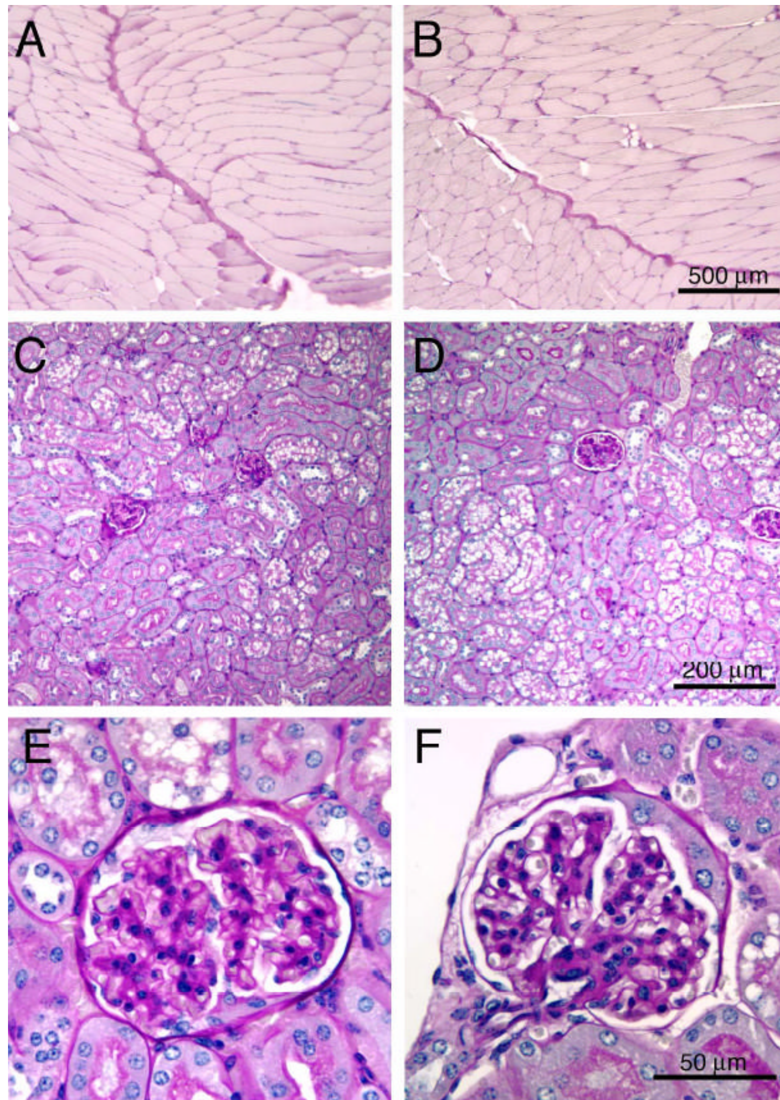

Fig. 9.

Histological analysis of skeletal muscle and kidney from aged control (A,C,E) and *Lamb2*−/−; MCK-B2; NEPH-B2 (B,D,F) mice. Paraffin sections were stained with PAS. No obvious pathology was observed in either skeletal muscle (A,B) or kidney (C,D; glomeruli at high power in E,F) from control or transgene-rescued mutant mice. Scale bar in B, 500 µm; in D, 200 µm; in F, 50 µm.