

Erratum

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We mistakenly gave one of the authors of this paper, Dr Andrey Irintchev, the incorrect first name (Audrey). We sincerely apologise to Dr Andrey Irintchev for this error.

The extracellular-matrix protein matrilin 2 participates in peripheral nerve regeneration

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Summary

Matrilins are adaptor proteins of the extracellular matrix involved in the formation of both collagen-dependent and collagen-independent filamentous networks. Although their molecular structure and binding partners have been characterized, the functional roles of the four matrilin family members *in vivo* are still largely unknown. Here, we show that matrilin 2, expressed in pre-myelinating Schwann cells during normal development, profoundly influences the behaviour of glial cells and neurons *in vitro*. When offered as a uniform substrate, matrilin 2 increased neurite outgrowth of dorsal root ganglia (DRG) neurons and enhanced the migration of both cell line- and embryonic DRG-derived Schwann cells. Vice versa, axonal outgrowth and cell migration were decreased in DRG cultures prepared from matrilin-2-deficient mice compared with wild-type (wt) cultures. In stripe assays, matrilin 2 alone was sufficient to guide axonal growth and, interestingly, axons favoured the combination of matrilin 2 and laminin over laminin alone. *In vivo*, matrilin 2 was strongly upregulated in

injured peripheral nerves of adult wild-type mice and failure of protein upregulation in knockout mice resulted in delayed regrowth of regenerating axons and delayed time-course of functional recovery. Strikingly, the functional recovery 2 months after nerve injury was inferior in matrilin-2-deficient mice compared with wild-type littermates, although motoneuron survival, quality of axonal regeneration, estimated by analyses of axonal diameters and degrees of myelination, and Schwann cell proliferation were not influenced by the mutation. These results show that matrilin 2 is a permissive substrate for axonal growth and cell migration, and that it is required for successful nerve regeneration.

Supplementary material available online at
<http://jcs.biologists.org/cgi/content/full/122/7/995/DC1>

Key words: Schwann cells, Axonal outgrowth, Extracellular matrix, Coating substrates, Cell migration, Peripheral nerve regeneration

Introduction

Since its discovery a decade ago (Deak et al., 1997), matrilin 2 has attracted the attention of researchers because of its wide distribution in the extracellular matrix (ECM) of different tissues and interesting molecular structure predicting functionally important molecular interactions. Matrilin 2 shares a similar modular structure with the other matrilin family members, matrilin 1 and matrilin 3 primarily expressed in cartilage and the ubiquitously expressed matrilin 4 (Wagener et al., 2005). Analysis of mouse and human matrilin-2 cDNA sequences has revealed that the precursor protein contains a putative signal peptide, two von Willebrand factor A (VWA)-like modules separated by ten epidermal growth factor (EGF)-like motifs, a unique segment not identified in other proteins and a coiled-coil (CC) oligomerization domain (Deak et al., 1997). This structure, in particular the vWFA modules, have predicted possibilities for versatile protein-protein interactions. And indeed, in tissue extracts and cell culture medium, matrilin 2 exists as mono-, di-, tri- and tetramers (Piecha et al., 1999). In addition, biochemical and biophysical characterization of matrilin CC domains have revealed the potential for heterotrimeric interactions between matrilin 2 and matrilin 1 or matrilin 4 (Frank et al., 2002). In addition, several other ECM ligands for matrilin 2, such as different types of collagen, fibrillin 1, fibrillin 2, laminin and fibronectin, have been identified using surface plasmon

resonance and electron microscopy (Piecha et al., 2002). Thus, matrilin 2 is a widespread ECM component that can interact with itself and with other collagenous and non-collagenous matrix molecules. This broad interaction repertoire, together with its oligomeric structure, render matrilin 2 a good candidate as an adapter or mediator molecule for interactions between other matrix macromolecules during the assembly of the ECM.

Despite this knowledge, the specific functions of matrilin 2 are currently unknown because the recently generated matrilin-2-knockout mouse has not revealed any apparent phenotypes (Mates et al., 2004). Based on previous data on the expression of matrilin 2 in the perineurium of peripheral nerves, among various other mouse organs and tissues (Deak et al., 1997; Piecha et al., 1999), and our own data demonstrating the expression in developing dorsal root ganglia and in Schwann cells (SCs) after nerve injury, we hypothesized that matrilin 2 might be important for cellular interactions and repair processes in the peripheral nervous system. To test this hypothesis, we devised experiments using a spectrum of approaches to characterize the influences of matrilin 2, or its absence, on the behaviour of SCs and peripheral neurons *in vitro*. These experiments showed that matrilin 2 profoundly influences cell migration and axonal outgrowth *in vitro*. Taking advantage of a recently developed femoral nerve injury paradigm allowing

precise evaluation of motor recovery and subsequent morphological evaluation of nerve regeneration in the same animals (Irintchev et al., 2005; Simova et al., 2006), we compared functional recovery in adult matrilin-2-deficient mice and wild-type littermates. The combined results of our analyses provide novel evidence for the functional importance of matrilin 2 in nerve regeneration.

Results

Matrilin 2 is expressed in Schwann cells

In order to determine the cell types expressing matrilin-2 mRNAs in the developing PNS we used, in addition to wild-type mice, *ErbB3*-deficient embryos. These embryos lack SC precursors along peripheral nerves (Britsch et al., 1998; Riethmacher et al., 1997), a feature that aids expression profiling in the PNS. Using in situ hybridization, we found that matrilin 2 was expressed in DRG and along peripheral nerves throughout development of wild-type embryos (Fig. 1C,E,F), whereas in *ErbB3*-deficient embryos, no matrilin 2 was detectable in peripheral nerves, and the expression level was much lower than in wild-type embryos within the DRG (Fig. 1D). This result was confirmed by semi-quantitative RT-PCR of cDNA prepared from DRG where the signal was severely reduced in samples from *ErbB3*-deficient embryos compared with the wild-type control (Fig. 1A). By immunohistochemistry matrilin 2 was found in developing DRG and in the endoneurium of developing spinal nerves, as well as in the spinal dura mater and the epineurium (supplementary material Fig. S3). Furthermore, the expression of matrilin 2 was analyzed in different cell types by semi-quantitative RT-PCR. Matrilin 2 was found to be expressed by at least two SC types: S16 cells (an immortalized SC line from rat sciatic nerve) and primary embryonic SCs (ESCs) (prepared from DRG of mouse embryos), whereas the precursor of embryonic SCs, neural crest stem cells, do not express matrilin 2 (Fig. 1B).

Matrilin 2 promotes adhesion and migration of immortalized S16 Schwann cells in vitro

Next, we sought to identify effects of matrilin 2 on SC behaviour in vitro. Using a cell adhesion assay, we found that matrilin 2 strongly promotes S16 SC adhesion compared with uncoated plastic substrate. This effect was, however, less pronounced than the degree of laminin- or fibronectin-mediated adhesion (Fig. 2A). We then analyzed the ability of matrilin 2 to promote SC migration, as opposed to other ECM substrates present in peripheral nerves, such as laminin or fibronectin, using the Varani migration assay (Varani et al., 1978). This assay measures the migration of cells from a high-density population of cells contained in an agarose drop away from it with time (for details see Materials and Methods). SCs began to migrate out of the drop within 2 hours of plating and continued to migrate radially for a number of days, producing a uniform 'corona' of cells around the drop. Migration was quantified daily by measuring the distance between the leading edge of the corona and the margin of the agarose drop. The results showed that matrilin 2 significantly promotes SC migration. Unexpectedly, the degree of promotion by matrilin 2 even exceeded the effects of laminin or fibronectin that are known promoters of SC migration (Milner et al., 1997b) (Fig. 2B,C).

Matrilin 2 promotes migration of ESCs from DRG in vitro

As matrilin 2 is expressed in ESCs, we next investigated the effect of matrilin 2 on ESC migration. DRG cultures were prepared from wild-type embryos (E12) and grown on different ECM substrates, including matrilin 2, PDO (poly-D-ornithine), laminin or the

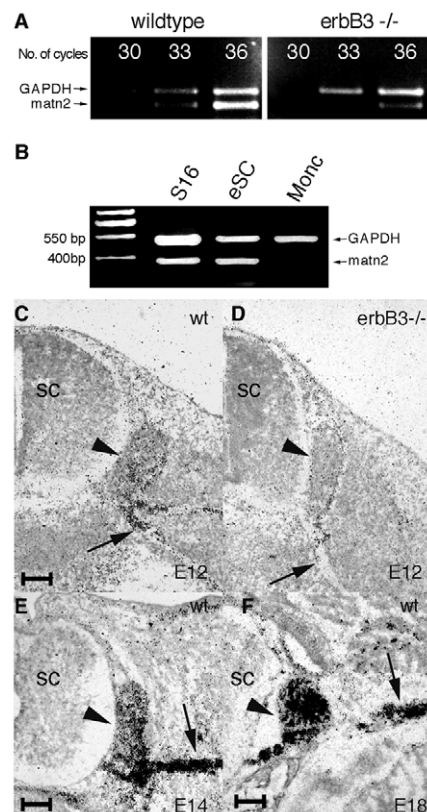


Fig. 1. Different expression pattern of matrilin 2 in wild-type and *ErbB3*^{-/-} embryos, and analysis of matrilin-2 expression levels in different cell types. (A) Semi-quantitative RT-PCR using cDNA prepared from DRG of wild-type and *ErbB3*-mutant embryos. GAPDH was used as an internal control. At comparable GAPDH levels, much higher levels of matrilin-2-specific amplicons were present in wild type compared with mutant extracts. (B) Analysis of matrilin-2 expression by semi-quantitative RT-PCR. Matrilin 2 is expressed in the rat SC line (S16) and primary embryonic SCs, whereas there is no expression of matrilin 2 in neural crest stem cells (Monc) (precursors of embryonic SCs). (C-F) In situ hybridization with ³⁵S-labelled matrilin-2-specific riboprobe on transverse cryosections of mouse embryos (E12-18). Arrowheads indicate the expression of matrilin 2 within DRG of wild-type embryos, whereas the arrows indicate the expression in SCs aligning axonal projections. Note the absence of the expression along axonal projections in the *ErbB3* mutant embryo at E12 (arrow in D). As almost all *ErbB3*-deficient embryos die at E12.5 and the PNS in surviving mutants is progressively degenerating, no older stages were analysed. Scale bars: 100 μ m (in C for C,D); 125 μ m (E); 175 μ m (F).

combination of matrilin 2 and laminin. ESCs were visualized by fluorescence microscopy after incubation with a Sox10-specific antibody (Fig. 3A). We found enhanced migration of ESCs, both in terms of numbers of migrating cells and distances of migration, in cultures grown on either matrilin 2 or the combination of matrilin 2 and laminin, when compared with other substrates (Fig. 3A-C).

In order to investigate whether ablation of matrilin 2 influences ESC migration, we isolated DRG from wild-type and matrilin-2-deficient embryos (E12) and cultured them in dishes coated with PDO, laminin or matrilin 2 in serum-free medium without neurotrophic factors for 72 hours. Immunostaining with anti-Sox10 antibodies and nuclear counterstaining with DAPI revealed a significant decrease of the number of migrating ESCs in cultures from matrilin-2-deficient embryos on PDO (data not shown) and laminin (Fig. 3D,E), which is in good agreement with our previous results. However, when DRG were grown on matrilin-2-coated surfaces, no significant

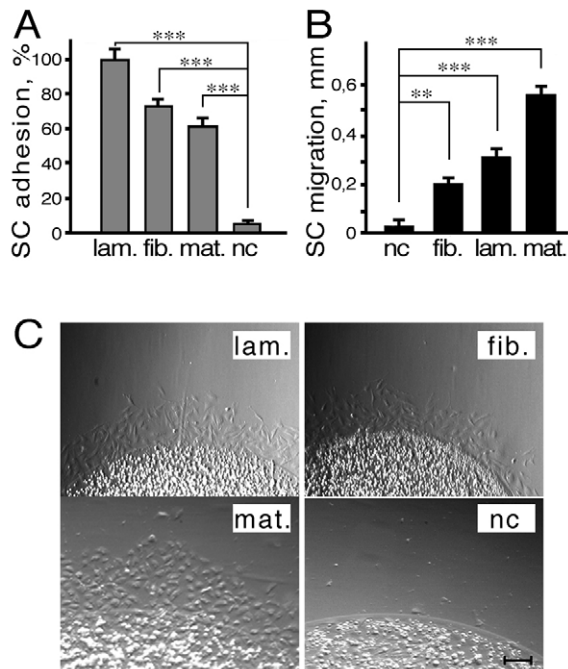


Fig. 2. Comparison of migration and adhesion of S16 cells on different extracellular matrix substrates. (A) Effect of matrilin 2 on SC adhesion. SC suspensions were incubated for 30 minutes in wells of 24-well tissue culture dishes coated with different ECM substrates. After washing, the adherent cells were fixed and counted using phase-contrast microscopy. Results are expressed as a percentage of the number of cells adhering to laminin, which was normalized to 100%. Statistical analysis showed a significant increase ($***P<0.001$) of the numbers of cells adhering to different substrates versus non-coated (nc) dishes. Note that also the differences in adhesion between laminin and fibronectin or matrilin 2 are significant. (B,C) S16 SCs migration on different coating substrates determined by the agarose drop migration assay. (B) Mean distances of SCs migration on different ECM substrates, fibronectin, laminin and matrilin 2. All ECM molecules promoted SCs migration compared with the uncoated control substrate ($***P<0.001$). In addition, the distances of migration on matrilin-2 surfaces were significantly increased ($***P<0.001$) in comparison with laminin- or fibronectin-coated surfaces. $**P<0.01$. (C) Cell migration was analyzed using phase-contrast microscopy. Scale bar: 100 μ m.

difference in the migration behaviour of ESCs could be observed between cultures from wild-type and matrilin-2-deficient embryos (Fig. 3D). In addition, if matrilin 2 was added to the medium of matrilin-2-deficient DRG seeded on laminin-coated surfaces, the number of migrating ESCs was significantly increased and was similar to migrating ESCs numbers in wild-type cultures on laminin (Fig. 3D,E). This indicates that adding matrilin 2 to the medium or growing the DRG on matrilin-2-coated surfaces can rescue the migration defect of matrilin-2-deficient ESC in our culture assay. Addition of matrilin 2 to the medium of cultures grown on matrilin-2-coated surfaces had no significant enhancing effect (Fig. 3D). Similar results regarding SC migration were obtained when cultures were grown in the presence of neurotrophic factors.

Matrilin 2 promotes axonal outgrowth but not axonal branching of DRG neurons

To analyze the effects of matrilin 2 on outgrowth and branching of sensory neuron neurites, we cultured DRG explants or dissociated DRG neurons on different ECM substrates: matrilin 2, laminin or the combination of laminin and matrilin 2 in serum-free medium

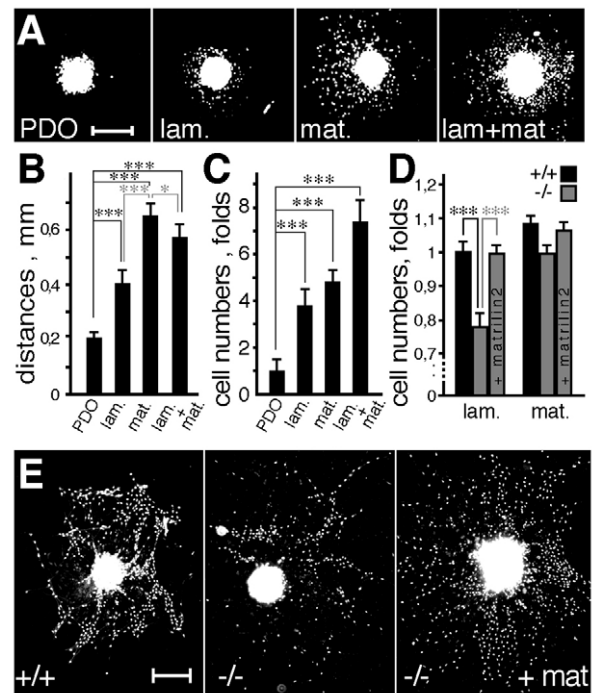


Fig. 3. Matrilin 2 promotes SC migration from embryonic DRG. (A) Cell migration from wild-type DRG on different ECM substrates. Cells allowed to migrate for 24 hours on different substrates were visualized by fluorescence microscopy after incubation with Sox10-specific antibodies. More cells migrate out of DRG on matrilin 2 and a mixture of matrilin 2 + laminin compared with just laminin. Scale bar: 250 μ m. (B) Mean distances of SC migration on different substrates. Migration was quantified by measuring the distance from the edge of a ganglion to the leading edge of migrating SCs on photomicrographs. Significant differences are indicated ($***P<0.001$; $*P<0.05$). (C) Mean numbers of migrating cells expressed as fold difference compared with PDO. Significant differences are indicated. In addition, the numbers of migrating cells on a mixture of laminin and matrilin 2 are significantly elevated ($***P<0.001$) compared with migration on laminin or matrilin 2 alone. (D) Migration of ESCs from DRG prepared from matrilin-2-deficient and wild-type embryos. Cells were allowed to migrate on laminin and matrilin-2-coated surfaces with or without addition of matrilin 2 (2 μ g/ml) to the medium for 72 hours. Mean numbers of migrating cells expressed as fold difference compared with wild-type on laminin. Significant differences are indicated ($***P<0.001$). Addition of matrilin 2 to the culture medium had no significant effect on wild-type cultures (data not shown). (E) DRG from wild-type (+/+) and matrilin-2-deficient (-/-) embryos grown on laminin for 72 hours without and with the addition of matrilin 2 to the culture medium (+mat). Note the increase in migrating SCs (Sox10-positive) in matrilin-2-deficient cultures on laminin after addition of matrilin 2 (+mat) compared with the mutant culture without matrilin 2. Scale bar: 250 μ m.

containing growth factors. As shown in Fig. 4A, we found a significant increase of axonal length on a matrilin-2-coated surface compared with PDO-coated surface. Interestingly, matrilin 2 was almost as potent in promoting neurite outgrowth as laminin, one of the most potent enhancers of axonal growth.

To determine whether axonal outgrowth is decreased in DRG of matrilin-2-deficient animals, we prepared DRG cultures from wild-type and matrilin-2-deficient embryos, and cultured them for 48 hours on PDO, laminin or matrilin-2-coated surfaces. Indeed, quantitative analysis revealed that axonal length was significantly decreased in matrilin-2-deficient cultures in comparison with wild-type on PDO (data not shown) and laminin (Fig. 4B). However, when DRG were cultured on matrilin-2-coated surfaces, no significant difference in

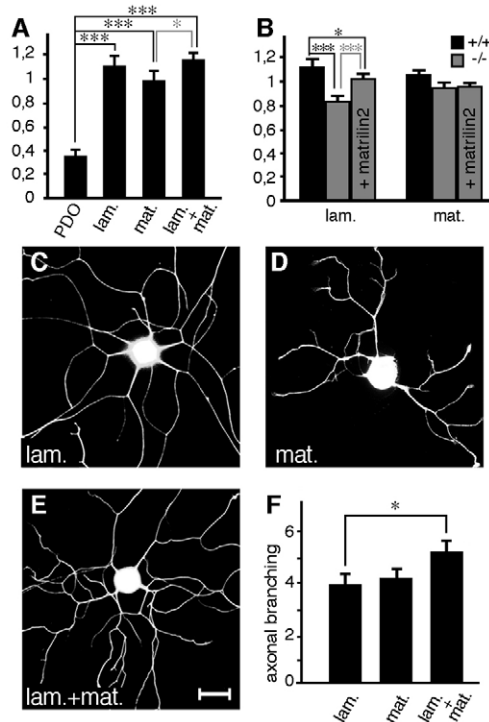


Fig. 4. Matrilin 2 promotes axonal outgrowth of DRG neurons but does not affect branching. (A) Axonal outgrowth from wild-type DRG cultures on different ECM substrates. Axons were allowed to grow for 24 hours on different substrates and were then visualized with NF160-specific antibodies. Significant differences are indicated (** $P < 0.001$; * $P < 0.05$). (B) Axon outgrowth from DRG neurons prepared from wt (+/+) and matrilin-2-deficient (-/-) embryos. Axons were grown for 48 hours on laminin (lam.) or matrilin-2-coated surfaces (mat.) with (+matrilin 2) or without the addition of matrilin 2 to the culture medium. After visualization using NF200-specific antibodies, the lengths of axons were measured. Significant differences are indicated (** $P < 0.001$; * $P < 0.05$). Note that axonal outgrowth on matrilin-2-coated surfaces showed no statistical differences and that addition of matrilin 2 to the medium on laminin could not fully rescue the mutants. (C-E) Axonal branching of wild-type DRG neurons on different ECM substrates. Dissociated DRG neurons were prepared from wild-type pups (P0-P3). Morphology of neurites of dissociated sensory neurons grown for 12 hours on laminin (C), matrilin 2 (D) and mixture of laminin and matrilin 2 (E) visualized by β -tubulin (Tuj1) immunostaining. Scale bar: 25 μ m. (F) Quantitative analysis of the mean number of branching points per main neurites demonstrated similar ratios of branching points. Note, however, that there is a slight difference in branching points between laminin and a mixture of laminin and matrilin 2 (* $P < 0.05$).

axonal length could be detected by comparing wild-type and matrilin-2-deficient cultures (Fig. 4B). When matrilin 2 was added to the medium of matrilin-2-deficient DRG grown on laminin, we could observe a significant increase in axonal length compared with matrilin-2-deficient DRG grown without added matrilin 2 (Fig. 4B). However, there still was a small reduction of axonal length compared with wild-type cultures, indicating that addition of matrilin 2 cannot completely rescue this phenotype.

When plated on uniform growth-permissive substrates such as laminin or matrilin 2, dissociated adult DRG neurons started to elaborate processes within a few hours *in vitro*. We compared axonal branching of DRG neurons from young postnatal wild-type mice (P3) within 12 hours of plating on the following ECM substrates: matrilin 2, laminin and matrilin 2 + laminin. We selected this time-point for analysis because the neurite trees were already well developed and

quantitative analysis could still be performed in a straightforward way (Fig. 4C-F). Previous studies have shown that laminin stimulates neurite extension rather than neurite branching. In the present study, we found that the effect of matrilin 2 on branching was similar to that of laminin. On matrilin-2-coated surfaces, DRG axons were slightly shorter than on laminin (Fig. 4C,D), but the number of branching points was comparable with those observed on laminin (Fig. 4F). However, a mixture of matrilin 2 and laminin seems to enhance branching slightly compared with laminin alone (Fig. 4F).

Matrilin 2 promotes axonal growth of DRG neurons

We next compared the ability of matrilin 2 and laminin, fibronectin and poly-L-lysine (PLL), to promote axonal growth in a classical stripe assay. These experiments were carried out in the presence of NGF to support neuronal survival and to elicit axonal outgrowth. As shown previously (Vielmetter et al., 1990), in our experiments DRG neuron axons grew more successfully on laminin compared with PLL (Fig. 5A). When DRG axons were exposed to alternating stripes of matrilin 2 and PLL, they always grew more successfully on matrilin 2 (Fig. 5B), although this effect was not as strong as the one observed for laminin. When DRG neuron axons were confronted with alternating stripes of laminin and matrilin 2, or fibronectin and matrilin 2, axons grew equally well on all substrates (Fig. 5C and data not shown). Interestingly, when exposed to alternating stripes of laminin alone and a mixture of laminin and matrilin 2, growth promotion of DRG neuron axons was enhanced on the mixture of laminin and matrilin 2 compared with laminin alone (Fig. 5D).

Injury of peripheral nerves in adult mice causes re-expression of matrilin 2

When we monitored the expression of matrilin 2 in the developing PNS, we found that the expression is downregulated postnatally (data not shown). Immunohistochemical and immunoblotting analyses revealed that matrilin 2 is still expressed in adult peripheral nerves but at very low levels (Fig. 6B,D). As matrilin 2 is strongly expressed in the developing PNS and then downregulated in the adult, we next investigated whether the expression is upregulated following peripheral nerve injury. Interestingly, at 3 or 5 days after transection of the femoral or sciatic nerve respectively, we observed a strong upregulation of matrilin 2 analysed by RT-PCR (data not shown), immunohistochemistry (Fig. 6A,C) and by western blot analysis (Fig. 6G). The upregulation of matrilin 2 in the distal stump of the injured nerves appeared slightly stronger than in the proximal part. Expression of matrilin 2 was observed in the perineurium of lesioned and intact sciatic nerve, as well as in endoneurium of damaged sciatic nerves (Fig. 6C,D). Interestingly, the perineurium of the femoral nerve was either not or only very weakly immunoreactive for matrilin 2 and exhibited no upregulation even after lesion (Fig. 6A,B). As other members of a family can often compensate if expression of one member of the family is lost, we looked at the expression of matrilin 1, matrilin 3 and matrilin 4 in nerves from wild-type and matrilin-2-deficient animals after lesion. We found no expression of matrilin 1 and matrilin 3 in wild-type or matrilin-2-deficient nerves before or after lesion by RT-PCR analysis (data not shown), indicating that neither matrilin-1 nor matrilin-3 expression show a compensatory upregulation when matrilin-2 expression is lost. When we looked at matrilin 4 immunohistochemically, we found that it was strongly upregulated in nerves of wild-type animals after lesion, similar to matrilin 2 (Fig. 6E,F). Additionally, the upregulation of matrilin 4 demonstrated by RT-PCR analysis is significantly stronger in lesioned nerves from matrilin-2-deficient animals

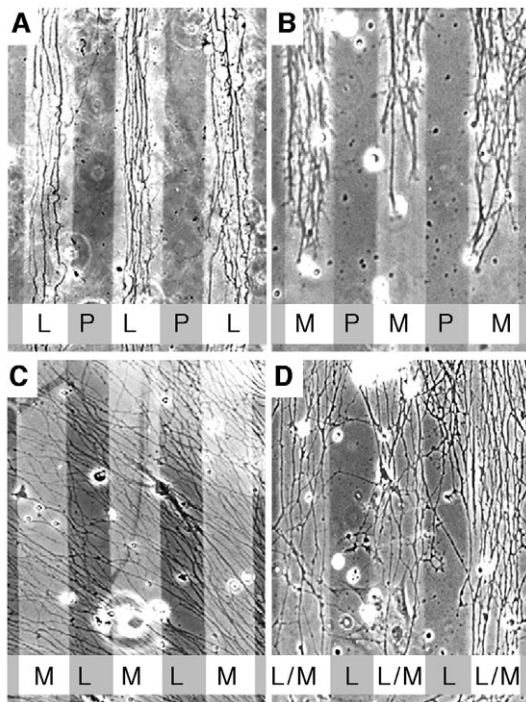


Fig. 5. Growth patterns of DRG neuron axons on different substrates analysed by a stripe assay. DRG explants were grown for 24 hours on 22 mm glasses that had been coated in stripes with laminin (L), poly-L-lysine (P), matrilin 2 (M), or laminin and matrilin 2 (L/M), fixed and immunostained using NF antibodies. Within one experiment, DRG axons were grown on slides coated with alternating stripes of laminin and poly-L-lysine (PLL) (A), matrilin 2 and PLL (B), matrilin 2 and laminin (C) or laminin and a mixture of laminin and matrilin 2 (D).

compared with wild-type animals (Fig. 6H) 5 days after lesion. A difference in matrilin-4 expression is also detectable 7 days after lesion (data not shown).

To further elucidate the tissue distribution of matrilin 2 in the injured nerve, we performed colocalization analyses using cell type- and tissue-specific markers (Fig. 7A-H). These studies revealed that matrilin 2 is not present in axons (Fig. 7A,B), as was expected from our previous experiments demonstrating that matrilin 2 is expressed in SCs. Colocalization using antibodies against myelin basic protein and matrilin 2 clearly showed that matrilin 2 is not incorporated into the myelin sheaths (Fig. 7C,D). By contrast, a high degree of colocalization was found between matrilin 2 and p75, the low-affinity NGF receptor expressed by SCs, (Taniuchi et al., 1986), (Fig. 7E,F) and nidogen 1, a basal lamina marker (Paulsson et al., 1987) (Fig. 7G,H). These results indicate that matrilin-2 expression is upregulated in SCs close to the injury site, both in the proximal and distal nerve stumps. Furthermore, the secreted protein is deposited in the endoneural tubes consisting of basal lamina and is associated with ECM components that guide regenerating axons after peripheral nerve injury. Similar results were obtained for matrilin 4 (supplementary material Fig. S2).

Impaired functional recovery of adult matrilin-2-deficient mice after femoral nerve repair

The finding of a strong upregulation of matrilin 2 in the injured peripheral nerve, its localization in endoneural tubes known to support axonal regeneration and its permissive properties for axonal

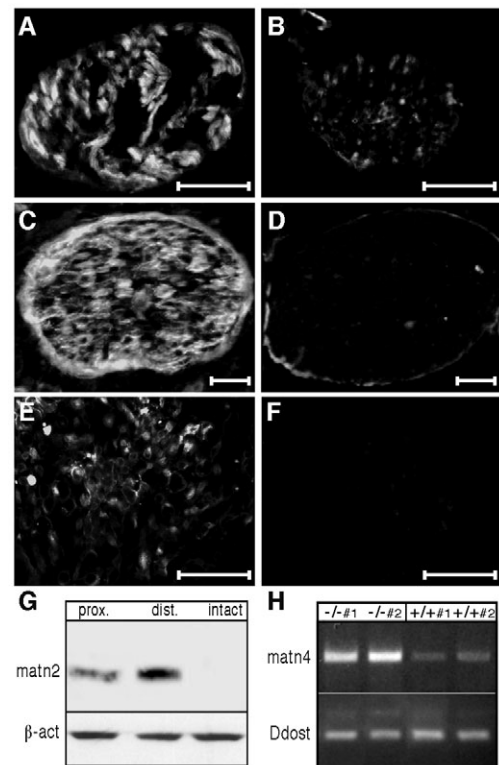


Fig. 6. Upregulation of matrilin 2 and 4 in nerves after lesion. Matrilin-2 expression analysed by immunofluorescence staining of transverse sections from damaged (A,C) and intact (B,D) femoral (A,B) and sciatic (C,D) nerves dissected from adult wild-type mice 3 or 5 days after transection of the femoral or sciatic nerve, respectively. Matrilin-4 expression analysed by immunofluorescence staining of transverse sections from damaged (E) and intact (F) sciatic nerve 7 days after transection. (A-F) Note the strong upregulation upon injury ($n=4$ mice per experiment). Scale bars: 100 μ m. (G) Upregulation of matrilin 2 in damaged sciatic nerves 7 days after nerve injury revealed by western blotting. Samples from intact nerves, as well as proximal and distal stumps of injured nerves, were analysed using antibodies against matrilin 2 and β -actin. (H) RT-PCR analysis was performed on lesioned femoral nerves from wild-type (+/+) and matrilin-2-deficient adult (-/-) animals 5 days after nerve lesion. Note the higher expression of matrilin 4 in samples from lesioned nerve from mutant animals compared with wild-type animals. Expression of Ddost served as an internal control.

regrowth in vitro, indicated that matrilin 2 might be functionally important during nerve regeneration. To address this issue, we used the femoral nerve injury paradigm and single-frame motion analysis for numerical evaluation of muscle function (Irintchev et al., 2005). Two parameters were used for evaluation, the heels-tail angle (HTA) and foot-base angle (FBA). These two parameters, evaluated from video recording of beam walking trials, reflect the ability of the quadriceps muscle to keep the knee extended during single support phases of the gate cycle and, for the FBA, to counteract in addition the rotational moments in the hip caused by the swing of the contralateral extremity. These abilities were severely compromised after femoral nerve transection and repair in both matrilin-2-deficient and wild-type mice, as seen from changes of the values of HTA and FBA between 0 days (prior to operation) and 1 week after operation (Fig. 8A,B). In the subsequent recovery period, however, restoration of function, as evaluated by the HTA, a parameter most directly reflecting knee joint dysfunction, was delayed, by 2 weeks, over time and was by far less complete at 2 months in matrilin-2-deficient

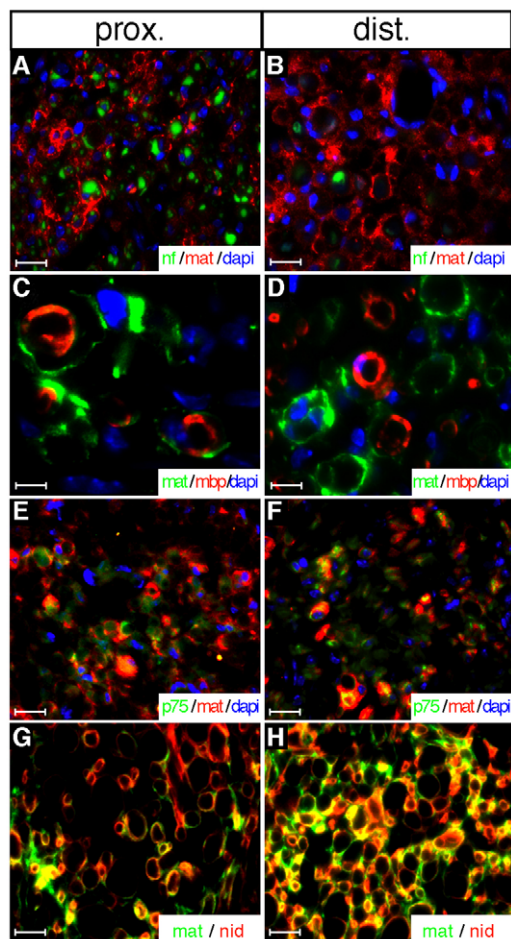


Fig. 7. Immunohistochemical analysis of matrilin-2 expression following nerve injury. Transverse sections from the proximal (A,C,E,G) and distal (B,D,F,H) nerve stumps 7 days after nerve transection in adult mice. (A,B) Co-immunostaining using anti-NF antibody to visualize axons (green) and anti-matrilin-2 (red) antibodies. Note the mutually exclusive pattern revealing the absence of matrilin-2 expression in axons. (C,D) Matrilin 2 (green) is also not detectable in myelin sheaths visualized with an antibody against myelin basic protein (MBP, red). (E-H) Matrilin 2 (red) colocalizes with the SC marker p75 (green) (E,F) and matrilin 2 (green) is also detectable in basement membranes, as indicated by its colocalization with nidogen 1 (red) (G,H). In A-F, cell nuclei were visualized with DAPI (blue). Shown are representative staining examples performed on sections from four independent wild-type mice. Scale bars: 20 μ m in A,B,E,H; 10 μ m in C,D.

mice compared with wild-type littermates (Fig. 8A). No differences between the genotypes were seen for the FBA. When the changes in individual functional abilities after the first post-operative week were normalized to the individual animal degree of walking impairment 1 week after injury by calculating individual recovery indices for both the FBA and the HTA, and the mean of these, the stance recovery index (Fig. 8C), it became apparent that knee function is, indeed, less successfully restored in matrilin-2-deficient mice compared with wild-type littermates.

Impaired functional recovery of matrilin-2-deficient mice is not associated with a regeneration failure of motoneurons, deficient axonal regeneration or reduced Schwann cell proliferation
To identify possible reasons for the impaired functional recovery in matrilin-2-deficient mice, we performed retrograde tracing

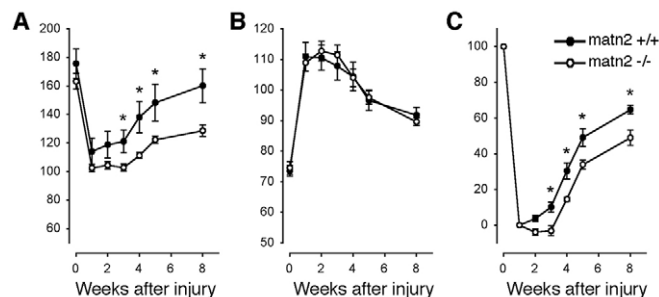


Fig. 8. Time course and degree of motor recovery after femoral nerve lesion in matrilin-2-deficient (matrilin 2^{-/-}) and wild-type (matrilin 2^{+/+}) mice. Shown are heel-tail angles (A), foot-base angles (B) and stance recovery indices (C) at different time-points after nerve transection and repair. Asterisks indicate significant differences between matrilin-2-deficient and wild-type mice ($n=7$ per group) at the respective time point ($*P<0.05$).

experiments for motoneurons that had successfully regenerated and morphometric analyses of their axons in mice used for functional evaluations. Analyses of back-labelling experiments revealed that similar numbers of motoneurons in both groups (matrilin-2-deficient and wild type) had sent axons into only the inappropriate saphenous (skin) branch of the femoral nerve, or into only the appropriate quadriceps (motor) branch, or into both branches (Fig. 9A). In addition, the total numbers of back-labelled motoneurons, reflecting the degree of motoneuron survival in this experimental paradigm (Simova et al., 2006), were similar in matrilin-2-deficient and wild-type mice. Analyses of axonal diameters and g-ratios, estimates of the degree of axonal remyelination, in regenerated nerves also did not reveal significant differences between the genotypes (Fig. 9B,C). Thus, the incomplete recovery of function in matrilin-2-deficient mice was not related to failure of motoneurons to re-innervate peripheral targets or deficits in axonal regeneration.

We also looked at the proliferation capacity of SCs after nerve lesion. Mice were injected with BrdU 4 hours before they were sacrificed 3, 5 and 7 days after nerve lesion. Numbers of BrdU/p75 double-positive cells or Ki67/p75 double-positive cells were determined on transverse sections. For each time point and each genotype, at least three animals were analysed and 10-30 sections were evaluated per animal. We could not observe a significant difference in the numbers of double-positive cells comparing sections from wild-type and matrilin-2-deficient animals at any of the three time points analysed (Fig. 9E; supplementary material Fig. S5). This finding demonstrates that the proliferation capacity of matrilin-2-deficient SCs during regeneration is not impaired.

Delayed functional recovery in matrilin-2-deficient mice is associated with retardation of axonal regeneration

As different *in vitro* approaches revealed that matrilin 2 is a permissive substrate for axonal growth, we asked the question is axonal regeneration also slowed down in matrilin-2-deficient mice. Rather than measuring the speed of axonal regrowth, we counted the regenerated axons at different distances from the lesion site at one time-point, to estimate the overall progression of regeneration. A similar approach has been used before where the process of asynchronous regrowth of individual axons over prolonged time-periods was analysed in great detail (Brushart et al., 2002). According to results of our pilot experiments using femoral nerves that were allowed to regenerate for 3, 4, 5, 6 and 9 days, respectively (data not shown), we selected a 5-day survival period as optimal

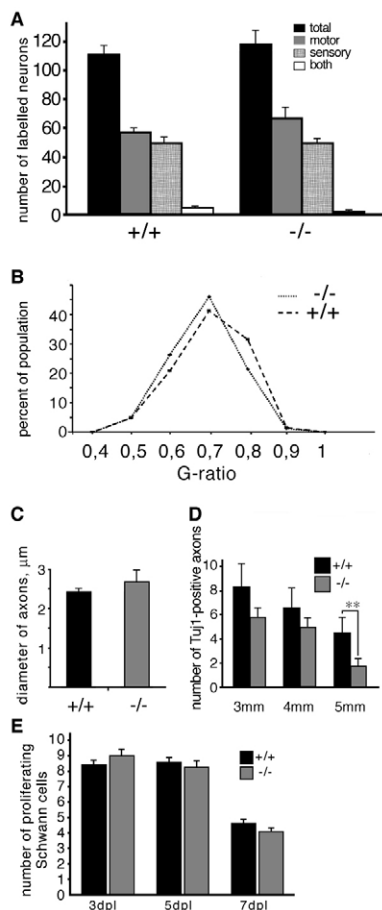


Fig. 9. Morphological evaluation of muscle reinnervation and speed of regeneration and Schwann cell proliferation. (A) Numbers of motoneurons innervating the motor or sensory branches only, or both branches, as well as the total number of regenerated motoneurons 3 months after nerve repair. (B) Relative degrees of myelination estimated by g-ratios (axon/fibre diameter ratios) and diameters of regenerated axons (C) evaluated in semi-thin sections from regenerated nerves cut ~2 mm distal from the site of transection 3 months after nerve repair. (D) Analysis of axonal regrowth 5 days after nerve lesion. Numbers of regenerated axons, identified by β -tubulin immunofluorescence, present in transverse sections of femoral nerves collected at distances of 3, 4 and 5 mm distal to lesion site. Asterisks indicate significant differences between matrilin-2-deficient and wild-type mice (** $P < 0.01$) in numbers of Tuj1-positive axons 5 mm distal from the site of transection. (E) Numbers of proliferating SCs in the distal stumps 3 days (3 dpl), 5 days (5 dpl) and 7 days (7 dpl) after lesion in wild-type (+/+) and matrilin-2-deficient (-/-) adult mice. Animals were sacrificed following a 4-hour pulse of BrdU applied by injection. Transverse sections of femoral nerves distal to the site of lesion were incubated with antibodies against p75 (SC marker) and BrdU. Only cells positive for both signals were counted. No statistically significant differences were observed between mutant and control at any time point. Similar results were obtained when antibodies against Ki-67 (proliferation marker) were used instead of BrdU.

time-point for our analysis. The counting of regenerated axons, visualized by β -tubulin immunohistochemistry, in transverse sections from regenerated nerves revealed lower numbers of axons in matrilin-2-deficient mice compared with wild-type littermates at 3 to 5 mm from the lesion site (Fig. 9D; supplementary material Fig. S1). This observation indicates that axonal regeneration is retarded in the absence of matrilin 2 and correlates well with the delay in functional recovery of matrilin-2-deficient mice and with the data obtained from our *in vitro* studies.

Discussion

Our work provides, for the first time, insights into the functions of matrilin 2. We show that matrilin 2, which is produced and secreted into the ECM by SCs in the peripheral nervous system, potentially enhances axonal growth and SC migration *in vitro*. *In vivo*, these functions appear to be important during peripheral nerve regeneration, a conclusion based on the finding of defective restoration of function in mice incapable of re-expressing matrilin 2 after nerve injury.

Expression pattern of matrilin 2 in the peripheral nervous system

It had been previously reported that matrilin 2 is expressed in a variety of tissues, including the CNS dura and pia mater, and the PNS endoneurium (Piecha et al., 1999). In this study, we show that, in the PNS, matrilin 2 is expressed by SCs. Co-labelling experiments using antibodies against the SC marker p75 and the basal lamina marker nidogen1 in combination with matrilin-2-specific antibodies revealed that matrilin 2 is expressed in non-myelinating SCs and is secreted into their basal lamina tubes. In the PNS, we found high levels of matrilin-2 expression by semi-quantitative RT-PCR and immunohistochemistry in embryonic tissue throughout development, whereas only low levels of expression were detectable by immunoblotting and immunohistochemical analyses in adult peripheral nerves. Upon injury of the sciatic or femoral nerves a strong upregulation was observed. Our finding adds a novel molecule to the long list of SC products. Characteristic for many of these SC-derived molecules is that, in rodents, they are highly expressed during early development, downregulated during postnatal life to very low or undetectable levels in the adult PNS and are re-expressed following nerve damage. Thus, our observations indicate a similar developmental and injury-related regulation of matrilin-2 expression. The progeny of once myelinating SCs in the distal nerve stump initially acquires a non-myelinating SC phenotype. Myelin genes are downregulated (LeBlanc and Poduslo, 1990; Trapp et al., 1988), whereas neurotrophic factors and ECM molecules (laminin, fibronectin, collagens), which are important for fasciculation, axonal prolongation and SC migration are strongly upregulated (Bixby et al., 1988; Brodkey et al., 1993; Bunge, 1983; Daniloff et al., 1986; Martini, 1994). Interestingly, the injury-related upregulation of matrilin-2 expression was more pronounced in the distal stump into which axons regenerate than in the proximal nerve stump. Similarly, following nerve injury SCs proliferate by far more in the distal than in the proximal nerve stump (Bunge, 1987; Clemence et al., 1989). So it appears that an environment that stimulates dedifferentiation and proliferation of SCs is also favourable for high levels of matrilin-2 expression. We did not observe any influence of matrilin 2 on SC proliferation during development (supplementary material Fig. S4) or peripheral nerve regeneration; however, other major events occurring in regenerating peripheral nerves such as axonal growth and SC migration can be stimulated by matrilin 2. It is likely that the colocalization of matrilin 2 with laminin and collagen IV, main components of the basal lamina tubes, is of functional relevance during the regeneration process.

Effects of matrilin 2 on cell migration and axonal outgrowth

The development of the PNS and its functionality later in the adult are strongly influenced by the ECM produced by SCs. Of particular functional importance are ECM proteins that participate in the formation of the endoneurial tubes (basal laminae) around individual

axons as these are implicated in developmental and repair processes such as axonal growth, cell migration, axonal branching and myelination (Chernousov and Carey, 2000). The best example is laminin, which is known for its potency to support cell migration and axonal outgrowth, as well as its essential role in myelination (Chen and Strickland, 2003). As we show here, matrilin 2, similar to laminin, is capable of promoting axonal growth, as well as migration and adhesion of SCs. Remarkably, when used as a uniform coating substrate *in vitro*, matrilin 2 supports migration of both, cell line- and embryonic DRG-derived, SCs better than laminin. Under choice conditions in stripe assays, matrilin 2 is a very good substrate and supports growth of DRG neuron axons almost as efficiently as does laminin. This is especially noteworthy considering that laminin is one of the most permissive substrates for axonal growth (Snow et al., 1996; Vielmetter et al., 1990). Interestingly, DRG neuron axons show a higher preference for growth on a mixture of laminin and matrilin 2 when compared with surfaces coated with laminin alone, arguing for a unique matrilin-2-mediated growth-promoting activity. These findings are further substantiated by the deficits in axonal growth and SC migration observed in DRG culture assays employing matrilin-2-deficient embryos. These deficits can be rescued by adding matrilin 2 to the cultures or using it as a coating substrate, demonstrating the direct effect on SC migration and axonal outgrowth.

It is known that laminin influences cellular behaviour via binding to cell surface receptors such as dystroglycan (Yamada et al., 1996) or integrins (Chernousov et al., 2007; Feltri et al., 2002); by contrast, the mechanisms underlying the observed effects of matrilin 2 are currently poorly understood. Except for matrilin 4, all matrilins mediate cell attachment, but the binding does not lead to the formation of focal contacts and reorganization of the actin cytoskeleton. Cell surface proteoglycans may promote the attachment, as cells deficient in glycosaminoglycan biosynthesis adhere less well to matrilin 3 (Mann et al., 2007). We can thus only speculate that multiple interactions of matrilin 2, enabled by the common matrilin domain structure, are important in mediating the effects reported here.

Matrilin 2 in peripheral nerve regeneration

Using a recently described functional assay (Irintchev et al., 2005), we provide direct evidence that matrilin 2 is important for recovery after peripheral nerve injury in adult animals. Recovery of the knee extension function of the quadriceps muscle after femoral nerve transection and surgical repair in matrilin-2-deficient mice was both delayed in time and finally less complete compared with wild-type littermates. The delay in the time-course of functional recovery is entirely explainable by the *in vitro* findings that matrilin 2 promotes SC migration and axonal elongation. We can assume that absence of matrilin 2 in the denervated basal lamina tubes causes slower regeneration by reducing the rate of axonal outgrowth and, simultaneously, the rate of migration of the SCs that guide the tips of regenerating peripheral axons (Son and Thompson, 1995). This interpretation is supported by the observation of lower numbers of regenerated axons distal to the nerve repair site in matrilin-2-deficient mice compared with wild-type animals 5 days after surgery. More difficult is the explanation of the finding that functional recovery at 2 months after injury is less complete in matrilin-2-deficient than in wild-type mice. This time-period is sufficient for maximum recovery, reaching 60–70% of the preoperative values in wild-type mice (Ahlborn et al., 2007; Eberhardt et al., 2006; Simova et al., 2006), and long enough to surpass the delay in recovery. This poor outcome was not associated with failure of regeneration as

numbers of back-labelled regenerated motoneurons, as well as diameters and degree of myelination of regenerated axons, were similar in knockout and wild-type mice. We can speculate that matrilin 2 also has beneficial functions during nerve regeneration that have not been revealed by this study. Although matrilin 2 has no effect on SC proliferation, it is possible that it improves the functional outcome of nerve regeneration by, for example, influencing the expression pattern of regeneration-related molecules by SCs or binding to yet unidentified receptors on the regenerating axons and thus exerting, directly or indirectly, 'trophic' effects on the injured motoneurons. In addition, impacts of matrilin 2 on the regeneration of sensory neurons and subsequent afferent-related influences on recovery of motor functions have to be considered. We can also speculate that after nerve injury in wild-type mice, matrilin 2 is expressed by SCs at denervated endplates and promotes endplate reinnervation when motor axons regrow into the muscle. Disturbances of endplate reinnervation by absence of matrilin 2 in knockout mice may lead to a significant and long-lasting muscle malfunction that limits motor recovery. The plausibility of the latter notion is supported by observations indicating that aberrant endplate reinnervation is a factor that limits restoration of function after nerve lesion (Guntinas-Lichius et al., 2005b). Here, we did not exploit the effect of matrilin-2 deficiency on endplate reinnervation, considering previously encountered technical difficulties in visualization of mouse axonal arborizations by immunohistochemistry (Guntinas-Lichius et al., 2005a). However, further studies that aim to elucidate the role of matrilin 2 at endplates appear apparently warranted.

Matrilin 2 is not the first basal lamina component shown to be essential for peripheral nerve regeneration. Deficient axonal regeneration has also been observed after genetic ablation of the laminin $\gamma 1$ chain in SCs, which leads to downregulation of most laminin isoforms in the peripheral nerves, or ablation of the laminin receptor $\alpha 7\beta 1$ integrin, which is upregulated in axotomized motoneurons (Chen and Strickland, 2003; Werner et al., 2000). However, intact laminin-deficient mice have motor deficits (tremor, ataxia) and impaired myelination in the PNS (Chen and Strickland, 2003; Wallquist et al., 2005), abnormalities that have been observed in neither this nor in previous studies on the matrilin-2-deficient mouse (Mates et al., 2004). Therefore, we can assume that laminin and matrilin 2 have both overlapping and distinct functions *in vivo*, e.g. promotion of axonal elongation and myelination, respectively.

It had been shown before that in matrilin-2-deficient animals other family members are not significantly upregulated, and the expression levels and distribution of matrilin-2 interaction partners are not grossly changed (Mates et al., 2004). Surprisingly, in peripheral nerves of matrilin-2-deficient animals, we observed a compensatory upregulation of matrilin 4 upon injury to levels significantly higher than in wild-type littermates. However, the impaired regeneration in matrilin-2 mutants demonstrates that this upregulation is not able to rescue the absence of matrilin 2, which can also be explained by the differences in the domain structures between these family members.

Our study reveals the beneficial impact of matrilin 2 on peripheral nerve regeneration. Beyond its contribution to understanding the roles of matrilin 2 *in vivo*, this knowledge might have implications in clinical settings. For example, it is conceivable that matrilin-2 peptides would be beneficial if used as coating substrates for artificial nerve conduits that bridge large nerve gaps, a reconstructive surgical approach typically associated with poor functional recovery.

Materials and Methods

RNA analysis

Total RNA was isolated from embryonic DRG and different types of mammalian cells by RNeasy mini kit (Qiagen). For semi-quantitative RT-PCR, 1–5 µg of total RNA was transcribed into cDNA using the SuperScript II Rnase H-reverse transcriptase (Invitrogen). For PCR reactions, one unit of AmpliTaq DNA polymerase (Invitrogen) and 0.5–2.0 µl of cDNA were used in 25 or 50 µl amplification buffer. The primer pairs used were: matrilin 2, 5′-TGCAAGAGATGCACTGAAGG-3′ (fwd.) and 5′-ATTTCTTGGCTGAGCTGAA-3′ (rev.); GAPDH (glyceraldehyde-3-phosphate dehydrogenase) 5′-ACCACAGTCCATCCATCAC-3′ (fwd.) and 5′-TCCACCACCTGTTGCTGTA (rev.); matrilin 4 5′-GACTCGTATTCTGTC-GTTGCC-3′ (fwd.) and 5′-AACCATCCACCAGGAGAACAAG-3 (rev.); and Ddost 5′-GGTCCCCTTTGATGGTGATGAC-3′ (fwd.) and 5′-TGCTGAAGATGA-AGAGCCCG-3′ (rev.). For every time point and every genotype, tissue from two or three different animals was used. In situ hybridization was performed essentially as described previously (Sonnenberg et al., 1991). A 592 bp fragment corresponding to the unique segment and small part of VWA2 domain of mouse matrilin 2 was cloned from RZPD clone (I.M.A.G.E. G_p998F188539Q3) using following primers: 5′-CCACGCAGGTACCCAGAGTA-3′ (fwd) and 5′-TCAGCGGCCGCTCTGTAT-TTT-3′ (rev). ³⁵S-labeled riboprobes were prepared following standard protocols.

Expression and purification of recombinant matrilin 2

The fragment containing a precursor of the full-length matrilin 2 was recovered from the RZPD clone (IMAGE G_p998F188539Q3), by suitable restriction enzymes and then inserted into pcDNA3.1-Myc/His₆(C). The recombinant plasmid was introduced into Chinese Hamster Ovary (CHO) cells using Lipofectamin 2000 reagent, according to the manufacturer's instructions (Invitrogen). The transfected cells were selected using 800 µg/ml G418 (Geneticin, Invitrogen) and grown to confluence. Secretion of matrilin 2 into the culture medium was verified by immunocytochemistry and SDS-PAGE with immunoblotting, using antibody against the Myc epitope. The protein was purified using Ni-NTA technology under denaturing condition. In parallel, matrilin-2 recombinant protein with His₆-tag on 5′-end was purified using native conditions. Both proteins were used in different experiments and showed similar activities.

Cell culture

In all cell culture experiments, 12 mm or 22 mm glass coverslips were coated first with poly-D-ornithine (PDO, 10 µg/ml, overnight incubation at room temperature), washed once in distilled water, air-dried and coated with either laminin (20 µg/ml) (Roche), fibronectin (20 µg/ml) (Sigma), matrilin 2 (20 µg/ml) (R. Wagener) or a mixture of laminin and matrilin 2 (20 µg/ml of each) by overnight incubation at 4°C.

Neural crest stem cells (Moncl) were cultured as described previously (Rao and Anderson, 1997). Cultures of DRG explants were prepared as described previously (Lumsden et al., 1981). Cultures of dissociated adult DRG neurons were prepared as described by previously (Svenningsen et al., 2003). The branching of neurites was analysed essentially as described (Bouquet et al., 2004).

The agarose drop migration assay was performed as described by Milner (Milner et al., 1997a). In this assay, immortalized rat S16 SCs were allowed to migrate for 24–48 hours. The migration was monitored using a phase-contrast microscope. The adhesion assays were performed according to published protocols (Chernousov et al., 2001; McGarvey et al., 1984; Milner et al., 1997a). All experiments were carried out in triplicate.

Cell migration and neurite outgrowth from DRG explants

DRG (E12) explants were placed on the dishes coated with different ECM substrates. Cell migration assay from DRGs was performed as described (Chernousov et al., 2001). Sox10-specific antibodies (a marker for embryonic SCs) and DAPI (cell nuclear staining) were used and samples analysed by immunofluorescence. The number of cells and the distance from the edge of a ganglion to the leading edge of migrating SCs was determined. Four measurements were made in different directions of radial migration for each ganglion and averaged. To analyse neurite outgrowth, ganglia were incubated in complete culture medium supplemented with NGF (Invitrogen, 20 ng/ml) for 24 hours, followed by immunostaining with anti-neurofilament (NF) antibodies or combination of anti-neurofilament and anti-sox10 antibodies.

Stripe assay

Stripe assays were performed as described previously (Vielmetter et al., 1990). Silicon matrix was obtained from Jürgen Jung (Max-Planck Institute, Tübingen, Germany). DRG explants and dissociated cells were grown for 24 hours on 22 mm glasses coated with different proteins as described above, fixed and immunostained with NF antibodies. All experiments were carried out in triplicate and performed at least three times independently.

Immunohistochemistry, antibodies and determination of proliferation rates

Immunohistochemistry analyses on cryosections or culture explants were performed as described previously (Sonnenberg-Riethmacher et al., 2001). The following

antibodies were used for immunohistochemistry at optimal dilutions: rabbit polyclonal against mouse matrilin 2 [(Piecha et al., 1999) 1:100], goat polyclonal against p75 (Santa Cruz, 1:30), rat monoclonal against MBP (1:200) and nidogen 1 (Chemicon, 1:40), mouse monoclonal against NF160, rabbit polyclonal against NF200, neuron-specific class III tubulin (Tuj1), β-actin (all Sigma, 1:200), Myc (9E10, Upstate, 1:5000), and mouse monoclonal against Ki-67 (Novocastra, NCL-Ki67-MM1, 1:200) and Sox10 hybridoma supernatant (K. Kuhlbrodt and M. Wegner, 1:5). Secondary antibodies against mouse, rat, rabbit and goat conjugated to Cy2 or TRITC were obtained from Dianova (Dianova, Hamburg) and applied at optimal dilutions as required.

In order to determine the numbers of proliferating SCs, mice were killed 3, 5 and 7 days after nerve lesion. Four hours prior to sacrifice, mice were injected with BrdU (40 µg/g body weight). Transverse sections (10 µm) of the femoral nerve distal to the lesion were cut and stained with anti-p75 and in some experiments co-labelled with anti-Ki67 antibodies. BrdU-positive nuclei were identified using mouse monoclonal anti-BrdU antibody (Roche, Clone BMC 9318). At least triplicates were used for each genotype at every time point.

Animals and surgical procedures

For cell culture experiments and expression assays, we used C57Bl/6 mice, ErbB3 and matrilin-2-deficient mice. The generation of the ErbB3 and matrilin-2 mutant mice has been described previously (Mates et al., 2004; Riethmacher et al., 1997). In addition to adult animals (3 months old), mice at different stages of embryonic (E12, E14, E16, E18) and early postnatal (P0, P1, P2, P3) development were studied. All animals were bred at the central animal facility of the University Medical Center Hamburg-Eppendorf. The mice were treated according to the German law on protection of experimental animals after approval by the responsible committee of The State of Hamburg.

For surgery, the 3-month-old matrilin-2-deficient mice and wild-type (wt) littermates were anesthetized by intraperitoneal injections of 0.4 mg/kg fentanyl (Fentanyl-Janssen, Janssen, Neuss, Germany), 10 mg/kg droperidol (Dehydrobenzperidol, Janssen) and 5 mg/kg diazepam (Valium 10 Roche, Hoffmann-La Roche, Grenzach-Wyhlen, Germany). The surgeries to lesion left femoral or sciatic nerves were performed as described previously (Simova et al., 2006).

Analysis of motor function, retrograde labelling of regenerated motoneurons and analysis of regrowth of regenerating axons

Functional recovery after femoral nerve repair was assessed by single frame motion analysis (SFMA) as described (Irintchev et al., 2005). Retrograde labelling and counting of back-labelled cells were performed as described (Simova et al., 2006).

To analyze the speed of regeneration, nerve injury was performed in matrilin-2-deficient and wild-type mice, and 5 days later the animals were perfused, the femoral nerves were removed, post-fixed overnight, incubated in 30% sucrose in buffer overnight, frozen and cut transversely (serial sections of 10 µm) on a cryostat. After staining with anti-tubulin (Tuj1) antibodies, sections were examined under a fluorescence microscope (Zeiss) with appropriate filter sets. Numbers of Tuj1-positive axons were measured 3, 4 and 5 mm distal to the transection site.

Morphometric and statistical analysis

Femoral nerves were dissected from animals fixed by perfusion (see above) and further analysed as described (Simova et al., 2006). In all experiments, statistical analysis was performed using 'GraphPad Prism 4' or MiniTab (Release 15) software by one-way ANOVA test for repeated measurements. Tukey's multiple comparison test was used as a post-hoc test for multiple comparisons of group mean values. Data are shown as mean values with standard errors of the mean.

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