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Regular Article

# Desmoplakin is involved in organization of an adhesion complex in peripheral nerve regeneration after injury

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ABSTRACT

Peripheral nerves have the unique capability to regenerate after injury. Insights into regeneration of peripheral nerves after injury may have implications for neurodegenerative diseases of the nervous system. In this study, we analyzed the expression and function of desmoplakin in peripheral nerve regeneration. Desmoplakin was upregulated in spinal cord motoneurons after sciatic nerve injury. Conditional ablation of desmoplakin in motoneurons demonstrated that desmoplakin is necessary for normal motor regeneration. SiRNA and desmoplakin deletion-constructs revealed a role of desmoplakin in neurite extension *in vitro*. A complex of N-cadherin, plakoglobin, desmoplakin and vimentin was shown in motoneuronal cell cultures and peripheral nerves after injury *in vivo*. Motor nerve fiber regeneration and localization of N-cadherin and vimentin to axonal growth fronts were reduced in conditionally desmoplakin-ablated mice.
These data indicate a function of desmoplakin in motor nerve regeneration by linking N-cadherin to intermediate filaments in regenerating motor axons.



## Introduction

Peripheral nerves have the unique potential to regenerate after a nerve lesion. After a process called Wallerian degeneration that leads to the degradation of compact myelin and axons, the axons of a lesioned nerve regenerate and grow out into the distal part of the nerve (Waller, 1850). Apart from being a natural mechanism of posttraumatic repair, this process represents a model for neuroregeneration that has been studied for many years in rats, mice, drosophila and C. elegans (Osterloh et al., 2012; Perry et al., 1990; Roytta et al., 1987; Xie et al., 2011). Animal models have led to the discovery of several genes that suppress Wallerian degeneration or improve regeneration, such as *Wlds* and *Sarm1* (Meyer zu Horste et al., 2011; Osterloh et al., 2012; Perry et al., 1990). In our previous work, we analyzed gene regulation of spinal cord motoneurons of transgenic mouse models of Charcot Marie Tooth (CMT) disease (Nattkamper et al., 2009). Among the regulated genes, we found several genes like *Atf3* and *Gap43* known to be involved in neuronal injury and repair (Kim and Moalem-Taylor, 2011; Verhaagen et al., 1988). A novel candidate – *Bex1* – was shown to be required for correct peripheral nerve regeneration in a subsequent study (Khazaei et al., 2010). In our previous study we also showed upregulation of Desmoplakin (*Dsp*) and Vimentin (*Vim*) in motoneurons of CMT mouse models by micro-array, real-time PCR and immunohistochemistry (Nattkamper et al., 2009). Vimentin had previously been found to be upregulated in other studies on gene regulation in motoneurons from mouse models of motoneuron disorders (Lobsiger et al., 2007; Verhaagen et al., 1988). Vimentin is known to be involved in regeneration of peripheral nerves by translocation of phosphorylated ERK through dynein and importins (Perlson et al., 2005). Desmoplakin, on the other hand, interacts with intermediate filaments such as keratins and vimentin (Meng et al., 1997; Stappenbeck et al., 1993). Further, an adhesion complex of N-cadherin, gamma-catenin/plakoglobin, desmoplakin and vimentin was found in eye lens and lymph nodes (reviewed in (Franke et al., 2009)). Similar interactions between N-cadherin, plakoglobin and desmoplakin were detected in hippocampal neurons (Tanaka et al., 2012).

Because of these previous findings, we were interested to study a potential role of desmoplakin in regeneration of the peripheral nervous system. We used peripheral nerve crush injuries as a model system for peripheral nerve regeneration in wild-type as well as conditional Desmoplakin-ablated mice. Here we show evidence of desmoplakin upregulation in spinal cord motoneurons after sciatic nerve crush. Further, we provide evidence of the interaction of desmoplakin with N-cadherin, plakoglobin and vimentin in motoneuronal axons with functional significance for axon regeneration and outgrowth *in vitro* and *in vivo*. These results could lead to a better understanding of peripheral nerve regeneration after injury and regenerative mechanisms in peripheral nerve disease. Furthermore, these results imply the existence and functional relevance of a novel adhesion complex in the motoneuron system.

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## Materials and methods

### *Transgenic mouse lines*

Design and genotyping of *Dsp*$^{flox}$ and *HB9*$^{Cre}$ have been described (Arber et al., 1999; Vasioukhin et al., 2001). Mice were genotyped from tail cuts. Primer sequences and PCR protocols can be obtained upon request. All mice were bred on a C57/Bl6 background. Mice were housed under standard conditions in compliance with German legislation. All animal experimentation was done in accordance with German legislation; permission for animal experimentation was obtained from the North-Rhine-Westphalian (NRS) ministry of nature, environment and consumer protection (LANUV) (No: 84–02.04.2013.A211).

### *Sciatic and quadriceps femoris nerve injury*

Mice were anesthetized with ketamine and xylazine (100 mg/kg body weight and 8 mg/kg body weight) and the sciatic nerve or quadriceps femoris nerve exposed. The nerve was crushed with a flattened forceps for 30 s. Preparation and crush of the quadriceps femoris nerve was done with a binocular preparation microscope. In transection experiments the nerve was cut using fine scissors. The crush site was marked with a non-resorbable thread and the skin was clipped. Mice were treated with naproxen immediately, and one and two days after the injury to reduce postoperative pain. Mice were killed 7, 14, 21, 28 and 35 days after crush.

### *Behavioral tests and electrophysiology*

All behavioral tests and electrophysiological measurements were performed by a trained investigator blinded towards the genotypes of the mice.

#### *Gait analysis*

Gait analysis was conducted using a walking track 1 m in length and 10 cm in width laid out with paper strips. Mice were trained one or two times before the first measurement. The hindpaws of mice were dipped in nontoxic paint (Pelikan). From the foot prints, the stride lengths of at least six strides were measured. Only regular walking intervals (no stopping or curving) were scored. Sciatic functional index was calculated from walking tracks as described previously (de Medinaceli et al., 1982). Sciatic functional index was measured 1, 2, 3, 7, 11, 15, 21, 28 and 35 days after nerve crush.

#### *Toe pinch score*

The toe pinch reflex was used to assess the recovery of sensory function as described previously (Seeds et al., 1997) by pinching the most distal portion of the last three toes (third, fourth and fifth) of the injured hindlimb with a flattened forceps. Foot withdrawal was recorded as positive responses indicative of recovery 1, 2, 3, 7, 11, 15, 21, 28 and 35 days after nerve crush. Digits 1 and 2 were not tested since they are partially innervated by the saphenous nerve (Navarro et al., 1995).

#### *Electrophysiology*

Electrophysiology was performed on mice 1, 7, 14, 21, 28, 35 and 42 days after crush as described (Gess et al., 2011). Mice were anesthetized with ketamine hydrochloride and xylazine hydrochloride (100 mg/kg body weight and 8 mg/kg body weight) and temperature controlled using a heat pad and a rectal thermal probe. Two pairs of steel needle electrodes (Schuler Medizintechnik) were placed subcutaneously along the nerve at the sciatic notch and above the ankle (distal stimulation). Supramaximal square wave pulses lasting 100 ms were delivered using a Toennies NeuroScreen system (Jaeger). Compound muscle action potential (CMAP) was recorded from the intrinsic foot muscles using steel electrodes. Both amplitudes and latencies of CMAP were determined. The distance between the two sites of stimulation was measured alongside the skin surface with fully extended legs and nerve conduction velocities (NCVs) were calculated automatically from sciatic nerve latency measurements.

### *Histology*

In deep ketamine/xylazine anesthesia mice were intracardially perfused with isotonic saline followed by a fixative solution (4% PFA, 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4). Sciatic and quadriceps femoris nerves were dissected, postfixed for 24 h at 4 °C, osmicated for 2 h in 2% osmium tetroxide at room temperature, washed in distilled H2O several times, dehydrated in ascending acetone, and finally embedded in Spurr's medium. Semi-thin sections (0.5 μm) were cut using an Ultracut 200 microtome (Leica), stained with alkaline toluidine blue, and subsequently analyzed by measuring axon diameter, fiber diameter and fiber densities per mm$^2$ on three sections per sciatic nerve.

### *Cell culture*

#### *NSC34 cells*

NSC34 cells were cultured in DMEM with 10% fetal bovine serum (FBS), L-glutamine and penicillin/streptomycin (1:100). For differentiation, NSC34 cells were incubated the differentiation medium (DMEM: HAM's F12 medium 1:1, 0.5% FBS, non-essential amino acids (1%), penicillin/streptomycin (1:100)) as described previously (Matusica et al., 2008).

#### *Primary motoneuron cell cultures*

Primary embryonal mouse motoneurons were cultured as described (Wiese et al., 2010). Briefly, embryos at gestational age 12.5–13.5 were dissected and spinal cords prepared. The meninges and dorsal root ganglia were removed. The spinal cords were dissociated in 0.1% Trypsin/0.04% EDTA and 0.004% DNase for 8–10 min at 37 °C. Motoneurons were selected by immunopanning using P75$^{NTR}$-antibody coated panning plates. After washing, motoneurons were detached by incubating with depolarisation buffer containing 30 mM Kcl, 2 mM CaCl and 0.8% (wt/vol) NaCl for 1 min. Motoneurons were cultured on poly-DL-ornithin and laminin coated glass cover slips in medium (Neurobasal medium, 5% horse-serum, B27 supplements (1:50), CNTF (10 ng/ml) and Glutamin (1:400)).

### *SiRNA and recombinant vector transfections*

SiRNA oligonucleotides against desmoplakin and nonsense control oligonucleotides were purchased from Dharmacon. SiRNA transfection was performed as described previously (Gess et al., 2008). Successful knockdown was checked by western blots of desmoplakin compared to β-actin.

Recombinant desmoplakin constructs were designed by fusion of the complete human *DSP* gene to the YFP gene in the expression vector pYFP-N1 (DSPfull, Clontech). An N-terminally deleted DSP mutant construct was designed by PCR amplification of the C-terminal region and cloning of the fragment into the pYFP-N1 vector in frame (DSPΔNterm). Constructs were transfected into NSC34 cells using Turbofect reagent (Fermentas). The correct fusion was verified by sequencing and expression was checked after transfecting HEK293 cells and performing Western blot analysis using anti-GFP antibodies (Clontech). Successful transfection in NSC34 cells was visualized by YFP fluorescence and Western blots using anti-GFP antibodies.

### *Immunohistochemistry*

For immunohistochemistry, mice were anesthetized with ketamine/xylazine and killed by cervical dislocation. Sciatic and quadriceps femoris nerves were dissected and embedded in Tissue-Tek OCT