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GDF-15 deficiency induces a progressive Schwann cell loss *in vivo* and regulates their survival and migration *in vitro*

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Summary

Schwann cells are the glial cells of the peripheral nervous system (PNS) with multifunctional roles. Myelinating Schwann cells wrap axons with multilayered myelin sheaths providing electrical insulation and rapid impulse propagation. They also play a key role in the immune response after nerve injury and play an active role during nerve repair by contributing to a surrounding growth environment that allows peripheral nerve axons to regenerate (Rodrigues, Rodrigues et al. 2012).

GDF-15, a neurotrophic factor and member of the TGF-β superfamily has been shown to support nerve regeneration (Mensching, Borger et al. 2012; Charalambous, Wang et al. 2013). Schwann cells express GDF-15 and seem to be an important source of GDF-15 in peripheral nerves (Strelau, Strzelczyk et al. 2009). Interestingly, analysis of GDF-15 deficient mice showed a severe and progressive motoneuron loss accompanied by a loss of axons. Moreover, studies in our laboratory showed a hypermyelination in adult mutant mice (Dr. J. Strelau, unpublished data). Together these data suggest that GDF-15 affects Schwann cell.

In this study, I first investigated the putative effect of GDF-15 deficiency on Schwann cells and showed for the first time that GDF-15 loss reduces Schwann cell numbers in adult mice. To address the question whether this observation is correlated with an up or down regulation of other important signaling molecules I analyzed several gene expression patterns in adult peripheral nerves and spinal marrow associated with Schwann cells and/or GDF-15. Interestingly, IL-6 expression, a key regulator of the immune response is upregulated in peripheral nerves. However, *in vitro* studies failed to proof the direct effect of GDF-15 knockdown on IL-6 expression in Schwann cells.

For functional studies including GDF-15 dependent proliferation, cell death and migration of Schwann cells I next established a method to produce highly enriched adult Schwann cell cultures, showing that GDF-15 is a survival factor for Schwann cells and that GDF-15 deficient Schwann cells fail to migrate. Both defects were rescued by treatment with recombinant GDF-15.

Since GDF-15 specific receptors are not unequivocally identified, I began to investigate the underlying signaling cascades well known to control myelin sheath growth, most notably the putative interaction of GDF-15 with ErbB2 receptor tyrosine kinase, expressed on Schwann cells. Here I showed the activation of the ErbB2 receptor by recombinant GDF-15 in Schwann cells for the first time.

Taken together, this suggests that GDF-15 constitutes an important endogenous regulator of Schwann cell functions in the adult.

Zusammenfassung

Schwann Zellen sind die Gliazellen des PNS. Sie sind zuständig für die Bildung und den Erhalt der Myelinschichten. Ausserdem spielen sie eine wichtige Rolle in der Immunantwort nach einer Nervenverletzung so wie während des Regenerationsprozesses (Rodrigues, Rodrigues et al. 2012).
GDF-15, ein neurotropher Faktor der zur TGF-β Superfamilie gehört, unterstüzt die Nerven Regeneration (Mensching, Borger et al. 2012; Charalambous, Wang et al. 2013). Ausserdem hat die Phänotypanalyse einer GDF-15 defizienten Maus gezeigt das Motoneurone im adulten Tier absterben sowie das Axone verloren gehen. In dieser Studie wurde ebenfalls gezeigt das Schwann Zellen GDF-15 expremieren und es wird vermutet, das sie eine wichtige Quelle von GDF-15 in peripheren Nerven darstellen (Strelau, Strzelczyk et al. 2009). Weitere Untersuchungen des Phänotypes der GDF-15 defizienten Maus in unserem Labor haben ergeben, das es im adulten Stadium zu einer Hypermyelinisierung der peripheren Nerven kommt (Dr. J. Strelau, unveröffentlichte Daten). Aufgrund dieser Daten haben wir vermutet, das GDF-15 im adulten Alter Schwann Zellen beeinflusst.
In dieser Studie habe ich das Zusammenspiel von Schwann Zellen und GDF-15 untersucht und konnte zum ersten mal zeigen, das GDF-15 Defizienz zu einem Verlust von Schwann Zellen im adulten Tier führt. Aufbauend auf diesem Ergebnis habe ich die hoch oder runter Regulation von verschiedenen Genen, die mit Schwann Zellen und/oder GDF-15 in Verbindung gebracht werden, in peripheren Nerven analysiert. Intressanterweise, war die Expression des Entzündungmarkers IL-6 erhöht. Allerdings konnten *in vitro* Studien nicht den direkten Effekt von runterreguliertem GDF-15 auf die IL-6 Expression in Schwann Zellen untermauern.
Desweiteren habe ich eine adulte Schwann Zell Kultur etabliert und funktionelle Studien ergaben das GDF-15 das Überleben von Schwann Zellen sichert so wie die Migration unterstüzt. Beide Defizite konnten durch Zugabe von rekombinanten GDF-15 reduziert werden.
Da der Signalweg von GDF-15 noch nicht vollkommen bekannt ist, habe ich noch Signalwege untersucht die dafür bekannt sind die Myelindicke zu regulieren und konnte zum ersten mal zeigen, das der ErbB2 Rezeptor in Schwann Zellen durch rekombinantes GDF-15 aktiviert wird.
Zusammengefasst zeigt diese Studie, das GDF-15 ein wichtiger endogener Faktor für adulte Schwann Zellen ist.

Chapter 1: Introduction

1.1 Peripheral nerve function and structure

A peripheral nerve is build up by fascicles (nerve bundles) containing several neurons (nerve fibers). Nerve fibers, the smallest functional compartment within a nerve, are mainly made by axons and Schwann cells. They are encased by a scaffold of connective tissue consisting of three parts. The endoneurium encloses several nerve fibers creating the fascicles. It protects nerve fibers by embedding them in collagenous matrix from trauma. The second part is the perineurium. It engulfs several fascicles and functions as a diffusion barrier. The whole nerve trunk is surrounded by the third part, the epineurium. The nerve fiber composition classifies a nerve as a sensory (e.g. optical nerve), motor (e. g. femoral nerve) or mixed (e.g. sciatic nerve) nerve. They extend through the entire body and provide information, motor, sensory or autonomous to nearly every organ and as well every tissue. For this reason development, maintenance and repair are critical for body functions (Squire 2012).

In contrast to the central nervous system (CNS), the peripheral nervous system (PNS) shows regenerative capability after injury as nerves residing in the periphery are more vulnerable to traumatic injury. The major glial cells of the PNS, the Schwann cells play a main role in nerve regeneration.

1.1.1 Glial cells of the peripheral nervous system

Schwann cells were first described by Theodor Schwann in 1839. There are two types of Schwann cells found within a peripheral nerve, non-myelinating and myelinating. The fate of Schwann cells depends on the size of the axons with which they make contact. Axons with less than 1 µm of diameter are engulfed by the cytoplasm of non-myelinating Schwann cells (Brinkmann, Agarwal et al. 2008). Several smaller axons are engulfed by one cell, building the so called Remak-Bundle (Fig.1.1). In contrary, myelinating Schwann cells can only wrap around one axon (Fig.1.1).

Other glial cells in the PNS are the olfactory ensheathing cells, resembling non-myelinating Schwann cells. They ensheath the PNS as well as the CNS part of

primary olfactory axons. In autonomic ganglia of the gut, a third type of glial cells is found, the enteric glia. Teloglia are found at nerve terminals and skeletal muscle. They support the stability of the neuromuscular junction.

1.1.2 Schwann cell development

Most Schwann cells originate from the neural crest. However, it was shown that some Schwann cells in ventral roots originate from the ventral tube and that some Schwann cells arise from the spinal cord after the migration of the neural crest is complete. The Schwann cell lineage in rodents, arising from the neural crest, starts with Schwann cell precursors appearing at embryonic day 14 (E14). During E15 to E17, Schwann cell precursors convert to immature Schwann cells. By finding contact with outgrowing neuritis, immature Schwann cells mature reversibly to non-myelinating or myelinating Schwann cells directly after birth (Jessen and Mirsky 1998).

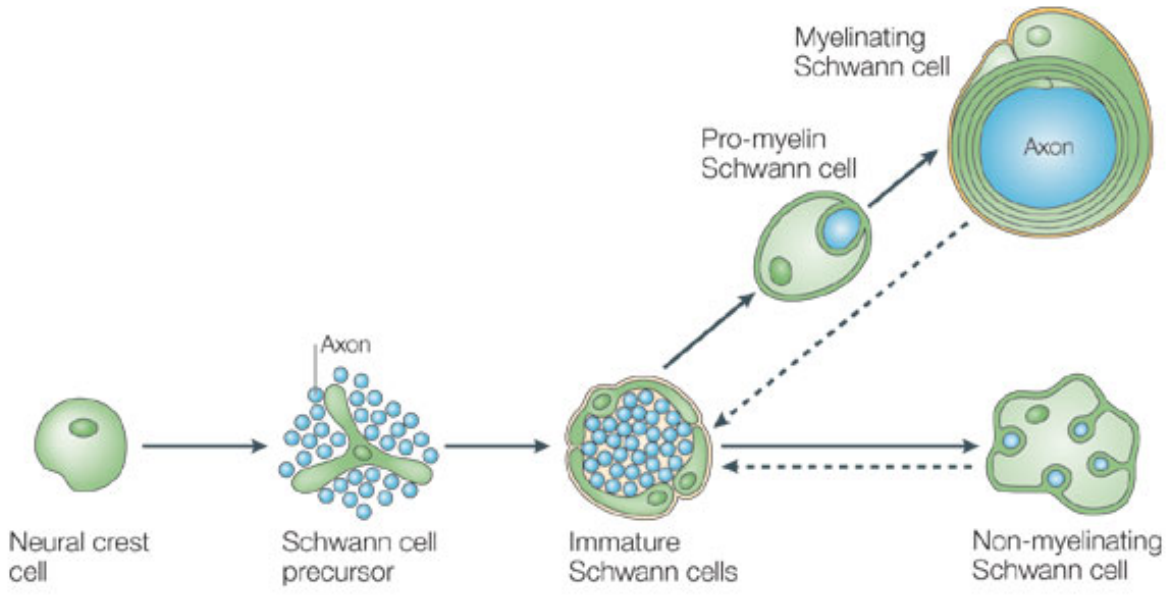


Figure 1.1: Schematic illustration of the Schwann cell lineage. Differentiation of Schwann cells to myelinating or non-myelinating types is reversible. Cartoon adapted from (Jessen and Mirsky 2005).

1.1.2.1 Differentiation, proliferation, survival and migration

Schwann cell differentiation, survival, proliferation and migration during development are controlled by different growth factors. One of the key factors is Neuregulin (encoded by *Nrg* 1-4), an epidermal growth factor (EGF)-like growth factor with a multifunctional role during Schwann cell development. In the early 1980s, Brocks and others demonstrated that cultured Schwann cells proliferate actively in the presence of Nrg1 whereas serum withdrawal inhibits proliferation (Brockes, Lemke et al. 1980). Today it is known that several aspects of Schwann cell development are influenced by Nrg1. In early Schwann cell lineage Nrg1 promotes glial differentiation from the neural crest and is required for survival of Schwann cell progenitors *in vitro.* Nrg signaling in Schwann cells is transmitted by epidermal growth factor (ErbB) 2/ErbB3 heterodimers (Garratt, Voiculescu et al. 2000) and Nrg/ErbB2 signaling is essential for survival of Schwann cell progenitors. Schwann cell progenitor numbers are clearly reduced in *ErbB2*, *ErbB3* or *Nrg1* mutant mice (Meyer and Birchmeier 1995; Riethmacher, Sonnenberg-Riethmacher et al. 1997; Morris, Lin et al. 1999).

The formation of immature Schwann cells from Schwann cell progenitors at ~E16 is accelerated by Nrg1, fibroblasts growth factor-2 (FGF-2) and Notch (Jessen and Mirsky 2005). Immature Schwann cells can, in contrary to the progenitor cells, support cell survival in an autocrine manner by expressing a mixture of survival factors. *In vitro*, this mixture contains neurotrophin-3, the platelet-derived growth factor-β and the insulin-like growth factor 2 (Meier, Parmantier et al. 1999).

Postnatally, Schwann cell numbers have to be adjusted to axonal numbers. There is evidence that the axons themselves regulate Schwann cell numbers by controlling proliferation. *In vivo,* axonal contact loss decreases proliferation of Schwann cells in newborn animals (Komiyama and Suzuki 1992). Nrg1 is a major axonal mitogen as it was shown in co-culture studies of Schwann cells and neurons (Morrissey, Levi et al. 1995). Notably, *in vivo* it was shown that deletion of the type II transforming growth factor-β (TGF-β) receptor decreases Schwann cell proliferation, demonstrating that TGF-βs are also a potential mitogen (Jessen and Mirsky 2005). *In vivo* an identified death signal after nerve injury in newborn animals involves the p75 neurotrophin receptor, possibly by binding of the neurotrophin, nerve growth factor (NGF) (Syroid, Maycox et al. 2000).

Immature Schwann cells mature to myelinating or non-myelinating Schwann cells, depending on the axon diameter. Axon derived Nrg1-type III is essential for Schwann cells to mature to myelinating Schwann cells *in vitro*. The transcription factors Krox20 and Oct-6 are also promoted by Nrg (Murphy, Topilko et al. 1996; Leimeroth, Lobsiger et al. 2002; Taveggia, Zanazzi et al. 2005).

Nrg1 also supports Schwann cell migration (Shah, Marchionni et al. 1994; Dong, Sinanan et al. 1999; Taveggia, Zanazzi et al. 2005; Aquino, Hjerling-Leffler et al. 2006). A zebrafish study from 2011 did not only show the importance of Nrg1 type III for Schwann cell migration; it also proved the importance of the ErbB2 receptors. In zebrafish, Schwann cell precursors, emerging from the neural crest, accumulate in the posterior lateral line ganglion (PLLg) and migrate as mature Schwann cells along the posterior lateral line nerve (PLLn). Knock down of Nrg1 reduces Schwann cell numbers along the PLLn. To prove the importance of the ErbB2 receptors, they made genetic chimeras. Interestingly, they found that reduced Schwann cell migration by lack of Nrg1 can be rescued by some wild type neurons in the PLLn and that the possession of ErbB2 receptors in Schwann cells is essential for the migration. Wild type Schwann cells cannot rescue their ErbB2 deficient neighbors thus proving that Schwann cell migration is dependent on Nrg1/ErbB2 signaling (Perlin, Lush et al. 2011).

1.1.3 Myelinogenesis

Myelinating Schwann cells form bands of Bunger next to the axons, engulf it with their complete cell body and spirally wrap it several times. The cell membranes, lying on top of each other, form the myelin sheaths. During this wrapping process, the Schwann cells cytoplasm is pushed to the outside, leading to a compaction of the myelin (Fig.1.2.b). One axon is engulfed by several Schwann cells in a row so that small gaps, the nodes of Ranvier, are formed between the single Schwann cells (Fig.1.2. a). Myelin, an electrical insulator, inhibits the excitation of the myelinated axonal parts. Transmission of the action potentials is only possible from node of Ranvier to node of Ranvier allowing even small axons to conduct rapidly. The leaping from node to node is called saltatory conduction (Squire 2008).

Myelin membranes built by Schwann cells in the PNS and oligodendrocytes in the CNS have a higher lipid to protein ratio in contrary to most other biological

membranes. The dry weight of myelin consists of ~70-80% lipids and only of ~20-30% proteins. The containing lipids are cholesterol, phospholipids, galactolipids and plasmalogen in a ratio of 3:3:2:1 to 4:3:2:,1 whereas cholesterol synthesis influences myelin thickness (Saher, Quintes et al. 2011).

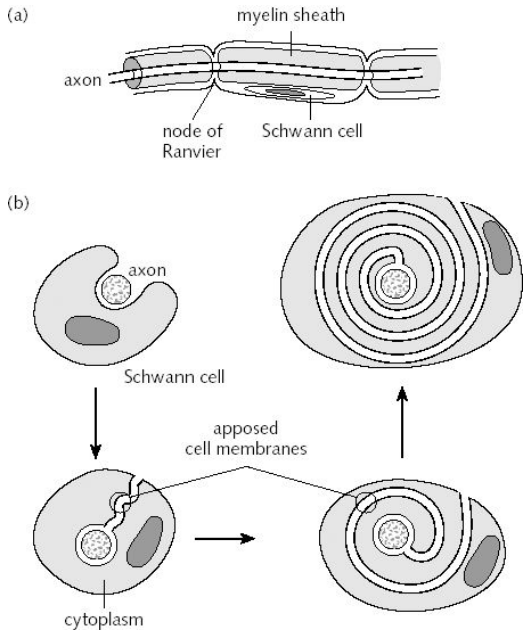


Fig 1.2. Schematic representation of a) a myelinated axon with indicated nodes of Ranvier and b) the development of the myelin sheath by a Schwann cell. Adapted from (Kettenmann and Ransom 2005).

As soon as immature Schwann cells are triggered to mature to myelinating Schwann cells they stay in a growth arrest (Johnson, Zoubos et al. 2005) and start the synthesis of four myelin associated main proteins. The glycoprotein Protein-0 (P0), peripheral myelin Protein-22 (PMP-22), myelin-associated glycoprotein (MAG) and the myelin basic protein (MBP) are the main proteins (Mirsky and Jessen 1996; Garbay, Heape et al. 2000; Mirsky, Jessen et al. 2002). Schwann cells, without axonal contact express basal levels of P0 as well as MBP. This expression is modulated by axonal contact *in vivo* and *in vitro* (LeBlanc and Poduslo 1990; Morgan, Jessen et al. 1994). P0 levels are also increased by increased cAMP *in vitro* especially if growth factors like fibroblast growth factor-2 (FGF-2) are abolished (Morgan, Jessen et al. 1994).

Other myelin associated proteins are proteolipid protein (PLP), CNPase and periaxin (Kurihara, Monoh et al. 1990; Dieperink, Oneill et al. 1992; Garbern, Cambi et al. 1997).

The organization of the myelination program is the focus of several groups and some transcriptional regulators have been identified over the last decades. The most famous ones, are the zinc-finger transcription factor early growth response gene 2 (Egr 2/Krox-20) and the POU transcription factor Oct-6 (Mirsky and Jessen 1996).
Krox-20 deficiency leads to the absence of Schwann cell myelination. It seems that Krox-20 is involved in the activation of the myelination program. However, as the promoters of myelin genes are missing the binding sites needed for Krox-20, intermediate genes are needed. Another explanation is that the absence of Krox-20 inhibits the Schwann cell lineage (Jessen, Brennan et al. 1994; Topilko, Schneider-Maunoury et al. 1994).
The phenotype of Oct-6 deficient mice has been studied by different groups with different outcomes. They agree on an altered myelination, while one group stated that the phenotype is similar to the Krox-20 deficient mice, another group said that the myelination onset is just delayed. In mice where the P0 promoter only drives a transcriptionally inactive form of Oct-6, myelin gene products are overexpressed leading to a hypermyelination (Mirsky and Jessen 1996).
Two other known transcription factors are Pax-3, which suppresses myelin differentiation as Oct-6 and c-Jun, in which levels are increased after loss of axonal contact (Mirsky and Jessen 1996).

1.1.3.1 Neurotrophic factors regulating myelination

During the last decades several growth factors regulating or influencing myelin are known. Among them are some neurotrophic factors. They are required for the survival of different neuronal populations and can be roughly classified into three major groups. One group is the TGF-βs, which includes TGF-β1, TGF-β2, TGF-β3, the glial cell line derived factor (GDNF) and the growth differentiation factor-15 (GDF-15). Several studies showed the inhibiting effect of TGF-βs on myelination. In purified Schwann cell cultures, cAMP induced differentiation to myelinating Schwann cells is inhibited by TGF-βs. It seems that TGF-βs can override axon induced as well as cAMP induced myelination (Mews and Meyer 1993; Einheber, Hannocks et al. 1995; Brown, Ward et al. 2003). The inhibition mechanism is still unclear, but it is suggested that TGF-βs might generate non-myelinating Schwann cells in developing

nerves or even act in the early Schwann cell lineage maintaining the non-myelinating Schwann cell phenotype. Thereby regulating myelination in an indirect way (Morgan, Jessen et al. 1994; Griner, Joshi et al. 2013). In axons, Schwann cells and their precursors, several different members of the TGF-β superfamily are found. TGF-β1, TGF-β2 and TGF-β3 are widely distributed and TGF-β1 is upregulated after nerve transection (Scherer, Kamholz et al. 1993; Rufer, Flanders et al. 1994; Einheber, Hannocks et al. 1995). Other TGF-β superfamily members, which are found in Schwann cells or their precursors, are GDNF (Henderson, Phillips et al. 1994), BMP-6 (Mirsky and Jessen 1996) and most importantly for this work GDF-15 (Strelau, Sullivan et al. 2000).

Another group of neurotrophic factors are the neurotrophins including among others NGF, brain-derived neurotrophic factor (BDNF) and neurotrophin-3. NGF shows a positive effect on myelination, whereas BDNF can both, inhibit and promote myelination depending on the different neuron types (Xiao, Kilpatrick et al. 2009). Interestingly, the positive effect of NGF is restricted to the receptor tyrosine kinase tropomyosin-related kinase A (TrkA) positive Dorsal root ganglion (DRG) neurons. Co-cultures of TrkA positive neurons and Schwann cells showed enhanced myelination after treatment with exogenous NGF. TrkA negative neurons are not affected by exogenous NGF (Chan, Watkins et al. 2004). Further *in vitro* analysis of TrkA positive neurons showed the enhanced myelin production by exogenous BDNF (Chan, Cosgaya et al. 2001). *In vivo*, overexpression of BDNF leads to hypermyelination and increased axonal diameters (Tolwani, Cosgaya et al. 2004). Interestingly, exogenous BDNF shows an inhibitory effect on myelin production in TrkB positive neurons (Xiao, Wong et al. 2009). Notably, it is known that NGF and BDNF upregulate the soluble forms of Nrg1 type I and type II in TrkA positive neurons *in vitro* (Taveggia, Zanazzi et al. 2005). Whether this regulation occurs on the type III isoform is not known.

1.1.3.2 Neuregulin is a key regulator of myelination

Today it is known that Neuregulin is not only an important growth factor involved in Schwann cell development, it is furthermore one of the key factors of myelination. Nrg1 deficiency leads to significant hypomyelination (lethal at P0), while Nrg1 overexpression leads to hypermyelination. The signaling pathway induced through

the epidermal growth factor receptor-2 (ErbB2) is important. Schwann cell-specific deletion of *ErbB2* as well as *Nrg1* deletion leads to hypomyelination (Garratt, Voiculescu et al. 2000; Michailov, Sereda et al. 2004; Chen, Velardez et al. 2006). As mentioned in Chapter 1.1.3, cholesterol, phospholipids, galactolipids and plasmalogen in a ratio of 3:3:2:1 to 4:3:2:1 constitute the myelinlipid composition whereas Cholesterol is 26% to 28% of dry weight in myelin (Saher, Quintes et al. 2011). Cholesterol synthesis is essential for myelin synthesis and Schwann cells can synthesize the needed amount of cholesterol *in situ* (Saher, Brugger et al. 2005). The rate-limiting step in cholesterol synthesis is the formation of mevalonate which is catalyzed by the enzyme HMG-CoA reductase (3-hydroxy-3-methyl-glutaryl-CoA, encoded by *Hmgcr*). Lipoprotein-deficient media induces Hmgcr expression in cultured Schwann cells, indicating the ability of Schwann cells to counteract low cholesterol levels (Fu, Goodrum et al. 1998). In 2007, Pertusa and colleagues analyzed the correlation of Nrg1/ErbB2 signaling and cholesterol synthesis in different cell lines and primary rat Schwann cells. They found out that (1) Nrg receptors ErbB2, ErbB3 and ErbB4 are transactivated by low cholesterol levels, (2) *Hmgcr* transcriptional activity is enhanced by overexpression of ErbB2 or ErbB3 and the combination of both, (3) treatment with recombinant Nrg1 type III increases ErbB2 and ErbB3 phosphorylation, (4) the use of ErbB inhibitors diminish *Hmgcr* promoter activity and (5) that NRG1 increases the steady-state levels of *Hmgcr* mRNA in primary rat Schwann cells. These findings prooved that Nrg1/ErbB2 signaling controls myelin thickness by regulating cholesterol biosynthesis on a transcriptional level (Pertusa, Morenilla-Palao et al. 2007).

1.1.4 Nerve regeneration

Understanding the mechanisms of nerve regeneration is of high clinical importance. Between 13 and 23 per 100 000 persons each year suffer under peripheral nerve injuries, and their recovery is proportional to the severity of damage. Understanding the mechanism of nerve regeneration is the basis for a complete cure. Even though, Schwann cells are known as the cells responsible for myelination in the PNS, they also play a major role in the immune response after peripheral nerve injury and regeneration. They are not only involved in the remyelination stage; their active role

in nerve regeneration starts directly after nerve injury. Schwann cells become activated and re-enter the cell cycle again. Myelin and axonal debris must be cleared of the injured site to support axonal growth which is done by infiltrating macrophages and Schwann cells. In fact, the expurgatory of myelin debris is an essential stage during regeneration. Myelin contains axonal growth inhibitors as NOGO-A and MAG (David and Lacroix 2003) and therefore, diminishes axonal regrowth. After cleaning the injury or inflammated site, Schwann cells form the bangs of Bunger guiding the newly formed axons towards the denervated targets. An extracellular matrix, consisting of collagen and laminin produced by Schwann cells further supports axonal regrowth and guidance (Evans 2001). After guidance the axon, the Schwann cells differentiate into myelinating Schwann cells and start to myelinate the newly formed axon (Stoll, Jander et al. 2002). This regeneration process is not only seen after traumatic injury; it has been observed in neurodegenerative disorders such as Alzheimers and Parkinson diseases and in demyelinating diseases such as multiple sclerosis (Coleman 2005).

However, identification of the mechanisms inducing the repair response or just leading the Schwann cells to maintain the myelin sheaths in the adult organism is still at its beginning and is partly not unequivocally discussed. For example, in late 2012 it was shown that Nrg1 overexpression in Schwann cells restored normal myelination after nerve injury (Stassart, Fledrich et al. 2013). But in early 2013 it was shown that Nrg1 is not essential for remyelination, as it seems that in late stages of remyelination other pathways compensate for Nrg1 deficiency (Fricker, Antunes-Martins et al. 2013).

Surgical intervention in peripheral nerve injuries is mostly necessary to align the axonal stumps. One method is the tubulisation of the injured nerves with e.g. collagen, showing an enhanced repair outcome by an additional treatment with BDNF. Interestingly, the form of BDNF submission also alters the experimental outcome. Collagen tubulisation with BDNF delivered to the repair site by an osmotic minipump or crosslinked to the collagen matrix was compared. BDNF cross-linked to the collagen matrix showed the highest nerve repair. Combined treatment with CNTF and BDNF further enhanced functional nerve regeneration (Lewin, Utley et al. 1997).

Another surgical method is the insertion of a nerve graft (Rodrigues, Rodrigues et al. 2012). The use of acellular nerve allografts (ANAs) is limited as they depend on host Schwann cells. The host Schwann cells have to proliferate rapidly, leading to

senescence. The lengths of ANAs are limited, such limitation is associated with increasing Schwann cell senescence (Saheb-Al-Zamani, Yan et al. 2013). Therefore, the use of nerve grafts containing Schwann cells overexpressing neurotrophins as BDNF, CNTF, NT-3 or GDNF is tested (Godinho, Teh et al. 2013; Santosa, Jesuraj et al. 2013)

In spinal cord injuries, Schwann cell transplantations are the up-to-date standard test system. Neuregulin and the TGF-β related cytokine GDNF have been shown to support Schwann cell migration within these test systems and enhance the repair outcome (Zhang, Zhao et al. 2010; Deng, Deng et al. 2013). GDNF is a very potent neurotrophic factor compared to other neurotrophins. GDNF is 75-fold more potent on supporting survival of cultured motoneurons (Zurn, Baetge et al. 1994). It was shown by Deng and coworkers that GDNF enhances spinal cord injury repair. The newly formed axons grew along the GDNF overexpressing Schwann cells and host Schwann cells as well as the GDNF overexpressing Schwann cells remyelinated the regenerated axons (Deng, Deng et al. 2013). There is also evidence that GDF-15, also a potent neurotrophic factor for motoneurons enhances nerve regeneration (see 1.2.4.4).

1.1.5 Demyelinating diseases

Myelination disorders occur in the CNS as well as in the PNS. In the CNS the most recognized myelination disorder is Multiple Sclerosis (MS). In the PNS, the most common diseases are the Guillain-Barre Syndrome (GBS), the Charcot-Marie Tooth disease and Friedreichs Ataxia.

MS is characterized by the formation of lesions in the CNS. It is an autoimmune disorder leading to the destruction of oligodendrocytes resulting in demyelination of axons. Pro-inflammatory cytokines such as TNF-α have been associated with DNA fragmentation in oligodendrocytes at the site of MS lesions (Lucchinetti, Bruck et al. 2001).

Acute inflammatory polyneuropathies are collectively called GBS. It is an autoimmune disorder that attacks axons and the myelin sheaths, inducing a demyelination that leads to a rapid progressive paralysis. Different forms of GBS are known. Demyelinating forms of GBS are characterized by a demyelination induced by

macrophages associated with T-cell and plasma cell infiltration (Chiang and Ubogu 2013).

The Charcot-Marie Tooth disease (CMT), also known as hereditary motor and sensory neuropathies is subdivided into 4 main groups whereas CMT-1 and CMT-4 are myelination disorders. They are defined by a characteristically reduced nerve conduction velocity (NCV) with an accompanied axonal loss leading to a severe disability in adulthood (Kalaydjieva, Gresham et al. 2000; Nave, Sereda et al. 2007). During the second or third decade of life, neural deafness develops by muscle weakness and wasting, tendon areflexia and sensory loss affecting all modalities. At this point in time, Schwann cell dysfunctions are observed which are manifested by disturbed myelination such as hypomyelination and demyelination/remyelination leading to the reduced NCV. The Schwann cell dysfunctions are followed by a severe and progressive axonal loss. Further studies showed that not the demyelination itself but the axonal loss leads to neural deafness. Gene expression studies showed that a mutation in the N-myc downstream regulated gene 1 (NDRG1) is found in CMT patients (Kalaydjieva, Gresham et al. 2000). Furthermore, NDRG1 deficiency in mice leads to a similar phenotype as found in CMT patients (Okuda, Higashi et al. 2004). Interestingly for this work, NDRG1 expression is increased by GDF-15 overexpression (Tsui, Chang et al. 2012).

Another neurodegenerative, myelin-affecting disorder is Friedreichs ataxia. Primarily dorsal root ganglia, spinocerebellar pathways, the posterior column and the pyramidal tracts of the spinal cord are affected. Axons degenerate in the distal parts leading to a dying back manner towards the cell bodies (Hughes, Brownell et al. 1968; Said, Marion et al. 1986; Lu, Schoenfeld et al. 2009). Today it is known, that this autosomal recessive disorder is induced by a mutation of the frataxin gene and it has been shown that the cells mostly affected by the frataxin mutation are Schwann cells. Frataxin deficiency induces an inflammatory toxicity to Schwann cells. Schwann cells die, leading to the dying back of the axons (Lu, Schoenfeld et al. 2009).

1.2 Growth differentiation factor-15

Growth differentiation factor-15 is a distant member of the transforming growth factor-βs (TGF-β) family. TGF-βs are structurally related cytokines which are involved in the regulation of cell proliferation, differentiation, immune response and extracellular matrix formation. (Palladino, Morris et al. 1990; Sporn and Roberts 1990; Bottner, Krieglstein et al. 2000). The TGF-β superfamily can be subdivided into 4 subfamilies, the TGF-β-, Glial derived neurotrophic factor (GDNF)-, activin- and decapentaplegic-Vg-related (DVR)-families and Growth differentiation factor-15 (GDF-15) (Bottner, Suter-Crazzolara et al. 1999; Strelau, Bottner et al. 2000).
GDF-15 was discovered in the late 1990s by several groups simultaneously and is therefore known by different names as GDF-15 (Bottner, Suter-Crazzolara et al. 1999), macrophage inhibitory cytokine 1 (MIC-1)(Bootcov, Bauskin et al. 1997), non-steroidal anti-inflammatory drugs activated gene-1 (NAG -1)(Baek, Kim et al. 2001), PL74 (Morrish, Linetsky et al. 1996), placental bone morphogenetic protein (PLAB) (Hromas, Hufford et al. 1997), placental transforming growth factor-β (PTGF- β) (Lawton, Bonaldo et al. 1997), prostate-derived factor (PDF) (Paralkar, Vail et al. 1998) and transforming growth factor-β placental (TGF-βPL)(YokoyamaKobayashi, Saeki et al. 1997). In this work, the term GDF-15 is used.

1.2.1 Gene and protein structure

The human *gdf15* gene is located on chromosome 19 whereas the mouse *gdf15* gene is located on chromosome 8. It is consistent of two exons, separated by one intron within the prepeptide domain of the protein. A TATA-like motif is located near to the putative translation start codon (Fig.1.1) (Bottner, Laaff et al. 1999).
The human precursor protein is consistent of 308 amino acids (aa) whereas the preproteins of rat and mouse are only consistent of 303 aa. The GDF-15 sequence similarity in the mature protein is remarkably low between species. Between the three species, they share only 70% conserved residues whereas in other TGF-βs as TGF-β1 and bone morphogenetic protein-2 (BMP-2) share 99% and 100% conserved residues. The protein in all three species possesses a hydrophobic signal peptide at the N-terminal end, a dibasic cleavage site (RGRR) and a conserved pattern of

cysteine's in the C-terminal domain, the cysteine knot, a structural hallmark of the TGF-β superfamily (Fig.1.3) (Bottner, Laaff et al. 1999).

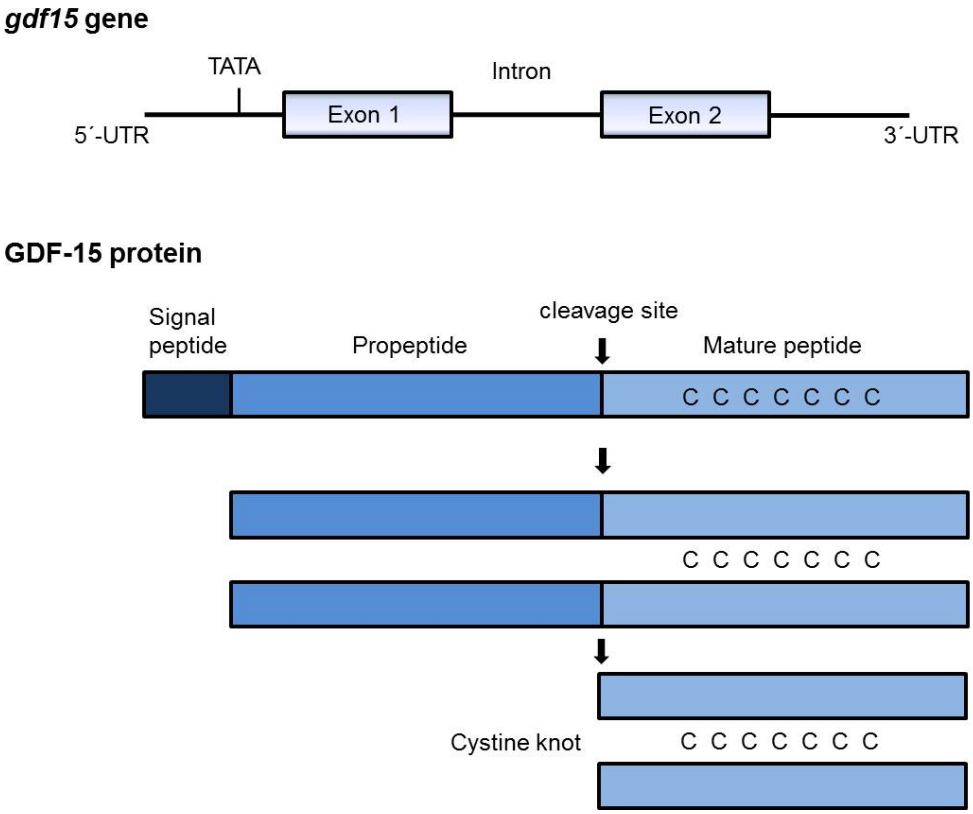


Figure 1.3: *gdf15* gene and GDF-15 protein structure. Note the cysteine knot, a structural hallmark of the TGF-βs in the mature protein.

1.2.2 Regulation of expression and tissue distribution

During the characterization of the *gdf15* genes in different mammals, the promoter region was analyzed as well, revealing several expression regulatory elements as five SP1, four AP-1, one AP-2 and NF-kB binding sites (Bottner, Laaff et al. 1999; Baek, Horowitz et al. 2001). Other studies showed that GDF-15 expression is upregulated by EGR-1(Baek, Kim et al. 2005) and p53 (Li, Wong et al. 2000). In 2003, Yang and colleagues showed that GDF-15 concentration can also serve as a biomarker for p53 pathway activation (Yang, Filipovic et al. 2003).

Alongside of the basal transcription mediators, several other factors, mediating GDF-15 expression were revealed. In the breast cancer cell line MCF-7, an overexpression of AKT upregulates GDF-15 expression (Campbell, Bhat-Nakshatri et

al. 2001) whereas an inhibition of PI3K and AKT induces GDF-15 in the cancer cell line HCT-116 (Yamaguchi, Lee et al. 2004).

The majority of TGF-β superfamily members are synthesized as an inactive precursor protein which dimerizes in the endoplasmatic reticulum. This is also true for GDF-15. The homodimer of the precursor protein is then transported to the golgi apparatus where it is cleaved at a furine-like cleavage site. The human mature GDF-15 consists of 112 aa, whereas the rodent mature GDF-15 contain 115 aa (Bottner, Laaff et al. 1999; Bauskin, Zhang et al. 2000).
GDF-15 is widely expressed in the adult organism. It is strongly expressed in the placenta and prostate. GDF-15 mRNA can be found in different epithelial cells as enteric, respiratory, ectodermal and endodermal epithelial cells and in activated macrophages (Lawton, Bonaldo et al. 1997; YokoyamaKobayashi, Saeki et al. 1997; Bottner, Suter-Crazzolara et al. 1999; Fairlie, Moore et al. 1999).
In the CNS, GDF-15 is highly expressed in the choroid plexus and secreted into the cerebrospinal fluid (CSF) (Strelau, Sullivan et al. 2000). GDF-15 expression in the adult rat brain is almost exclusively in the choroid plexus whereas in newborn rats, GDF-15 expression is also localized in ependymal cells lining the ventricles, in the striatal subventricular zone and in populations of non-neuronal cells of the thalamic/hippocampal lamina affixa (Schober, Bottner et al. 2001). In the PNS, GDF-15 is expressed in Schwann cells (Strelau, Strzelczyk et al. 2009).

1.2.3 GDF-15 signaling

As a distant member of the TGF-β superfamily, it was assumed that the signaling pathway of GDF-15 is similar to those of TGF-βs. However, this hypothesis could not be proved and the search for the involved receptors begun. As GDF-15 is involved in tumor progression and cell invasion in cancer and most GDF-15 publications focus on GDF-15 involvement in cancer, its signaling pathway has been studied by different groups in cancer cell lines. They showed that GDF-15 transactivates ErbB2 in human breast and cancer cells by activating AKT and extracellular signal-regulated kinase (ERK)-1/2 (Kim, Lee et al. 2008) or steroid receptor coactivator (Src) (Park, Lee et al. 2010). ErbB2 is also activated by Neuregulin, an important factor involved in early

myelin formation and peripheral nerve injury response (Calvo, Zhu et al. 2010; Newbern and Birchmeier 2010; Perlin, Lush et al. 2011; Chang, Shyu et al. 2013). AKT and ERK signaling is also involved in GDF-15 signaling in cerebellar granule neurons (CGN). It protects CGNs by activating AKT and inhibiting endogenously active ERK from low-potassium induced apoptosis (Subramaniam, Strelau et al. 2003). But still, the signaling pathway of GDF-15 in the PNS, especially in Schwann cells, is still unclear.

1.2.4 Functional roles for GDF-15

Since its discovery in the late 1990s, it was shown that GDF-15 is a multifunctional factor involved in inflammatory processes, cardiovascular diseases and cancer. Interestingly, it is also a very potent neurotrophic factor.

1.2.4.1 GDF-15 and inflammation

During inflammatory processes macrophages play a key role. During the search for factors involved in macrophage activation, Bootcov and colleagues simultaneously with other groups (see 1.2) discovered GDF-15, also known by the name macrophage inhibitory cytokine-1 (Mic-1). They showed that GDF-15 is upregulated by factors e.g. Interleukin-β or Interleukin-2 associated with macrophage activation (Bootcov, Bauskin et al. 1997). In atherosclerosis, a chronical inflammation of the aterial wall GDF-15 deletion as well as overexpression has protective effects (see 1.2.4.2).

1.2.4.2 GDF-15 and cardiovascular diseases

Myocardial GDF-15 is rapidly increased after ischemic injury (Kempf, Eden et al. 2006) and patients with acute myocardial infarction show elevated levels of circulating GDF-15 (Wollert 2007; Wollert, Kempf et al. 2007), showing the prognostic values of GDF-15 in cardiovascular diseases (Kehl, Iqbal et al. 2012).
In apolipoprotein (Apo) E-deficient mice, a mouse model for progressive atherosclerotic lesions, GDF-15 deficiency protects from atherosclerosis

progresssion (de Jager, Bermudez et al. 2011; Bonaterra, Zugel et al. 2012). At the same time, another study showed that GDF-15 overexpression has the same effect. ApoE-deficient mice overexpressing GDF-15 had reduced atherosclerosis than the control group (Johnen, Kuffner et al. 2012). However, the mechanisms in GDF-15 overexpression reducing atherosclerosis risk are unknown whereas the mechanisms in GDF-15 deficiency have been revealed. The Interleukin-6 (IL-6), an inflammatory marker, expression in ApoE deficient peritoneal macrophages is highly increased after stimulation with oxidized low-density lipoprotein (oxLDL). ApoE and GDF-15 deficient macrophages completely lack the IL-6 induction by oxLDL stimulation showing that GDF-15 deficiency regulates IL-6 dependent inflammatory response to vascular injury (Bonaterra, Zugel et al. 2012). IL-6 expression is not regulated by GDF-15 overexpression (Johnen, Kuffner et al. 2012).

1.2.4.3 GDF-15 and cancer

As mentioned before, GDF-15 is involved in the tumorigenesis of different cancer types. In gastric cancer and colorectal cancer patients, GDF-15 serum levels are increased (Brown, Ward et al. 2003; Baek, Yoon et al. 2009). An anti-tumorigenic effect of GDF-15 was shown in lung, pancreatic and prostate cancer (Eling, Baek et al. 2006; Kadara, Schroeder et al. 2006; Kim, Kim et al. 2008; Min, Zhang et al. 2012). In a cohort study with 94 patients suffering under intercranial tumors including gliomas and meningioma elevated GDF-15 protein levels were detected by ELISA in the cerebrospinal fluid and most interestingly, patients with elevated GDF-15 levels in the CSF and glioblastoma had a shorter survival showing the prognostic value of GDF-15 (Shnaper, Desbaillets et al. 2009).

1.2.4.4 GDF-15 and the nervous system

Despite the fact that GDF-15 is widely expressed in the organism, most publications focus on the involvement and effect of GDF-15 in cancer progression (Vanhara, Hampl et al. 2012; Griner, Joshi et al. 2013; Wang, Baek et al. 2013) and cardiovascular diseases (de Jager, Bermudez et al. 2011; Bonaterra, Zugel et al.

2012; Rohatgi, Patel et al. 2012). The elucidation about the physiological roles of GDF-15 in the CNS and PNS is still at its beginning.

In 2000 it was shown that GDF-15 is a trophic and neuroprotective factor for midbrain dopaminergic neurons *in vitro*. It promotes survival of cultured midbrain dopaminergic neurons, and furthermore, it protects dopaminergic neurons against cell death induced by iron intoxication. Strelau and others also showed that GDF-15 protects nigrostriatal neurons *in vivo* after receiving a 6-hydroxydopamine lesion (Strelau, Sullivan et al. 2000). By occlusion of the middle cerebral artery in mice, an ischemic lesion can be induced, which leads to a direct and drastical GDF-15 mRNA upregulation in the hippocampus and parietal cortex as well as an upregulation of GDF-15 immunoreactivity in almost all neurons in the granular layer of the dentate gyrus. Microglial GDF-15 immunoreactivity is moderately increased (Schindowski, von Bohlen und Halbach et al. 2011). Groschel and colleagues analysed GDF-15 blood levels in patients with symptoms for acute ischemic stroke and found out that GDF-15 blood levels can predict the outcome 90 days after stroke. Increased GDF-15 levels predict an unfavorable outcome (Groschel, Schnaudigel et al. 2012).

To further investigate the neurotrophic and neuroprotective effects of GDF-15, a GDF-15 deficient mouse was established (Strelau, Strzelczyk et al. 2009). Analysis of motoneuron numbers in the brainstem including trigeminal and facial motor nuclei and in spinal cord showed again the neuroprotective effect of GDF-15. Motoneuron numbers were decreased in GDF-15 deficient mice. At the age of 6 months, the deficit reached its maximum of ~20%, and most importantly for this work, it is accompanied by an axonal loss (~13%). Cell numbers in dorsal root ganglion were also reduced (Strelau, Strzelczyk et al. 2009). Another TGF-β, TGF-β2 is also known to promote motoneuron survival (McLennan and Koishi 2002). Trophic factors, required by motoneurons are mostly expressed by striated muscle and peripheral nerves. An mRNA study of peripheral nerves and gastrocnemius muscle indicated that peripheral nerves may be the most important source of GDF-15. GDF-15 immunoreactivity in dissociated adult mouse peripheral nerve cultures were most intense in S100 (a Schwann cell marker) positive cells, and determination of GDF-15 protein levels by ELISA of human Schwann cells showed that Schwann cells possess a high amount of GDF-15, suggesting that Schwann cells are an important source of GDF-15 in the peripheral nervous system. Furthermore, they showed that GDF-15 is

transported retrogradly along adult sciatic nerve axons (Strelau, Strzelczyk et al. 2009).

GDF-15 is not only important for the survival of motoneurons; it also plays a supporting role in peripheral nerve injury response. Charalambous and colleagues investigated the effect of GDF-15 on nerve regeneration. They showed that GDF-15 expression is increased one day after nerve crush. A slightly elevated level is existent until 28 days post crush (Charalambous, Wang et al. 2013). Another paper showed that GDF-15 substitution leads to an accelerated sensory recovery whereas the numbers of regenerated and myelinated axons were decreased. However, the recovered axons after GDF-15 treatment were further in progress showing higher myelinated areas and axonal diameters (Mensching, Borger et al. 2012).

1.3 Aim of the study

In the last years, it was shown that GDF-15 is a neurotrophic factor (Strelau, Sullivan et al. 2000) which also supports nerve regeneration (Mensching, Borger et al. 2012). Motoneuron and axonal numbers are reduced in adult GDF-15 deficient mice (Strelau, Strzelczyk et al. 2009). Furthermore, a hypermyelination is induced (Dr. J. Strelau, unpublished Data).
Schwann cells play a key role in maintaining peripheral nerves. They are involved in nerve immune responses (Wallerian degeneration)(Stoll, Jander et al. 2002), supporting nerve regrowth after injury and just by maintaining the correct myelin thickness. For example, in the neuropathy Charcot-Marie Tooth disease Schwann cell dysfunction leads to axonal loss which induces neural deafness (Kalaydjieva, Gresham et al. 2000). As GDF-15 deficiency induces an axonal loss and hypermyelination, I suggest a Schwann cell dysfunction occurring in adulthood of mice.

To prove this hypothesis, *in vivo* Schwann cell numbers were determined in a peripheral nerve of wild type and GDF-15 mutant mice of different ages as well as gene expression patterns of myelin associated genes in adult peripheral nerves.
In vitro gene expression studies were done with the help of a GDF-15 siRNA induced knockdown in the Schwann cell line RT4-D6P2T.
To further elucidate effects of GDF-15 deficiency on Schwann cells, a method to prepare highly pure primary Schwann cell cultures from adult (6 months) mice was established. The cultures were used for functional studies as proliferation, apoptosis and migration.

Chapter 2: Materials and Methods

2.1 Abbreviations

7-AAD	7-Amino-Actinomycin
µL	micro liter
µM	micro molar
µmol	micro mol
bp	base pairs
BPE	Bovine Pituitary Extract
Ca^{2+}	Calcium ion
cDNA	complementary DNA
CNS	Central nervous system
Ct	Cycle threshold
DMEM	Supplemented Dulbecco´s Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide Triphosphates
ECL	enhanced chemiluminiscence
e.g.	for example
et al.	and other (authors)
ErbB2	epidermal growth factor receptor 2
EtBr	Ethidium bromide
FACS	Fluorescent associated cell sorting
FCS	fetal calve serum
GDF-15	Growth differentiation factor-15
GDNF	Glial-Derived Neurotrophic Factor
gen.	genomic
h	hour
IL-6	Interleukin-6
$MgCl_2$	Magnesium chloride
mM	milli Molar
mRNA	messenger Ribonucleic acid

NDRG1	N-my downregulated gene 1
ng	nanogram
NGF	Nerve growth factor
Nrg	Neuregulin
NTF-3	Neurotrophin-3
PBS	Phosphate Buffer Saline
PCR	Polymerase chain reaction
PNS	Peripheral nervous system
pSC	primary Schwann cell cultures
PVP	Polyvinylpyrrolidon K30
qRT	Quantitative real-time
RNA	Ribonucleic acid
rpm	Rounds per minute
RT	Reverse transcription
SC	Schwann cell
Taq DNA polymerase	DNA Polymerase derived from *Thermophilus aquaticus*
TBE	Tris-borate-EDTA buffer
TGF-β	Transforming growth factor-β

2.2 Materials and Solutions

2.2.1 Materials and solutions used in the molecular biology

2.2.1.1 Materials used in molecular biology

β-Mercaptoethanol	*Merck*
Agarose	*Invitrogen*
Chloroform	*Merck*
DNA Ladder	DNA-Marker 2-Log, *New England Biolabs*
ECL-Kit	ECL Prime, *Amersham GE Healthcare*
EtBr	*Roth*
Ethanol	*Roth*
gen. DNA extraction	QuickExtract DNA Extraction Solution 1.0, Epicentre, *Biozym*
Glycogen	GlycoBlue™, *Ambion*
Glycerine	*Merck*
Isopropanol	*Appllichem*
$MgCl_2$	*Promega*
Nucleotide Mix	*Promega*
PCR Buffer	PCR-Buffer green 5x, *Promega*
PVDF membrane	Immobilon-P, *Millipore*
Taq Polymerase	GoTaq DNA Polymerase 5 Units/µl, *Promega*
TriFast	*Peqlab*
Tris-HCL	*Merck*
Tween20	*Sigma*

2.2.1.2 Antibodies used in molecular biology

2^{nd} α-mouse	True Blot α –mouse HRP, *Rockland*
2^{nd} α-rabbit	*Cell Signaling*
Phospho-ErbB2	Phospho-HER2/ErbB2 (Tyr877), *Cell Signaling*
ErbB2	Her2/ErbB2 (M45), *Cell Signaling*
β-actin	*Sigma*

2.2.1.3 Solutions used in molecular biology

2x Laemmli Loading Buffer

20.0%	Glycerine
6.6%	β-Mercaptoethanol
4.0%	SDS
125 mM	Tris pH8.8
10 mM	EDTA

2x Laemmli Running Buffer

0.25 M	Tris pH8.8
1.92 M	Glycerine
1%	SDS

10x Wet Blot Buffer

480 mM	Tris pH8.8
390 mM	Glycine

PBS 0.1 M

16 g	NaCl
0.4 g	KH_2PO_4
2.3 g	Na_2HPO_4

in 2000 ml Milli-Q-Water

TBS-T

15 mM	NaCl
1 mM	Tris pH8.8

Adjust pH to 7.5

Add 0.1% Tween20

2.2.2 Materials, solutions and media used for cell culture

2.2.2.1 Materials used in cell culture

Acetic acid	Acetic acid 100%, *VWR*
Aseptic filter	Sterile filter 0.2 µm, *Schleicher&Schuell*
BPE	Bovine Pituitary extract, *BD Biosciences*
ErbB2 inhibitor	EGFR/ErbB-2/ErbB-4 inhibitor, *Calbiochem*
Cell culture flask	cell culture flask T25 and T75, *Greiner*
Cell culture plates	cell culture plates 6-well, 12-well, 24-well, 48-well, *Greiner*
Cryotubes	Cryotubes 1.8 ml, *Nunc*
D-MEM	Dulbecco's Modified Eagle Medium *Gibco*
DMSO	Dimethylsulfoxide, *Sigma-Aldrich*
Disposable syringes	Discardit II 5 ml and 20 ml, *BD Biosciences*
GDF-15	rhGDF-15, *R&D systems*
Falcon tubes	falcon tubes 15 ml and 50 ml, *Greiner*
FCS	fetal calve serum, *Biochrom*
Glutamine	*PAA*
Hemocytometer	*Neubauer*
MGM	Melanocyte Growth Medium, *PromoCell*
NaCl	Sodium chloride, *AppliChem*
Neuregulin	rhHeregulin α, *R&D systems*
PBS	Phosphate buffered saline Dulbecco (1x) (without Ca^{2+}/Mg^{2+}), *Biochrom*
PenStrep	250 µg/mL Penicillin Streptomycin, *Roche*
Trypsin	Trypsin-EDTA solution (1x), *Gibco*

2.2.2.2 Solutions and media used in cell culture

Freezing medium	90% FCS + 10% DMSO
MCF-7 medium	D-MEM, 10% FCS, 1% PenStrep
pSC culture medium	Melanocyte Growth Medium, 5% FCS ,1% PenStrep, 5 µg ml^{-1} BPE

pSC predegeneration	D-MEM,10% FCS, 1% PenStrep
pSC dissociation solution	D-MEM,10% FCS, 1% PenStrep, 0.125% Collagenase, 1.25 U ml^{-1} Dispase
RT4-D6P2T medium	D-MEM, 10% FCS, 1% PenStrep, 2 mM Glutamine

2.2.2.3 Solutions and kits used for FACS analysis

7-AAD	*BD Biosciences*
Annexin V binding buffer	10x Annexin V binding buffer, *BD Biosciences*
APC Annexin V	*BD Biosciences*
Cell dissociation	Enzyme Free Cell Dissociation Solution, *Millipore*
Proliferation assay	BD Pharmingen™ BrdU Flow Kit, *BD Biosciences*

2.2.3 Materials and solutions used for Histology

2.2.3.1 Materials used for Histology

0.1 M Na-Cacodylatebuffer pH 7.2	*Merck*
Dimethylarsinacid-Na-Salt-Trihydrate	
DDSA	*Serva*
DMP30	*Serva*
Glutaraldehyde 25%	*Merck*
Glycidether	*Serva*
MNA	*Serva*
Na_2HPO_4-Dehydrate	*Merck*
NaH_2PO_4-Monohydrate	*Merck*
Maleinacid-Na-Salt	
Na-Maleate buffer pH 6.0	*Merck*
PFA	Applichem
Procainhydrochloride	*Merck*
PVP	*Roth*
Toluidine Blue	*Merck*

2.2.3.2 Solutions used for histology

Phosphate-Buffer 0.1 M

5.24 g NaH_2PO_4-dehydrate

28.82 g Na_2HPO_4-monohydrate

in 2000 ml Milli-Q-Water, pH 7.4

Paraformaldehyde 4%

40 g Paraformaldehyde

in 1000 ml Phosphate-Buffer 0.1 M

Toluidine Blue

1% Borax

1% Toluidine Blue

in Phosphate-Buffer 0.1 M

2.3 Methods

2.3.1 Mouse lines and IBF

In this work, P5 to 12 months old mice of a GDF-15 knockout/lacZ knockin (*gdf15-/-*) mouse line were used. The transfections of the embryonic stem cells were done at the European Molecular Biology Laboratory (EMBL, Heidelberg) and have been described in detail by Strelau et al. (Strelau, Strzelczyk et al. 2009).
The *gdf15-/-* mouse line was crossbred with a S100β-eGFP reporter mouse line which was established at the University of Texas and has been described in detail by Zuo et al. (Zuo, Lubischer et al. 2004). It was made available by Thomas Misgeld. Homozygous transgenic (t/t) and *gdf15-/-* or *gdf15*+/+ mice were used for tissue extractions and preparation of primary Schwann cell cultures.
Mouse lines were handled according to the Animal Welfare Act (TierSchG) and the breeding and care-taking were done in the Interfakultäre Biomedizinische Forschungseinrichtung (IBF), University of Heidelberg.

2.3.2 Molecular Biology

2.3.2.1 Genotyping

Genotyping by PCR

Mice genotyping was accomplished by extraction of genomic DNA from a tail snip and the amplification of a specific gene sequence.
Tail snips needed for genomic DNA extraction were prepared in the IBF. The tail snips were incubated in 200 µL “QuickExtract DNA Extraction Solution 1.0“ (Epicentre, Biozym) for 6 minutes at 65°C followed by 2 minutes at 98°C. 2 µl of this DNA solution was used for the amplification of a specific gene sequence.
The amplification was done with use of the polymerase chain reaction (PCR) technique, which is a method for amplification of short DNA fragments by the use of a heat stable DNA polymerase (*Taq* Polymerase) and the help of a primer pair. Normally, in one PCR reaction two primers (forward and reverse) are used to ensure an exponential amplification of the DNA fragment. In this case, a primer mix comsisting of two different forward and reverse primers was used.

One PCR reaction consisted of:

32.0	µL	Rnase free water		(Qiagen)
10.0	µL	PCR-Buffer green 5x		(Promega)
2.0	µL	$MgCl_2$	(1 mM)	(Promega)
1.0	µL	Nucleotide Mix	(0.8 mM)	(Promega)
2.5	µL	Primer Mix	(0.625 pmol/µl/Primer)	(Metabion)
0.5	µL	GoTaq DNA Polymerase 5 Units/µl	(2.5 Units)	(Promega)
48.0	µL			
+2	µL	gen.-DNA	(~ 200 ng)	

The primer mix consisted of:

NeoF1:	5‘-TCG CCT TCT TGA CGA GTT CT-3‘	(forward)
R20:	5‘-CCC AGT CTT GTA GAC AGA GCA A-3‘	(reverse)
F26:	5‘-ATG CGC ACC CAA GAG ACT-3‘	(forward)
R21:	5‘-GGC CAC CAG GTC ATC ATA AG-3‘	(reverse)

Product size: *gdf15*+/+: 690bp *gdf15*-/-: 320bp

PCR conditions used are listed below.

1. Initial denaturation		3:00 min	94°C
2. PCR Cycles (40x)			
	Denaturation	0:30 min	94°C
	Annealing	0:30 min	56°C
	Extension	1:00 min	72°C
3. Final extension		9:00 min	72°C
4. Cooling		∞	4°C

DNA fragments were separated by gel electrophoresis. Due to the negatively charged phosphate group, the PCR product is attracted to the positive pole of the electric field. The PCR products were transferred to a 1.5% agarose gel as well as a DNA ladder. After the gel electrophoresis (Horizon11*14, LifeTechnologies), the agarose gel was stained in an Ethidium Bromide bath (30µL EtBr + 500ml distilled water), and the PCR products were documented with an Intas UV System.

Genotyping by qRT-PCR

The genotyping for the transgenic mouse line expressing eGFP in S100β positive tissue was done with a qRT-PCR (see detailed description in 2.3.2.4). Shortly, genomic tail DNA was amplified according to the following instruction in a StepOne Plus Real-Time PCR system (Applied Biosystems):

The following primers were used:

ApoE (= Housekeeping gene)

oIMR180:	5‘-GCC TAG CCG AGG GAG AGC CG-3‘	(forward)
oIMR181:	5‘-TGT GAC TTG GGA GCT CTG CAG C-3‘	(reverse)

eGFP (= gene of interest)

eGFP-F:	5'-CAC ATG AAG CAG CAC GAC TT-3'	(forward)
eGFP-R:	5'-TGC TCA GGT AGT GGT TGT CG-3'	(reverse)

Each qRT-PCR reaction for the eGFP genotyping consisted of:

3.00	µl	gen. DNA	
15.00	µl	2x QuantiFast SYBR Green PCR Master Mix	(Qiagen)
0.15	µl	forward Primer (50 pmol/µl)	(Metabion)
0.15	µl	reverse Primer (50 pmol/µl)	(Metabion)
11.70	µl	RNase free water	(Qiagen)
30.00	µl		

The PCR conditions were composed of three steps:

1. PCR initial Activation step 5 min 95°C
2. PCR cycles (45x)

Denaturation	20 sec 95°C
Annealing	60 sec 62°C
Extension	35 sec 72°C

3. Dissociation curve analysis

The mean Ct (cycle threshold) values were used for the analysis of the data by the comparative ΔCt method (see detailed description in 2.3.2.4).

2.3.2.2 RNA extraction

Cells and tissues were used for the extraction of RNA. Tissues were homogenized in 1 ml TriFast by the use of PreCell tubes and Schwann cells were scraped off the culture plates after addition of 1 ml TriFast. The RNA extraction was done according to the user manual. The glycogen used to support the RNA precipitation was the GlycoBlue™.

2.3.2.3 Reverse Transcription PCR (RT-PCR)

Extracted RNA was measured in BioPhotometer (Eppendorf), and 1.5 µg of the RNA was used for one RT-PCR reaction. The RevertAid First Strand cDNA Synthesis Kit was used, and the RT-PCR was carried out in an Eppendorf Mastercycler.

One RT- PCR reaction was consistent of:

1.5	µg	RNA
0.5	µL	RiboLock RNase Inhibitor (20 u/µl)
0.5	µL	RQ1 DNase
		Nuclease-free water (Qiagen) (fill up to 13 µL)
13.0	µL	

The RNA, together with an RNase inhibitor as well as DNase I, was incubated for 20 minutes at 37°C followed by 10 minutes at 70°C before the enzyme solution was added.

4.0	µL	Buffer
1.0	µL	dNTP
0.5	µL	Oligo dT
0.5	µL	Random Hex
1.0	µL	Enzyme-Mix
7.0	µL	

After addition of the enzyme solution, the reverse transcriptase program was started:

10:00	min	25°C	} Transcription
25:00	min	50°C	}
5:00	min	85°C	Reaction stop

The cDNA were used for qRT-PCR or stored for longer periods at -20°C.

2.3.2.4 Quantitative real-time PCR (qRT-PCR)

The quantitative real-time PCR (qRT-PCR) allows a quantitative determination of the amount of a specific DNA or RNA sequence by measuring the amount of the PCR product after each cycle. Instead of measuring the total PCR amount at a terminal measurement as it is done in a semi-quantitative RT-PCR.
The synthesized cDNA was diluted 1:5 with RNase free water (Qiagen).

Each qRT-PCR reaction was composed of:

3.0	µl	diluted cDNA	
6.2	µl	2x QuantiFast SYBR Green PCR Master Mix	(Qiagen)
1.3	µl	QuantiTect Primer Assay	(Qiagen)
3.5	µl	RNase free water	(Qiagen)
14.0	µl		

For each sample, three reactions were prepared in a 96 well plate.
The StepOne Plus Real-Time PCR system from Applied Biosystems was used for the amplification and measuring of the PCR products.

The PCR conditions were composed of three steps:

1. PCR initial Activation step 15 min 95°C
2. PCR cycles (40x)
   - Denaturation 15 sec 94°C
   - Annealing 30 sec 55°C
   - Extension 35 sec 72°C
3. Dissociation curve analysis

The mean Ct (cycle threshold) values and mean standard deviation were used for the analysis of the data by the comparative ΔCt method. The Ct value is the cycle in which the fluorescence of a sample crosses the threshold. Thus, a smaller Ct value indicates a higher occurrence of the transcript in a sample because it is in an earlier cycle detectable than in samples where the Ct value for a transcript is higher because it is later detectable. For the comparative ΔCt method a control sample e.g. RNA from untreated cells is needed. The Ct values are normalized to a

housekeeping gene (RPLPO). The mean Ct values of the samples of interest are compared to the mean Ct values of the control sample and the difference (delta Ct, ΔCt) between the values indicates if the gene expression had changed. In a PCR the gene of interest is exponentially amplified which means that the amount of the amplified product is doubled after each cycle.

Name	Symbol	Detected transcript	Company
18s ribosomal RNA	Rn18S	NR_003278	Qiagen GmbH, Hilden, Germany
Interleukin-6	IL-6	NM_031168	Qiagen GmbH, Hilden, Germany
ribosomal protein, large, P0	Rplpo	NM_007475	Qiagen GmbH, Hilden, Germany
N-myc downstream regulated 1	Ndrg1	NM_008681	Qiagen GmbH, Hilden, Germany
3-Hydroxy-3-Methylglutaryl-Coenzym-A-Reductase	Hmgcr	NM_008255	Qiagen GmbH, Hilden, Germany

Figure 2.1: List of used predesigned qRT-PCR primers

The primer used for the Nrg1 type III qRT-PCR was a self-designed primer purchased at the company Metabion.

Nrg1 Type III primer sequence:
(forward) 5'-CCG AGG CAT ACA CTT CAC CT-3'
(reverse) 5'-TGT TTG TGG CTG AGT TCC TG-3'
Product size: 224bp

2.3.2.5 Western Blot

Western blots were done as previously described (Strelau, Strzelczyk et al. 2009). Cells were dissolved in electrophoresis buffer (2xLaemmli) and loaded onto an SDS-Polyacrylamide gel. After the electrophoresis (30 mA; 2 h) of the samples, they were transferred to a PVDF membrane by an electroblotting overnight (350 mA, 12h, Wet Blot System, Amersham Biosciences). The membranes were washed in TBS-T,

blocked in 5% BSA in TBS-T and incubated at 4°C for 12 hours with Her2/ErbB2 (M45) (1:1000), Phospho-HER2/ErbB2 (Tyr877) (1:1000) or β-actin (1:20000) respectively. Bound antibodies were detected by secondary antibodies and the ECL Prime Western Blotting according to the manual.

2.3.3 Cell culture

2.3.3.1 Collagen extraction from rat tails

Rat tails were kindly provided by the IBF and stored at -20°C until use. Before the extraction of the collagen, the rat tails had to be scrubbed and stored in 70% Ethanol for at least 30 minutes. After disinfection, the skin was cut several times in a distance of 0.5 cm. The caudal vertebra were separated and snapped. Meanwhile, the tail was pulled and twisted to resolve the collagen fibers. The tip of the tail was pulled of as well as the segmented part of the tail to expose the collagen fibers. With the help of forceps, the collagen fibers were removed from the tail segments and stored in 70% Ethanol. After the extraction of the fibers, they were washed twice with sterile MilliQ water and then transferred to 0.1% acetic acid (1ml 0.1% acetic acid/ 10 mg collagen fibers). After stirring the reagent for 3 days, it was centrifuged for 2 hours. The supernatant consistent of solved collagen was alliquotted and stored at 4°C.

2.3.3.2 Coating of cell culture plates

Some adherent cells especially primary cells need special coating of the cell culture plates to simulate a normal growth condition. The growth of primary mouse Schwann cells is best on collagen coated plates. Therefore, extracted collagen was diluted 1:200 in sterile 0.1% acetic acid and cell culture plates were covered with the diluted collagen for 30 minutes. After 30 minutes the collagen solution was aspirated and the plates were air dried. The plates were used directly or stored at 4°C (max. 24 h).

2.3.3.3 Primary Schwann cell cultures (pSC)

Primary adult Schwann cell cultures were prepared from peripheral nervous tissue from 6 months old mice. The mouse line S100β-eGFP crossbred with *gdf15-/-* mouse line was used. Therefore, both sciatic nerves were extracted and the epineurium was removed thoroughly under a stereomicroscope (StemiSV6, Zeiss) to reduce fibroblast contaminations. The use of fresh tissue results in poor Schwann cell yields. Therefore, extracted and purified sciatic nerve tissue was incubated for 10 days in 10% FCS, 1% PenStrep in D-MEM (Gibco) at 37°C and 5% CO_2. During the 10 days incubation, an *in vitro* pre-degeneration takes place which allows for the Wallerian degeneration process to begin. During a Wallerian degeneration, necrotic tissue and myelin debris are removed by macrophages and Schwann cells. Therefore, Schwann cells start to proliferate to form bands of Bungner. After 10 days, the pre-degeneration was stopped by dissociation of the nerves in pSC dissociation solution for 6 hours at 37°C and 5% CO_2. Tissue residues were mechanically separated into single cells by gentle trituration with a 1000 µl Variopipette. Cells were seeded into collagen (1:200) coated 12-wells plates with pSC culture medium for 48 hours (Figure 2.2, A´-A´´´´). Even so, the epineurum was thoroughly removed, a small number of fibroblasts were still present and Schwann cell cultures had to be purified. The used purification method is based on the different attachment properties of adult peripheral nerve cells. Fibroblasts grow mainly flat at the cell culture surface whereas Schwann cells grow on top of the fibroblasts. When the cells were covered with 500 µl 1xTrypsin-EDTA, Schwann cells detached easily within 1 minute which was observed through an Inversemicroscope (Axiovision, Zeiss). The reaction was directly stopped by adding the same amount of 100% heat-inactivated FCS. Plates with remaining fibroblasts were discarded (Figure 2.2, B´-B´´´´). The purified Schwann cells were seeded into new collagen coated 12-well plates and the purification was repeated in total three times with a time distance of 48 hours (Figure 2.2, C;D).

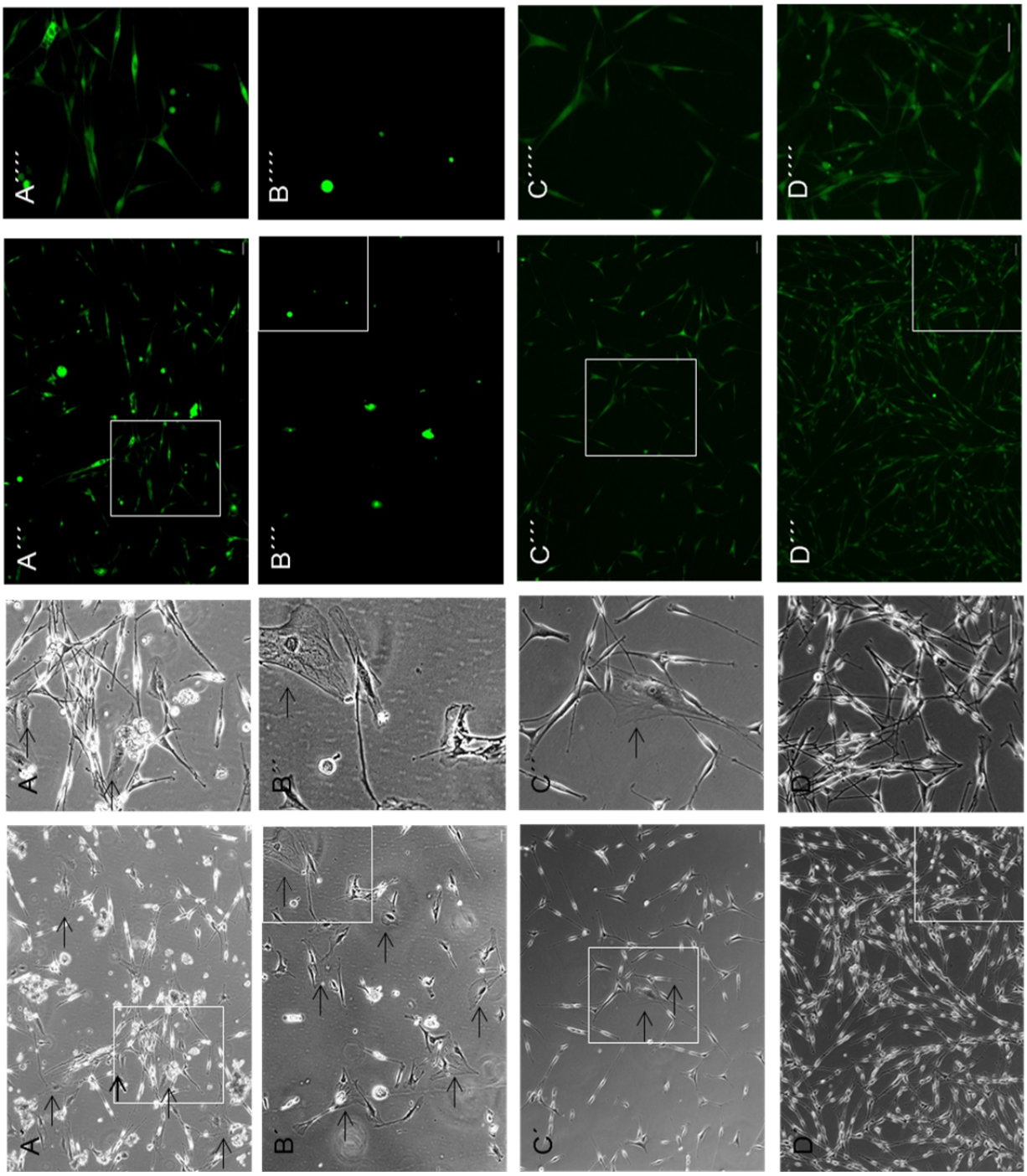


Figure 2.2: Purification of primary Schwann cells by trypsinisation leads to highly pure cultures. **A´)** 48 hours after plating of the predegenerated and dissociated nerves, the cultures consist of mostly Schwann cells and some Fibroblasts (black arrows). **A´´)** enlarged part of A´. **A´´´)** Schwann cell marker S100β is represented by eGFP expression. **A´´´´**) enlarged part of A´´´. **B´-B´´´´**) After trypsinisation most of the cells still attached to the plate are fibroblasts. **C´-C´´´´)** After one purification primary Schwann cell cultures only contain a few fibroblasts. **D´-D´´´´)** After three purification steps (8 days in culture), Schwann cells are ~95 % pure. scale bar: 50µm

2.3.3.4 Cell lines used

The cell lines MCF-7 (ATCC® HTB-22™) and RT4-D6P2T (ATCC® CRL-2768™) were used. The MCF-7 is a human cancer cell line, isolated from an adenocarcinoma. The RT4-D6P2T cell line is a schwannoma cell line derived from a rat peripheral neurotumor. MCF-7 cells are adherent and were cultivated in D-MEM, 10% heat-inactivated FCS and 1% PS at 37°C and 5% CO_2. 2 mM Glutamine was added to the medium when used for RT4-D6P2T. Both cell lines were splitted when reached a confluence of ~80% by a ratio of 1:5. For longer storage periods, cell lines were stored at -196°C in a liquid nitrogen tank. For freezing of the cells, 5% DMSO was added to the normal growth medium.

2.3.3.5 Proliferation assay by FACS analysis

The proliferation rate of primary Schwann cells was determined by a Fluorescent associated cell sorting (FACS) analysis with the BD Pharmingen™ BrdU Flow Kit. Bromodeoxyuridine (BrdU) is incorporated into newly synthesized DNA in cells, which are in the S-phase. Therefore, 500 000 Schwann cells were plated out in Melanocyte Growth Medium, 5% FCS and 1% PS and afterwards, cultivated with 1 mM BrdU (10 µl BrdU solution/ml medium) for 72 hours. As a control experiment, one sample was cultivated with PBS instead of BrdU. The cells were trypsinized after 72h and stained according to the user manual. After fixation and permeabilisation, the cells were stored in 1% FCS in PBS at 4°C until further use. Before the cells were analyzed by FACS they were stained with a specific APC conjugated Antibody according to the manufactures guide.

In a FACS analysis, cells are incorporated into a small liquid stream and by illumination with a laser beam, fluorescent light from positive cells give an electric signal. The amount of all cells and fluorescent positive cells respectively are measured. A FACS Calibur flow cytometer (BD Biosciences) was used and the FACS analyses were done by supervision of Dr. H. Peterziel at the german cancer research center (DKFZ). Quantification of data was done by the use of the FlowJo software.

2.3.3.6 Apoptosis assay by FACS analysis

Apoptosis is characterized by energy-dependent mechanisms and morphological changes including loss of membrane integrity. The translocation of the membrane phospholipid phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane is one of the earliest morphological changes that take place during apoptosis. The phospholipid binding protein Annexin V which can be conjugated to fluorochromes as APC binds to cells with exposed PS and early apoptotic stages can be determined by FACS. To differentiate between early and late apoptotic or necrotic stages, the cells of interest were counter stained with a viable dye, 7-AAD (7-Amino-Actinomycin). Cells positive only for Annexin V are early apoptotic and cells positive for both, Annexin V or 7-AAD are late apoptotic, already dead or necrotic. Thus, the staining with both allows a distinction between apoptosis and necrosis.

Therefore, 500 000 primary Schwann cells in Melanocyte Growth Medium, 5% FCS and 1% PS were plated into one well of a 6-well cell culture plate coated with collagen. After 72 hours under normal growth conditions with BrdU or as control without BrdU the medium was transferred to 15 ml Falcon tubes and the primary Schwann cells were rinsed with 1x Annexin V binding buffer (BD Biosciences). Primary Schwann cells were removed from the plates by adding 500 µl of Enzyme Free Cell Dissociation Solution (Millipore) for 10 minutes followed by a rinsing step of the wells with 1x Annexin V binding buffer. The cell solutions were centrifuged for 5 minutes. The pellets were suspended in 1 ml 1x Annexin V binding buffer and transferred to 1.5 ml reaction tubes. Before the staining with APC Annexin V and 7-AAD (BD Biosciences), the cell solutions were centrifuged and the pellets suspended in 100 µl 1x Annexin V binding buffer with 5 µl 7-AAD and 2.5 µl APC Annexin V.

To ensure that the staining is specific and to adjust the FACS analysis correctly, 5 different controls were needed.

Therefore, 3 wells with primary Schwann cells were killed by treating the cells with 0.8 mM H_2O_2 for 6 hours. Primary Schwann cells, treated with H_2O_2 for 6 hours were in a late apoptotic stage and were both, 7-AAD and APC Annexin V positive when stained with both.

Two samples, one with induced Apoptosis were only stained with 100 µl 1x Annexin V binding buffer and 5 µl 7-AAD, two samples, one with induced Apoptosis were only

stained with 100 µl 1x Annexin V binding buffer and 2.5 µl APC Annexin V and the last control, with induced Apoptosis was stained with 100 µl 1x Annexin V binding buffer with 5 µl 7-AAD and 2.5 µl APC Annexin V.
A FACS Calibur flow cytometer (BD Biosciences) was used and the FACS analyses were done by supervision of Dr. H. Peterziel at the DKFZ.

2.3.3.7 Migration assay

The used migration assay is based on the ability of cells to aggregate, to from spheres and on the space needed to grow. Primary Schwann cell spheres were formed by the hanging drop technique and plated onto collagen coated cell culture plates. One hour after plating out the spheres, a serial documentation of the spheres were started.
First, hanging drop cultures were prepared by placing 5000 primary Schwann cells in 15 µl Melanocyte Growth Medium, 5% FCS and 1% PS on the inside of the lid of a 60mm culture plate. The bottom was filled with 5 ml autoclaved distilled water to avoid desiccation of the Schwann cell solution. By transferring the lid onto the bottom plate, hanging drop cultures were achieved. Some cultures were treated with recombinant GDF-15 (rGDF-15; 100 ng/ml) or with an ErbB2 inhibitor (working concentration: 0.8 ng/ml) respectively.
The hanging drop cultures were incubated for 48 hours under normal growth conditions before one drop (one sphere) was transferred into one well of a 48 well plate coated with collagen. One hour after plating out the spheres the plate was transferred to a Zeiss LSM with an incubation chamber and a serial documentation for 36 hours was started. The area of the spheres was quantified after 0, 12, 24 and 36 hours by the use of the area tool of the Fiji Image analysis software. In each experiment, at least 4 spheres per genotype and condition were analyzed.

2.3.3.8 Treatment of cell lines and primary Schwann cell cultures with recombinant GDF-15 and/or NRG1 Type III

MCF-7 and RT4-D6P2T cell were used for a receptor study. Therefore, they were starved overnight. The serum concentration was reduced to 0.1%. Cells were treated with 100 ng/ml recombinant GDF-15 or Neuregulin 1 type III for 5 minutes before addition of 2xLaemmli. Protein lysates were than used for Western blot analysis.

2.3.4 Histological methods

2.3.4.1 Perfusion of mice

For the preparation of some histological slices, the fixation of the tissue of interest is needed. The most effective fixing method is the rinsing of the bloodstream of a narcotized mouse with freshly prepared 4% Paraformaldehyde (PFA) in 0.1 M Phosphate buffer.

Therefore, mice were transferred to a chamber with a few drops of Isofluran until the mice started gapping. The mice were removed from the chamber and an anesthesia mask was put on over mouth and nose. After loss of the toe pinch response, mice were placed on a rack in a dorsal decubitus position. The body cavity was opened by incisions along the Linea Alba and towards the distal end of the forelimbs. The thorax was opened by cutting the diaphragm laterally at both sides and the ribs towards the head. The sternum was lifted until the heart was easy to access. A clamp was put on the tip of the left ventricle and the incarcerated part of the left ventricle was incised. A blunt cannula of the infusion set was placed into the incision. The right atrium was snipped and the blood stream was perfused with PBS until the liver and kidneys were anemic. Afterwards, the mice were fixed with 4% PFA for 10 minutes. The whole fixated animals were used for tissue extraction.

2.3.4.2 *In vivo* Schwann cell quantification

The femoral nerves were removed from *gdf15*+/+ and *gdf15*-/- mice of different ages. Mice in the age of 3, 6 and 12 month were perfused with 4% PFA before the femoral nerves were removed. Mice of the age of 5 days were killed, pinned on a cork plate

and the femoral nerves were exposed (Figure 2.2). The mice were then, still pinned on the cork plate transferred into 4% PFA. After an overnight incubation, the femoral nerves were removed and as well as the adult femoral nerves post-fixed with 2% glutaraldehyde in 0.1 M sodium cocadylat buffer.
For the preparation of semi-thin sections the femoral nerves were embedded in an epoxy resin, Epon. The tissue embedding and the semi-thin sections were developed by Barbara Brühl according to the following embedding protocol:

• Rinsing with 0.1 M Cacodylat buffer, pH 7.2	6 x	10 min
• Post-fixation with 1% Osmium tetraoxide and 1.5% Potassium ferrycyanide	1 x	60 min
• Rinsing with 0.1 M Cacodylat buffer, pH 7.2	3 x	10 min
• Rinsing with Sodium-maleate buffer, pH 6.0	3 x	10 min
• Block staining in 1% uranyl acetate in Sodium-maleate buffer, pH 6.0	1 x	90 min
• Rinsing with Sodium-maleate buffer, pH 6.0	3 x	10 min
• Dehydration in graded ethanol (30% - 50% - 70% - 90% - 96% - 99%)	each 2x	5 min
• Dehydration in absolute Ethanol	4 x	15 min
• Propylene oxide as intermediary	2 x	5 min
• Propylene oxide + Epon mixture (1:1)	1 x	60 min
• Penetration with Epon	1 x	over night
• Embedding in freshly prepared Epon		
• Polymerisation of the Epon	60°C	24 h

Serial semi-thin sections with a distance of 20 µm were prepared at an Ultramicrotom (Leica) and stained with filtered Toluidine Blue. Images were taken at a Nikon Ni-E research microscope at the Nikon imaging centre (Bioquant Heidelberg).

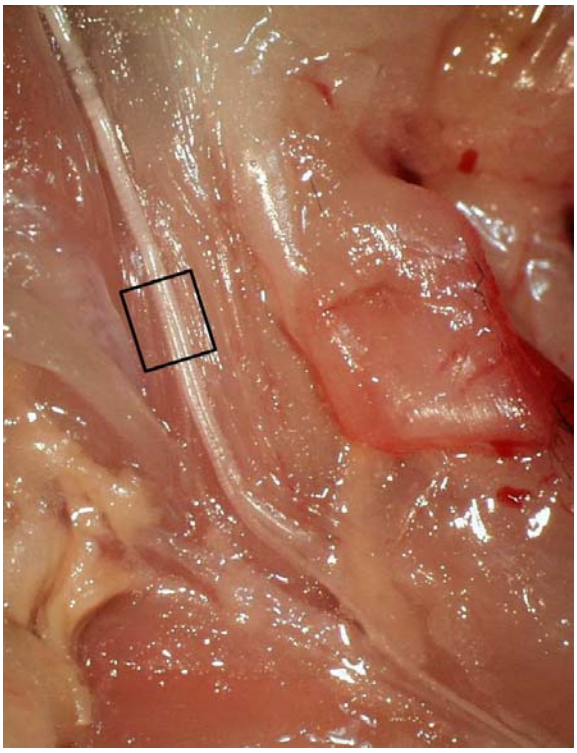


Figure 2.3. Perfused mice with exposed femoral nerve. The square marks the area used for serial semi-thin sections.

2.3.4 Statistical Analysis

Data analysis was done in Microsofts Excel 2010 and GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). Datasets with Gaussian distribution were subjected to a two-tailed unpaired t-test. For non-Gaussian distributions, between-group comparisons were made using Tukey's Multiple Comparison Test. P-values <0.05 were taken as statistical significant.

Chapter 3: Results

3.1 Phenotype analysis of *gdf15-/-* mice

Studies in our laboratory have shown that GDF-15 deficiency leads to a progressive loss of facial, spinal and trigeminal motoneurons, reaching a maximum of ~20% at 6 months and is accompanied by a loss of facial axons (Strelau, Strzelczyk et al. 2009). Because neuronal death and axon degeneration in debilitating diseases (including multiple sclerosis and Charcot-Marie-Tooth peripheral neuropathies) result from disruption of myelin, I started to address the question of whether myelin-forming Schwann cells have an impact on neuron loss in absence of GDF-15. Therefore, Schwann cell numbers in peripheral nerve were quantified and some genes of interest were investigated.

3.1.1 Schwann cell numbers are reduced in adult peripheral nerves

To prove the hypothesis that GDF-15 deficiency affects Schwann cells, Schwann cell numbers in *gdf15*+/+ and *gdf15-/-* mice were analyzed in a peripheral nerve. The femoral nerve possesses motor and sensory functions, whereas the sensory part splits off as the saphenous nerve. The femoral nerves of P5, 3.5, 6 and 12 months old *gdf15*+/+ and *gdf15-/-* mice were used for the preparation of serial semi thin sections (Figure 3.1).

The highest number of Schwann cells was counted in P5 old mice, when the myelination of the peripheral nerves is still at the beginning. A trend towards a reduced Schwann cell number in *gdf15-/-* mice starts at 3.5 months but at this time point, the difference did not reach significance. A significant Schwann cell reduction can be seen from 6 months (*gdf15-/-*: 68.87%) becoming more severe after 1 year (*gdf15-/-*: 47.31%) (Figure 3.2). Thus, GDF-15 deficiency leads to a Schwann cell loss in adult femoral nerves.

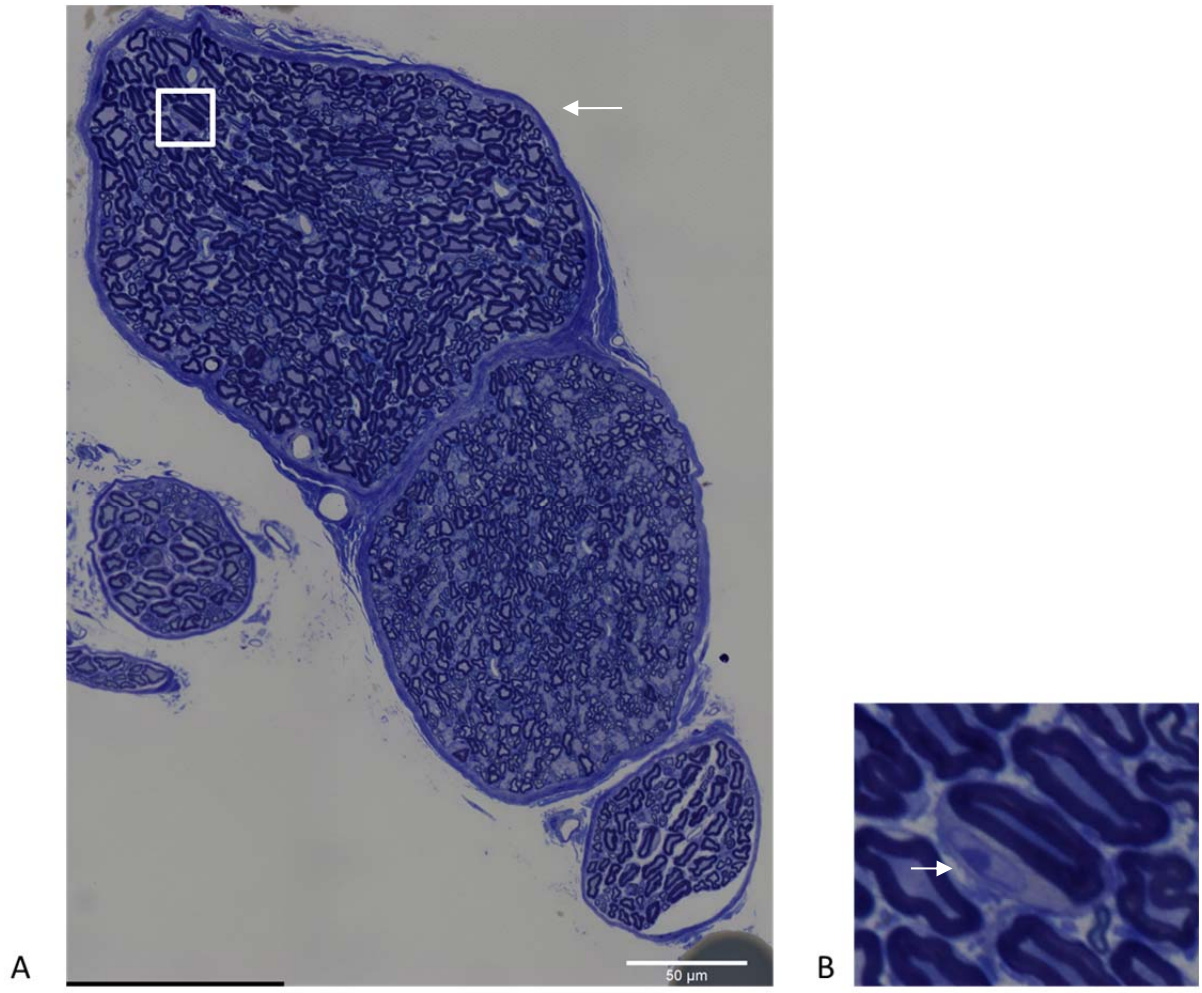
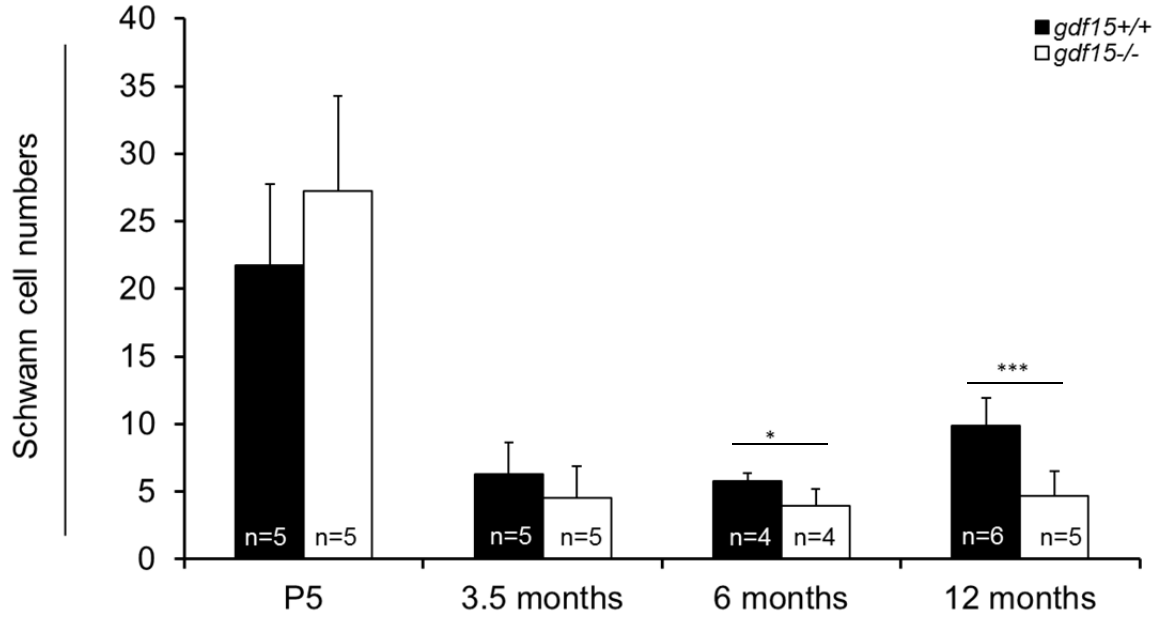


Figure 3.1: **A)** Picture of 6 months old femoral (white arrow) and saphenous nerves which are clearly separated by the epineurium. **B)** Enlarged part from panel A (white square) in which a myelinated axon with a Schwann cell nucleus (white arrow) is shown.



	P5	*s.e.m.*	***3.5 months***	*s.e.m.*	***6 months***	*s.e.m.*	***12 months***	*s.e.m.*
gdf15+/+	*21.75*	*2.45*	*6.27*	*1.06*	*5.75*	*0.32*	*9.87*	*0.60*
gdf15-/-	*27.24*	*3.14*	*4.54*	*1.05*	*3.96*	*1.23*	*4.67*	*0.83*
significance	*p>0.05*		*p>0.05*		*p=**		*p=****	

Figure 3.2: upper panel: Quantification of Schwann cell numbers in femoral nerves from mice of different ages and genotypes (black bars: *gdf15+/+*, white bars: *gdf15-/-*) show a significant reduction of Schwann cells in *gdf15-/-* mice starting at 6 months (student´s two-tailed T-test: *: p< 0.05, ***: p<0.001)

lower panel: Data of the SC quantification with s.e.m. and student´s two-tailed t-test (*: p< 0.05, **: p<0.01, ***: p<0.001)

3.1.2 *In vivo* gene expression study: Analysis of Neuregulin, ErbB2, Hmgcr, NDRG1 and IL-6

Myelination and maintenance of myelin sheath is essential for a functional nervous system. In the past few years, several factors involved in myelination have been discovered, but its complexity and its maintenance is still not completely understood. Here, several factors were analyzed *in vivo* that have been known to be involved in hyper- or hypomyelination or in myelin defects. Therefore, gene expression in different tissues such as sciatic, femoral and facial nerves and spinal marrow (n=3) was analyzed by quantitative real-time PCR.

3.1.2.1 GDF-15 deficiency has no effect on the Neuregulin 1 type III expression in nerve tissue

Neuregulin is a survival factor of Schwann cells during development, and it regulates myelin thickness. Nrg1 deficiency leads to significant hypomyelination, while Nrg1 overexpression leads to hypermyelination. Schwann cell specific deletion of *Nrg1* leads to hypomyelination (Garratt, Voiculescu et al. 2000; Michailov, Sereda et al. 2004; Chen, Velardez et al. 2006). The threshold level of Neuregulin is higher in myelinated nerves than in unmyelinated (Michailov, Sereda et al. 2004). Furthermore, Neuregulin enhances nerve regeneration. Neuregulin 1 type III overexpression allows remyelinated axons to regain normal myelin thickness (Stassart, Fledrich et al. 2013), similar to GDF-15 submission after nerve injury. It was shown that injured nerves treated with GDF-15 regenerated axons had a better quality. The myelination was better than in untreated animals (Mensching, Borger et al. 2012).
Neuregulin 1 type III expression was highest in spinal marrow. However, there was no detectable difference between wild type and mutant tissue (Fig.3.3).

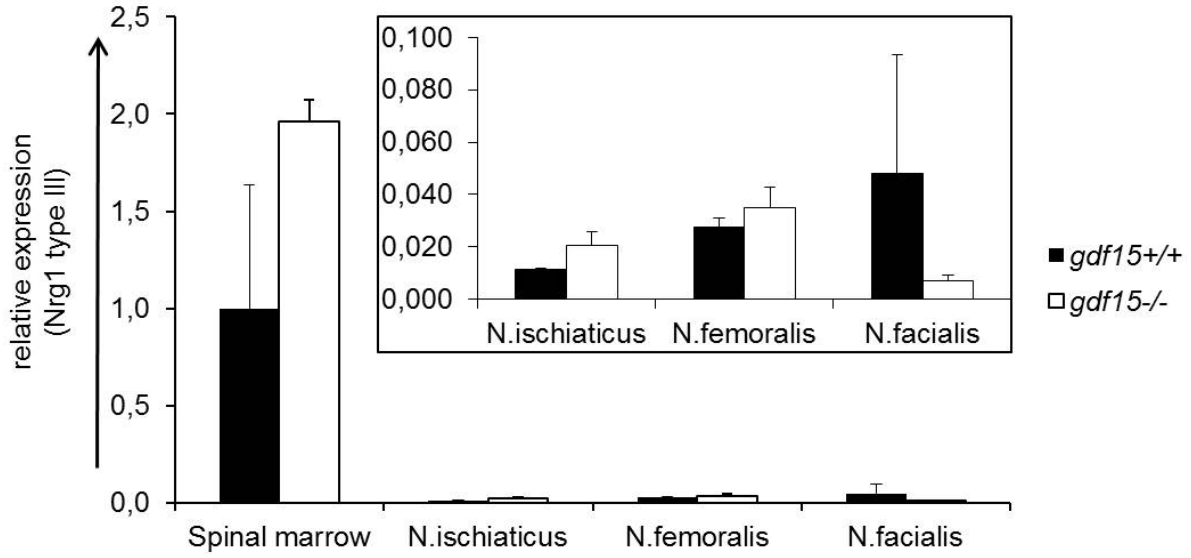


Figure 3.3: Expression of Neuregulin 1 type III in different tissues of 6 months old *gdf15*+/+ (black bars) and *gdf15*-/- (white bars) mice (n=3). The box shows an enlarged graphic of the Neuregulin expression in peripheral nerves. The *gdf15*+/+ spinal marrow was set to 1 as calibrator. Error bars indicate s.e.m.

3.1.2.2 GDF-15 deficiency increases the expression of ErbB2 in spinal marrow but has no effect on nerve tissue

In this regard, the ErbB2 receptor is of interest. It is known that ErbB2 signaling plays a key role in myelination (Pertusa, Morenilla-Palao et al. 2007; Perlin, Lush et al. 2011; Ma, Zhang et al. 2012; Chang, Shyu et al. 2013). Nrg signals through ErbB2 and Nrg/ErbB2 signaling regulates HmgCr expression on a transcriptional level (Pertusa, Morenilla-Palao et al. 2007). There are also indications that GDF-15 signals through ErbB2 in different cancer cell lines (Kim, Lee et al. 2008; Park, Lee et al. 2010; Joshi, Brown et al. 2011).

Interestingly, ErbB2 was highest expressed in the femoral nerve, but a difference between *gdf15*+/+ and *gdf15-/-* was only existent in the spinal marrow. It is significantly increased in *gdf15-/-* tissue (Fig.3.4).

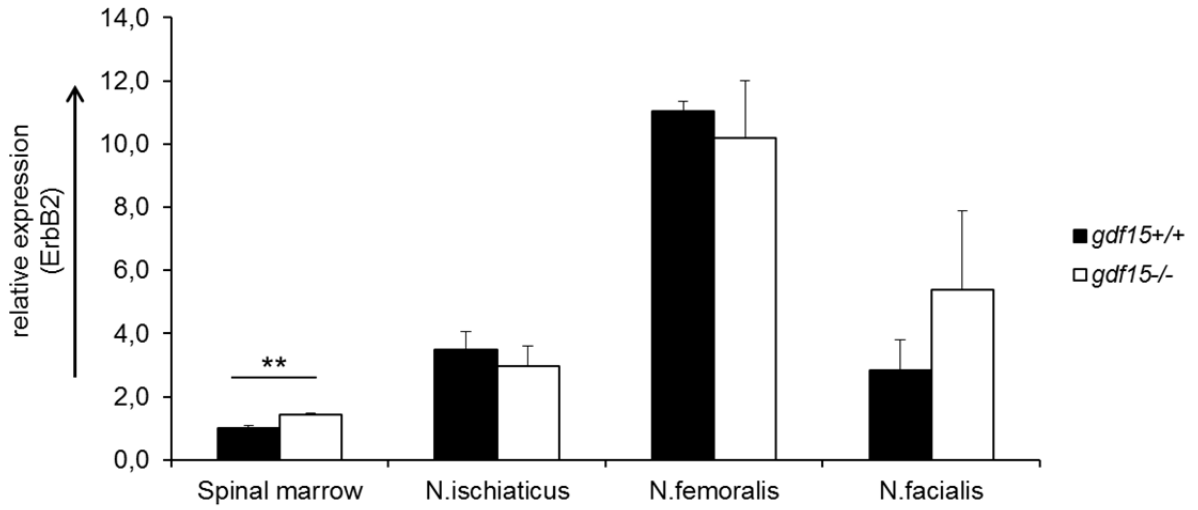


Figure 3.4: Nerves, especially the femoral nerve, express more ErbB2 than spinal marrow. But a difference between *gdf15*+/+ and *gdf15-/-* mice was only seen in the spinal marrow. ErbB2 expression is increased in the spinal marrow of *gdf15-/-* mice. The *gdf15*+/+ spinal marrow was set to 1 as calibrator. Error bars indicate s.e.m. (student´s two-tailed T-test **: p< 0.01)

3.1.2.3 GDF-15 deficiency has no effect on the Hmgcr expression in nerve tissue

Neuregulin, signaling through ErbB2, regulates myelin thickness by regulating the expression of 3-hydroxy-3-methyl-glutaryl-CoA reductase (encoded by *Hmgcr*). This factor is the rate-limiting step in cholesterol synthesis, and the importance of a functional cholesterol homeostasis on the myelination has been shown in several publications over the last years (Pertusa, Morenilla-Palao et al. 2007; Saher, Quintes et al. 2009).

Hmgcr expression level was highest in spinal marrow, and the lowest expression level was measured in sciatic and facial nerves. However, no significant differences were observed by comparing wild type and mutant mice (Figure 3.5).

Thus, it seems that GDF-15 deficiency has no effect on Hmgcr expression in nerve tissue *in vivo.*

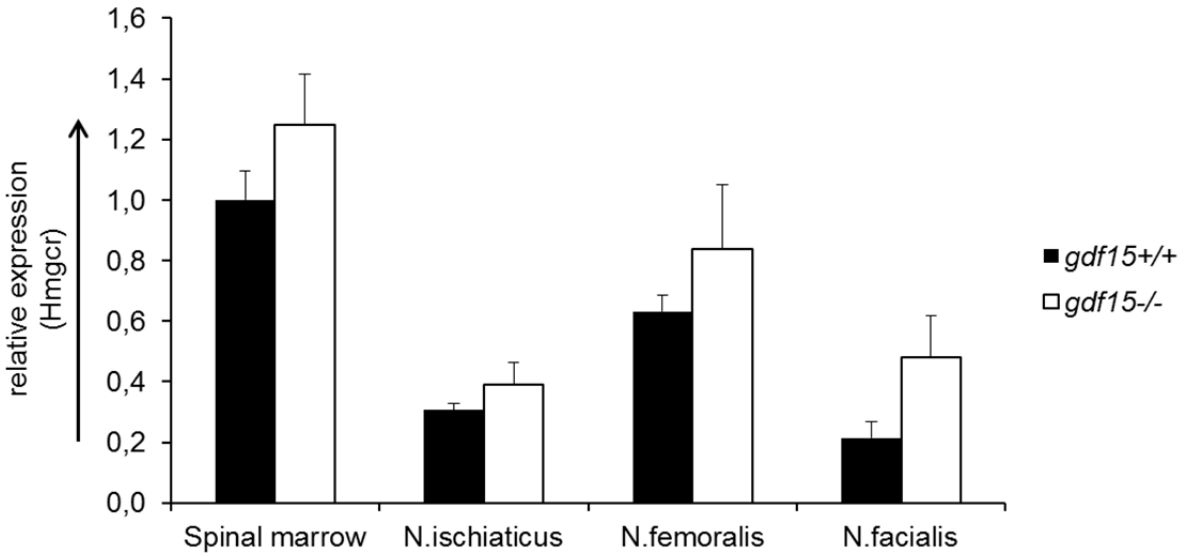


Figure 3.5: Expression of Hmgcr in different tissues of 6 months old *gdf15*+/+ (black bars) and *gdf15* -/- (white bars) mice (n=3). The *gdf15* +/+ spinal marrow was set to 1 as calibrator. Error bars indicate s.e.m.

3.1.2.4 GDF-15 deficiency has no effect on the NDRG1 expression in nerve tissue

N-myc downstream-regulated gene 1 (NDRG1) has been associated with the Charcot-Marie Tooth disease type 4D (CMTD-T4D), a myelination disorder (Kalaydjieva, Gresham et al. 2000). Furthermore, NDRG1 deficiency leads to both a defect in myelin maintenance and a Schwann cell dysfunction (Okuda, Higashi et al. 2004) and most interestingly GDF-15 overexpression induces an upregulation of NDRG1 expression in cancer cells (Tsui, Chang et al. 2012).

In this experiment, kidney tissue was also analyzed because NDRG1 is highly expressed in kidney tissue and GDF-15 levels are increased during chronic kidney disease (Breit, Carrero et al. 2012).

NDRG1 expression was as previously shown highest in kidney and femoral tissue. The lowest expression is in the spinal marrow. However, no significant differences were observed by comparing wild type and mutant mice (Figure 3.6).

Thus, it seems that GDF-15 deficiency has no effect on NDRG1 expression in nerve tissue *in vivo.*

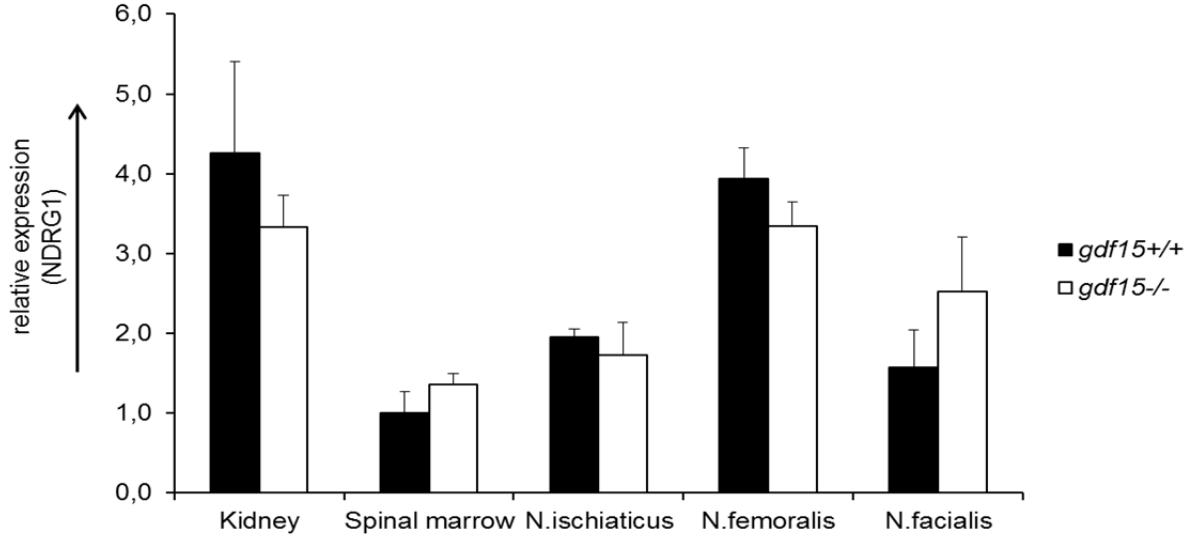


Figure 3.6: Expression of NDRG1 in different tissues of 6 months old *gdf15*+/+ (black bars) and *gdf15* -/- (white bars) mice (n=3). The *gdf15* +/+ spinal marrow was set to 1 as calibrator. Error bars indicate s.e.m.

3.1.2.5 GDF-15 deficiency leads to an increase of IL-6 in sciatic and femoral nerves *in vivo*

GDF-15 is a stress-responsive cytokine, and several publications over the last years have shown a correlation between GDF-15 and Interleukin-6 (IL-6) (Bonaterra, Zugel et al. 2012; Tsui, Chang et al. 2012). IL-6 is an important cytokine in the immune response (Naugler and Karin 2008) and in peripheral nerve injuries Schwann cells play an important role in the immune response. Therefore, it was tested if GDF-15 deficiency leads to a defect in Schwann cells and/or nerve immune responses by regulating IL-6 expression.

IL-6 expression was highest in spinal marrow but differences between *gdf15*+/+ and *gdf15-/-* were only seen in sciatic and femoral nerves. The IL-6 expression was significantly increased in *gdf15-/-* sciatic and femoral nerves. Thus, GDF-15 deficiency increases IL-6 expression in sciatic and femoral nerves, indicating an altered immune response *in vivo* (Figure 3.7).

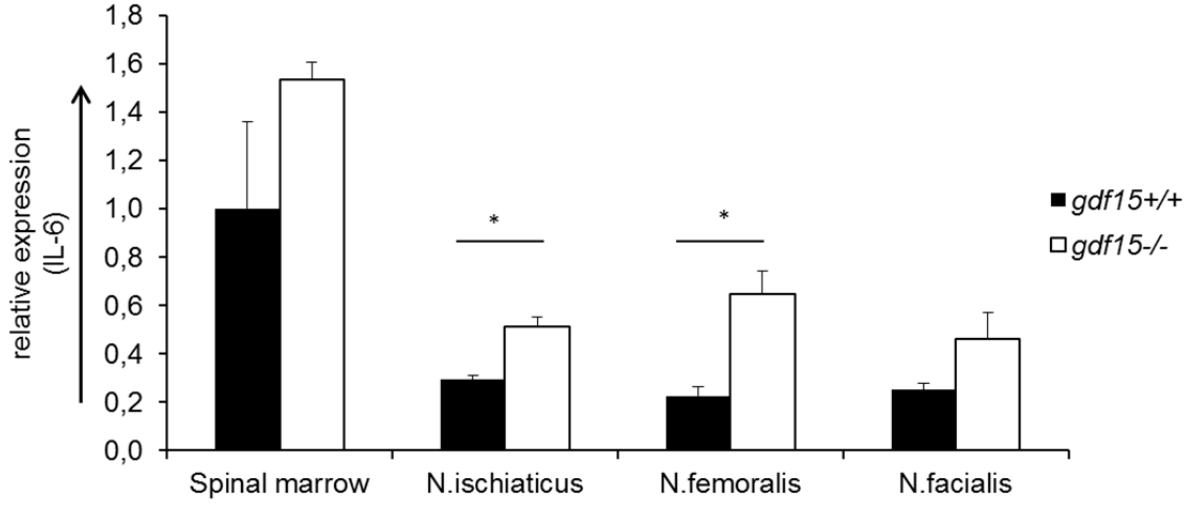


Figure 3.7: Expression of IL-6 in different tissues of 6 months old *gdf15*+/+ (black bars) and *gdf15-/-* (white bars) mice (n=3). The *gdf15*+/+ spinal marrow was set to 1 as calibrator. Error bars indicate s.e.m. (student´s two-tailed T-test *: p< 0.05)

3.2 *In vitro* gene expression study: *gdf15* knockdown in the Schwann cell line RT4-D6P2T and its effect on IL-6 and NDRG1 expression levels

By analyzing gene expression from nerve tissues, different cell types are analyzed and furthermore, the effect of GDF-15 deficiency on the expression level may no longer be measurable anymore. Therefore, a siRNA GDF-15 knockdown was induced in pure Schwann cells, the Schwann cell line RT4-D6P2T and gene expression of GDF-15, IL-6 and NDRG1 was measured 24h post transfection. In a publication from 2002, Hai et al. analyzed several Schwann cell lines for their myelin gene expression patterns and the RT4-D6P2T as well as the rat derived cell line S16 were most suitable (Hai, Muja et al. 2002).

3.2.1 Efficient *gdf15* knockdown by using two different siRNAs

Three different siRNA knockdown experiments with duplicates were done. RT4-D6P2T cells were transfected with a negative control siRNA and two different siRNAs called gdf-2 and gdf-3. As controls, untreated RT4-D6P2T cells and RT4-D6P2T transfected with an unspecific siRNA (negative control) were used. As GDF-15 is a stress-responsive cytokine, its expression was increased due to the stress that cells undergo during transfection. Nevertheless, the use of the siRNA gdf-3 and the combination of the siRNAs gdf-2 and gdf-3 induced a significant knockdown of GDF-15 expression 24h post transfection (Figure 3.8). Thus, RT4-D6P2T cells with siRNA induced GDF-15 knockdown are suitable as *in vitro* model system for gene expression analysis.

3.2.2 siRNA induced *gdf15* knockdown has no effect on IL-6 or NDRG1 expression

Even so, a strong and significant *gdf15* knockdown was induced by transfection with siRNAs; it had no effect on the expression of NDRG1 or IL-6.
The data gained from three independent siRNA knockdowns were similar, concerning the IL-6 expression. But by comparing all three experiments together and by

analyzing each experiment individually no significant differences were induced by GDF-15 knockdown.

Considering the NDRG1 expression, the data set from experiment 1 and 2 were similar but in the data set from experiment 3, the NDRG1 expression was greatly increased. However, by comparing the data from each experiment individually, no significant differences could be observed (Figure 3.8).

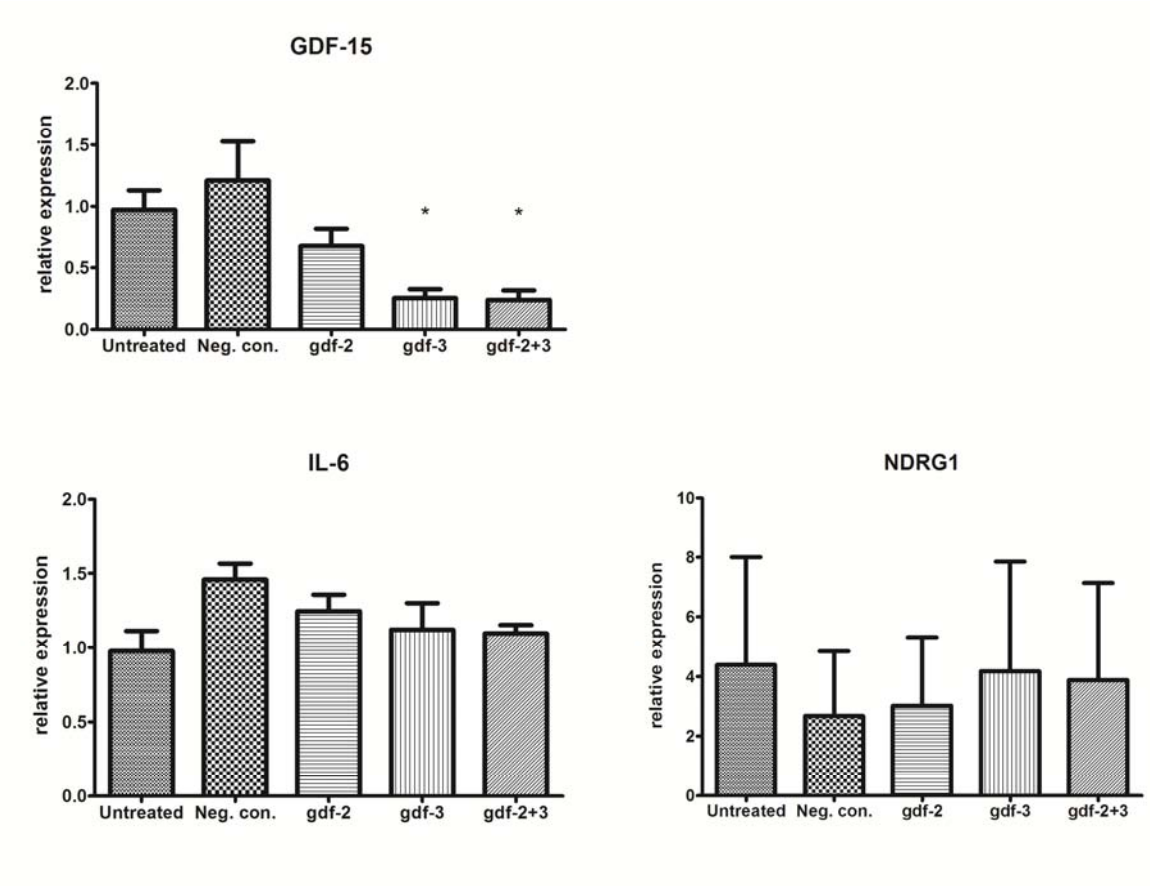


Figure 3.8: Upper panel: GDF-15 expression in untreated RT4-D6P2T cells and in siRNA transfected RT4-D6P2T cells. The transfection with an unspecific siRNA induced an increase in GDF-15 expression, but the GDF-15 expression was still significantly knocked down by the use of the siRNA gdf-3 and the combination of siRNAs gdf-2+3 (n=3). The untreated cells were set to 1 as calibrator. Error bars indicate s.e.m. (Tukey's Multiple Comparison Test: *: $p<0.05$).

lower Panel: *gdf15* knockdown has no effect on the expression of IL-6 and NDRG1 (n=3). Error bars indicate s.e.m.

3.3. Preparation and functional studies of adult mouse primary Schwann cell cultures

To further elucidate possible dysfunctions that led to a Schwann cell loss in GDF-15 mutant mice, I studied proliferation, apoptosis and migration of primary Schwann cells *in vitro*.

3.3.1 Predegeneration of adult sciatic nerves and purification by trypsinization leads to pure mouse adult primary Schwann cell cultures

First, primary Schwann cell cultures from mouse adult nerves were needed. Normally, Schwann cell cultures are prepared from sciatic nerves from neonatal animals, mostly rats. In the past few years, publications have shown preparations of adult rat or human Schwann cells (Haastert, Mauritz et al. 2007). Nevertheless, adult mouse primary Schwann cell cultures were needed and this method had to be established.

The method used here is based on the principle of the Wallerian degeneration and on the different attachment properties of fibroblasts and Schwann cells on coated culture plates. A detailed description of the established method can be found in chapter 2.3.3.3.

FACS analysis of 33 different Schwann cell cultures showed a Schwann cell purity of ~95% (Figure 3.9).

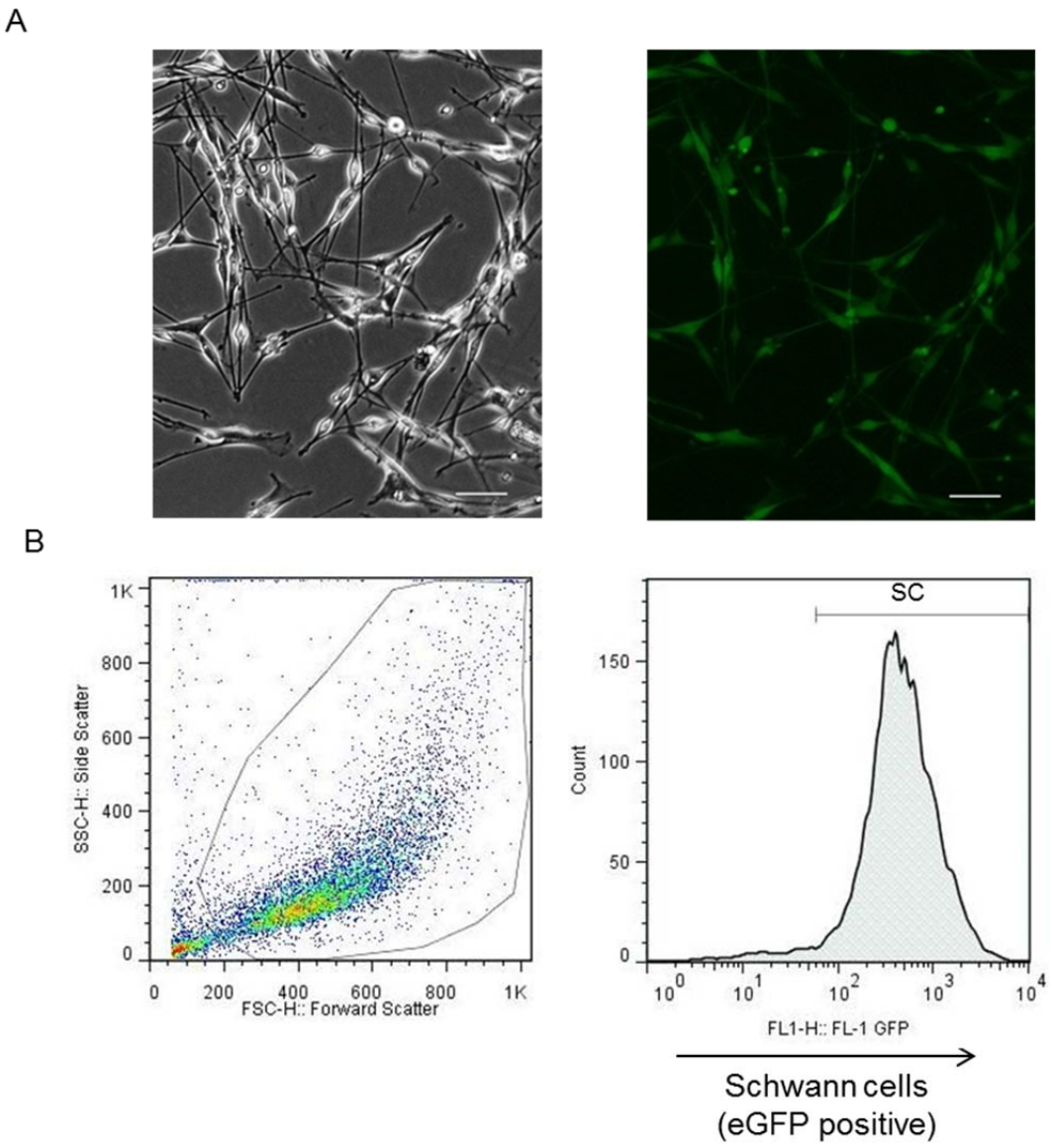


Figure 3.9: **A)** Schwann cell culture after 3 purification steps. No fibroblasts were remaining. Scale bar: 50µm **B)** 33 mouse adult primary Schwann cell cultures were tested. They have a purity of ~ 95% (s.e.m.: 0.79). In the example histograms, analysis of a Schwann cell culture is shown. The Schwann cell/fibroblast population was marked (left, 85%) and 96.1% were Schwann cells (eGFP positive).

3.3.2 Functional studies: Schwann cell proliferation, apoptosis and migration in vitro

Our previous data have shown that GDF-15 deficiency led to a significant decrease of Schwann cells *in vivo*. Schwann cell numbers are regulated by proliferation and apoptosis as well as their migratory potential, and members of the transforming growth factor-β (TGF-β) superfamily have been demonstrated to control these functions (Parkinson, Bhaskaran et al. 2004; Jessen and Mirsky 2005; Ribeiro-Resende, Koenig et al. 2009). Thus, it is reasonable to hypothesize that GDF-15 might affect Schwann cell numbers by regulating proliferation, death and/or migration. In this context, I analyzed primary Schwann cell cultures derived from wild type and GDF-15 mutant mice.

3.3.2.1 GDF-15 deficiency has no effect on cell cycle progression in primary Schwann cell cultures

During Schwann cell development, it has been shown that TGF-βs affect Schwann cell proliferation *in vivo (Jessen and Mirsky 2005)*. To analyze GDF-15 dependent cell cycle progression I started to evaluate BrdU incorporation into newly synthesized DNA (Figure,3.10, A) and DNA stained with 7-AAD (Figure 3.10, B).

In average, 5.17% *gdf15*+/+ Schwann cells and 4.87% *gdf15-/-* Schwann cells were BrdU positive and therefore, went through S-Phase of the cell cycle within 48h. However, the number of proliferating wild type and mutant Schwann cells was not different (Figure 3.10 A).

An investigation of the cell cycle by assessing 7-AAD staining (Figure, 3.10 B) revealed that, only two peaks could be seen clearly: one at around 200 representing cells in the G1/G0 phase and one at around 0, representing cells undergoing apoptosis (Sub-G1 peak). Cells in S, G2 or M phase are hard to distinguish and are referred to as S-G2-M. All cells behind the G1 peak are in S-G2-M. Cell cycle analysis confirmed that most cells are not entering cell cycle. However, an analysis of the data revealed a significant increase in the Sub-G1 peak in mutant Schwann cells, thus indicating an increase in apoptosis.

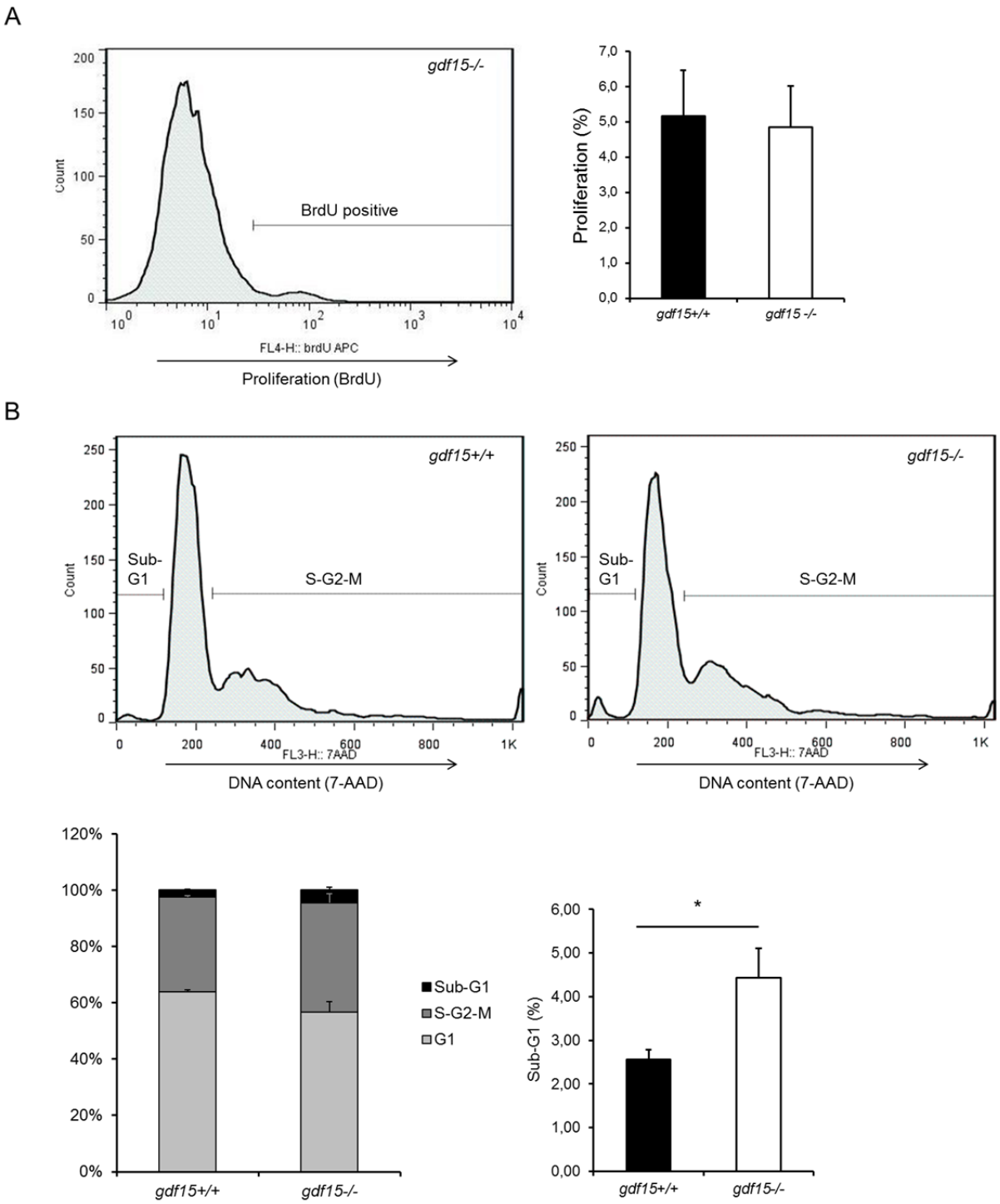


Figure 3.10: *gdf15*+/+ and *gdf15*-/- Schwann cells show no difference in their cell cycles **A)** Incorporation of BrdU into newly synthesized DNA did not show a difference in proliferation rates in *gdf15*+/+ (black bars) and *gdf15*-/- (white bars) Schwann cells. A measurement histogram is shown as an example in the left panel. **B)** Cell cycle analysis of *gdf15*+/+ and *gdf15*-/- Schwann cells based on the 7-AAD staining did not show a difference. However, an increase in the Sub-G1 peak was observed in the *gdf15*-/- Schwann cells indicating an increased apoptosis rate.

Data are shown from 5 independent experiments (student´s two-tailed T-test: *<0.05). Error bars indicate s.e.m.

3.3.2.3 GDF-15 deficiency leads to an increased apoptosis in Schwann cells *in vitro* and recombinant GDF-15 promotes Schwann cell survival

To further elucidate the increased Sub-G1 peak representing cells undergoing apoptosis, wild type and mutant Schwann cells were analyzed with respect to their apoptosis by staining with APC conjugated Annexin V. Apoptosis is characterized by morphological changes. One of the earliest morphological changes in apoptosis is the translocation of phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane. Annexin V binds to cells with exposed PS, and early apoptotic cells can be stained by Annexin V conjugated to APC.

By analyzing *gdf15*+/+ Schwann cells (Figure 3.11, A), it was observed that most Schwann cells were localized in the lower left quadrant. Those cells are negative for 7-AAD (a viable dye) as well as for APC-Annexin V; they are non-apoptotic. Only a few Schwann cells were localized in the lower right quadrant; they are positive for APC-Annexin V and are apoptotic. Schwann cells in the upper right quadrant are both 7-AAD and APC-Annexin V positive and are late apoptotic or necrotic.

A comparison of *gdf15*+/+ and *gdf15*-/- Schwann cells clearly reveals that the distinct population seen in *gdf15*+/+ Schwann cells is more scattered. This demonstrates that the *gdf15*+/+ cells have more APC-Annexin V positive cells (and therefore more apoptotic Schwann cells) which is reduced by a treatment with recombinant GDF-15 (100 ng/ml) (Figure 3.11, C). As GDF-15 is highly expressed by Schwann cells, wild type Schwann cells were not treated (Strelau, Strzelczyk et al. 2009).

Statistical analysis showed that the increased apoptosis (~44.4 %; s.e.m. 7.78%) in mutant Schwann cells reached significance in four out of five independent experiments and that mutant Schwann cells could be rescued by treatment with 100 ng/ml recombinant GDF-15, reaching significance in two out of three experiments.

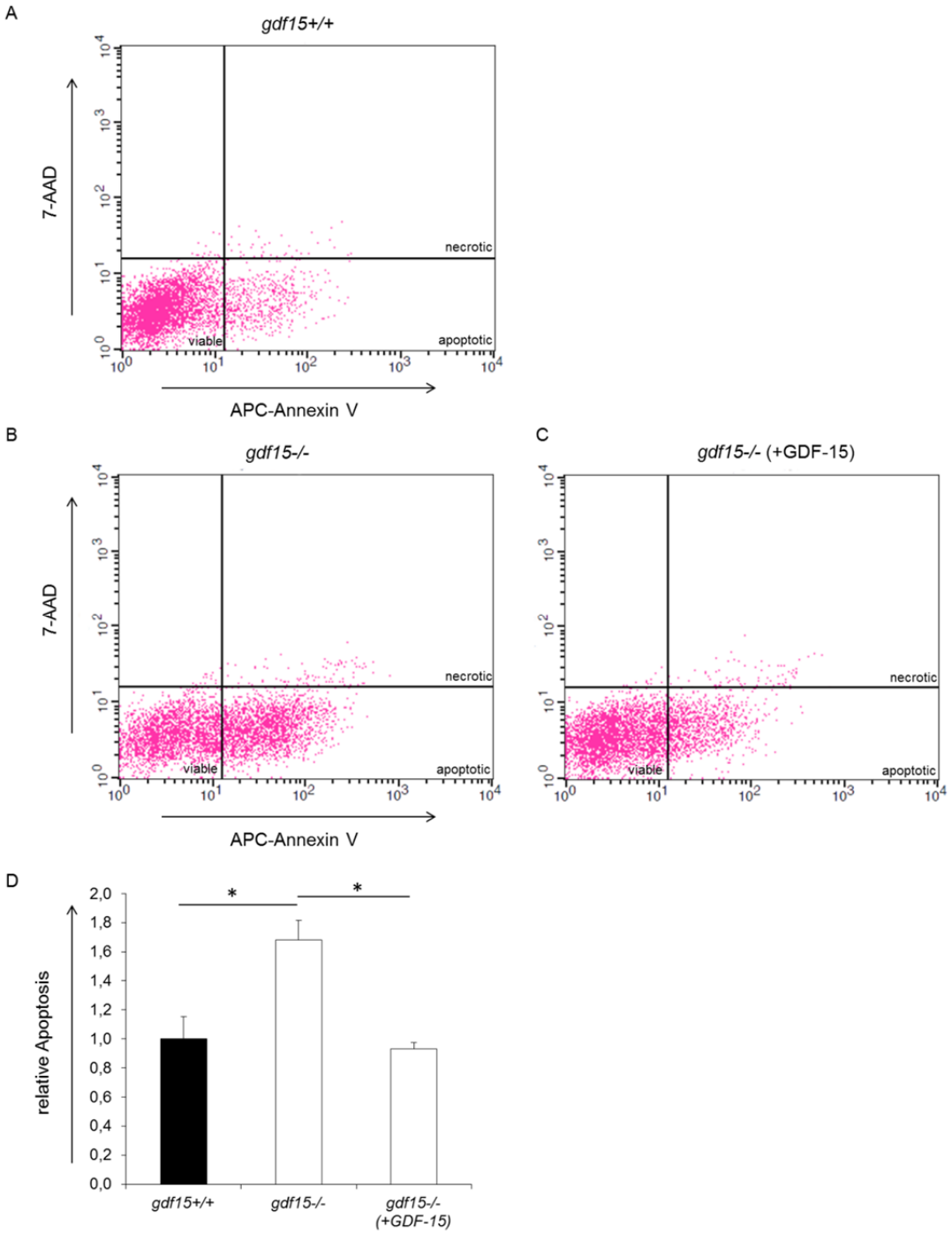


Figure 3.11: GDF-15 deficiency increases apoptotic cell numbers in Schwann cells. **A-C)** Dot plots of APC-Annexin V/7-AAD stained Schwann cells. **A)** Most cells are located in the lower, left quadrant (viable cells) whereas in **B)** cells are scattered in the lower left (viable cells) and right (apoptotic cells) quadrants. **C)** After treatment of mutant Schwann cells with 100 ng/ml recombinant GDF-15, the cell population is shifted towards the lower left quadrant (viable cells) **D)** Statistical analysis proofs an increased apoptosis in mutant Schwann cells which can be rescued by treatment with recombinant GDF-15.

Gdf15+/+ apoptosis rate was set to 1 calibrator. Error bars indicate s.e.m. (Tukey's Multiple Comparison Test: *: $p<0.05$)

3.3.2.2 GDF-15 deficiency reduces the migration of Schwann cells *in vitro*

Schwann cell migration is important during the nerve regeneration process. For example the formation of bands of Bunger (for which adequate Schwann cell migration is required) guides the newly formed axons. In 2009, Ribeiro-Resende et al. have shown that a combination of neuregulin (NRG), nerve growth factor (NGF) and TGF-β support the Schwann cell alignment during the formation of Bands of Bungner, thus supporting the migratory ability of Schwann cells (Ribeiro-Resende, Koenig et al. 2009). A zebrafish study demonstrated the importance of Neuregulin for the Schwann cell migration (Perlin, Lush et al. 2011) and another study showed that Neuregulin enhances nerve regeneration by supporting Schwann cell migration. As GDF-15 treatment also enhances nerve regeneration (Mensching, Borger et al. 2012), I suggest that GDF-15 controls Schwann cell migration.

Figure 3.12 shows wild type (left panel) and mutant (right panel) Schwann cell spheres at different time points after plating. Schwann cells start to migrate out and away from the spheres (expansion of spheres).

The area of the expanded spheres (Figure 3.13, A) differed greatly between wild type and mutant Schwann cells starting at time point 12h (Figure 3.13, B). After 36h, the area of wild type Schwann cell spheres were increased ~59.3 times whereas the area of mutant Schwann cell spheres was only increased ~30.5 times, a reduction of 45.8% compared to wild type Schwann cells. Moreover, treatment with 100 ng/ml exogenous recombinant GDF-15 partially restored the migration abilities. Compared to untreated mutant Schwann cell spheres, treated mutant Schwann cell spheres expanded ~138%. However, after four independent experiments, the partial rescue did not reach significance. By analyzing each experiment individually, the rescue reaches significance.

Additionally I compared the sphere diameters from the beginning (T: 0h) and at the end of the experiments (T: 36h) (Figure 3.13, C). Wild type Schwann cell spheres were significantly decreased after 36h (~53%), suggesting that Schwann cells went out of the spheres into the surrounding area, whereas mutant Schwann cell sphere sizes were not significantly reduced. In contrast, mutant Schwann cell spheres treated with exogenous recombinant GDF-15 showed reduced sphere diameters (~65.7%) comparable to wild type spheres, demonstrating that treatment with

exogenous recombinant GDF-15 restores the migration ability in mutant Schwann cells *in vitro* (Figure 3.13, C).

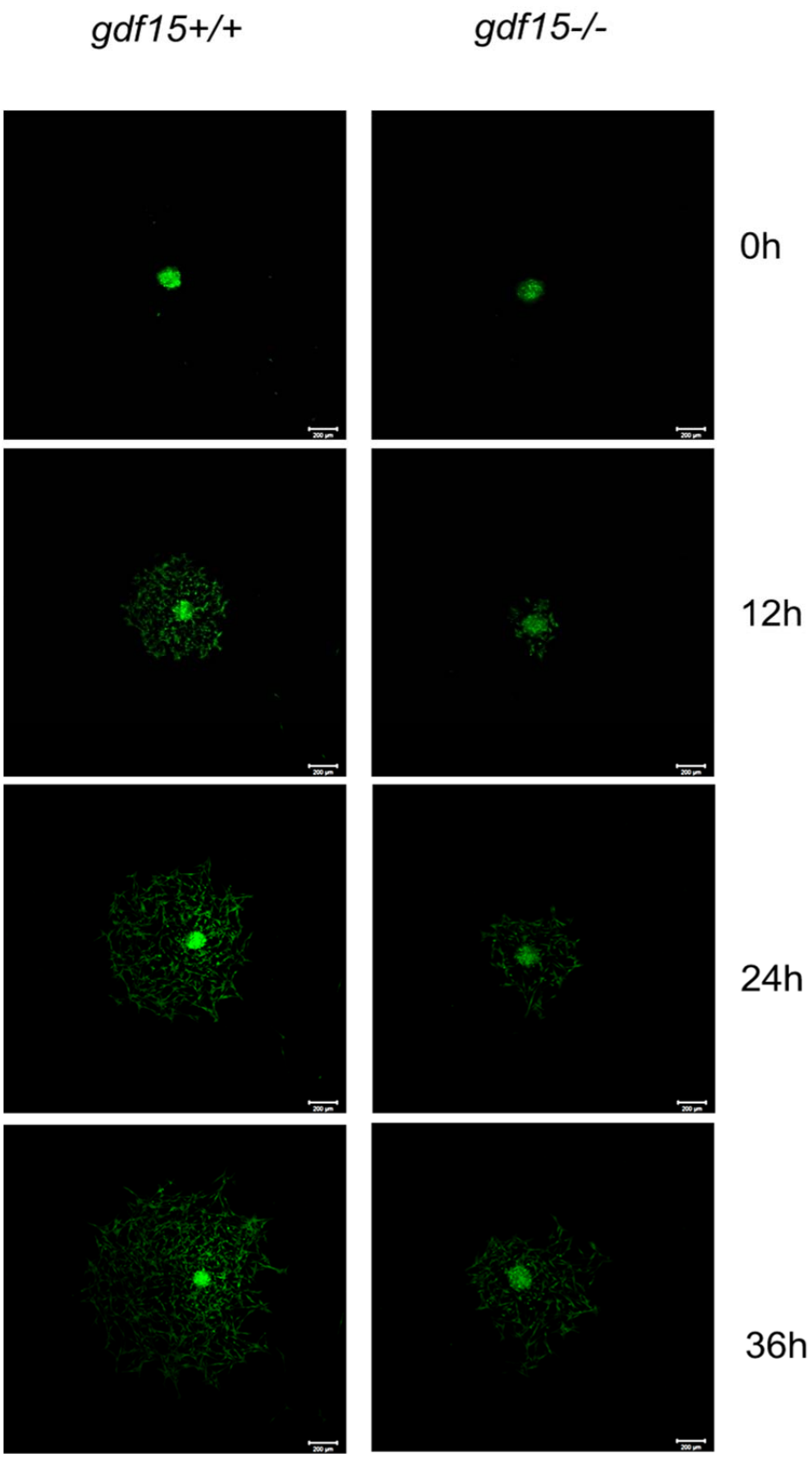
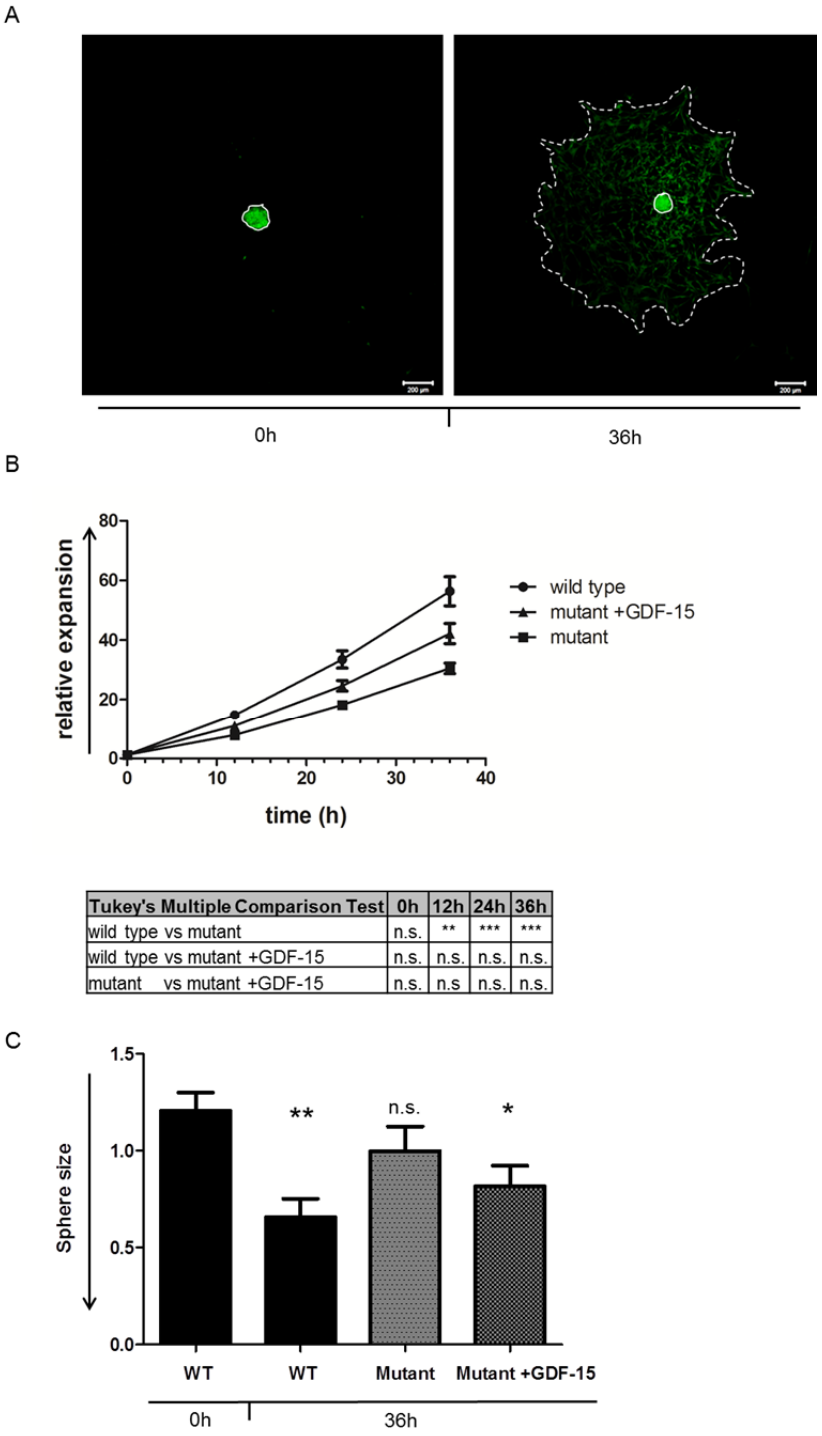


Figure 3.12: Images of Schwann cell spheres 0, 12, 24 and 36 hours after plating out. The left panel shows a *gdf15+/+* sphere and the right panel a *gdf15-/-* sphere. A reduced migration behavior can be clearly seen in the *gdf15-/-* sphere.



Tukey's Multiple Comparison Test	0h	12h	24h	36h
wild type vs mutant	n.s.	**	***	***
wild type vs mutant +GDF-15	n.s.	n.s.	n.s.	n.s.
mutant vs mutant +GDF-15	n.s.	n.s	n.s.	n.s.

Figure 3.13: **A)** Representative Schwann cell sphere images with marked spheres (white lines) and expansion of Schwann cells (dashed white line). **B)** GDF-15 deficiency (square marks, n=7) leads to a significant decreased expansion of Schwann cell spheres which is partially rescued by the treatment with 100ng/ml recombinant GDF-15 (triangle marks, n=4). **C)** After 36h, the sphere size is significantly decreased in *gdf15*+/+ Schwann cells (black bars) whereas the sphere size in *gdf15*-/- Schwann cells (grey bars) is only significantly decreased after treatment with 100 ng/ml recombinant GDF-15. Size of *gdf15*+/+ spheres at 0h was set to 1 as calibrator. Error bars indicate s.e.m. (Tukey's Multiple Comparison Test: *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$)

3.4 GDF-15 signaling in primary Schwann cells and in the Schwann cell line RT4-D6P2T

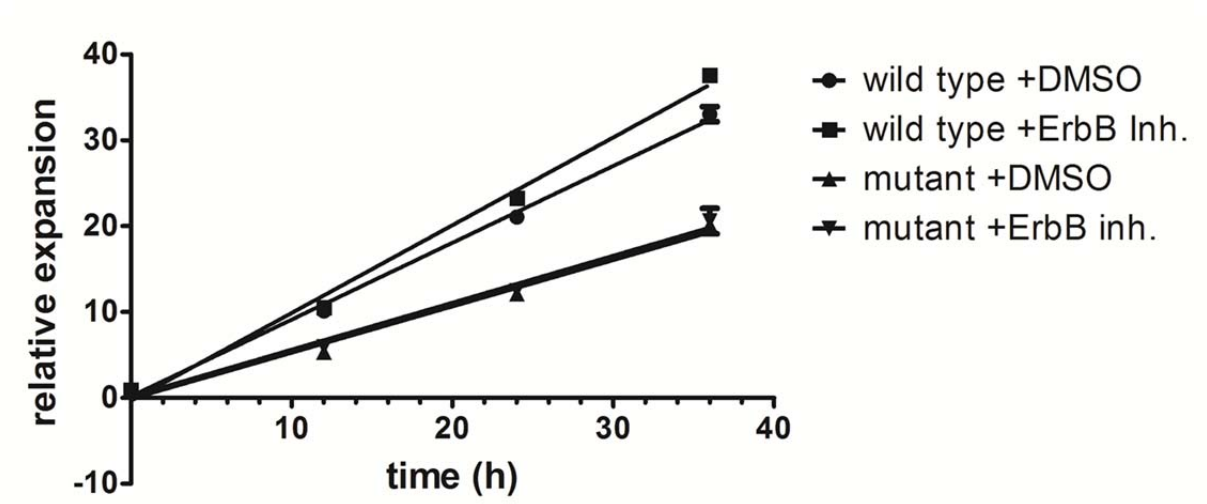
So far, we know that GDF-15 deficiency leads to a reduced Schwann cell number in peripheral nerves due to reduced migration ability and an increased apoptosis rate in Schwann cells. However, the analysis of the myelin associated factors NDRG1 and HmgCr did not show any difference, whereas the expression of the pro-inflammatory cytokine IL-6 was increased in GDF-15 deficient sciatic and femoral nerves *in vivo*. Thus, the mechanisms which lead to a reduced Schwann cell numbers and altered Schwann cell behavior are still not understood. Furthermore, it is still unclear which receptor is involved in GDF-15 signaling in Schwann cells. To further elucidate the involved mechanisms, a possible candidate, the ErbB2 receptor, was studied.

3.4.1 ErbB2 inhibition has no effect on Schwann cell migration

Several publications have shown that a treatment with recombinant GDF-15 activates the ErbB2 receptor in cancer cell lines, as SK-BR-3 (Kim, Lee et al. 2008; Park, Lee et al. 2010; Joshi, Brown et al. 2011). Furthermore, it was shown that ErbB2 signaling plays a key role in myelination (Pertusa, Morenilla-Palao et al. 2007; Perlin, Lush et al. 2011; Ma, Zhang et al. 2012; Chang, Shyu et al. 2013) and most importantly that Schwann cell migration depends on ErbB2 signaling in zebrafish (Perlin, Lush et al. 2011; Chang, Shyu et al. 2013).

Functional effects of ErbB2 signaling on primary Schwann cells were investigated by a migratory assay in which Schwann cells were treated with an ErbB inhibitor. In theory, the blockage of ErbB2 by the use of an ErbB inhibitor should alter wild type Schwann cell migration as GDF-15 deficiency does. Therefore, mutant and wild type Schwann cell spheres were treated with an ErbB Inhibitor which was also used for other mouse Schwann cell studies (Heermann, Schmucker et al. 2011).

Mutant Schwann cells again showed a decreased migration compared to wild type Schwann cells. However, the use of the ErbB inhibitor did not reduce wild type Schwann cell migration (Figure 3.14).



Tukey's Multiple Comparison Test	0h	12h	24h	36h
wild type +DMSO vs mutant +DMSO	n.s.	**	**	**
wild type +DMSO vs mutant +ErbB Inh.	n.s.	**	**	**
wild type +DMSO vs wild type +ErbB Inh.	n.s.	n.s.	n.s.	*
mutant +DMSO vs mutant +ErbB Inh.	n.s.	n.s.	n.s.	n.s.

Figure 3.14: Wild type and mutant Schwann cell spheres were observed in a time lapse experiment over 36h, and the expansion of Schwann cell spheres was measured after 0,12, 24 and 36h. Treatment with ErbB inhibitors did not reduce the migration compared to the DMSO treated control groups. Size of wild type spheres at 0h was set to 1 as calibrator. Error bars indicate s.e.m. (Tukey's Multiple Comparison Test: *: $p<0.05$, **: $p<0.01$)

3.4.2 ErbB2 activation is involved in the signaling mechanism of GDF-15 in the Schwann cell line RT4-D6P2T

ErbB2 phosphorylation was shown by western blotting of cell lysates from MCF-7 cells, a human cancer cell line and RT4-D6P2T cells, a rat Schwannoma derived cell line. Due to the lack of available phospho-ErbB2 antibodies, no cell lysates from primary Schwann cells of mice were used. The MCF-7 cells were used as positive control to establish a functional western blot method. As already shown, treatment with Nrg (a factor highly involved in early myelination) induced a phosphorylation of the ErbB2 receptor in the MCF-7 cells. However, treatment with GDF-15 did not activate ErbB2 in MCF-7 (Figure 3.15, A) but rather lead to an ErbB2 phosphorylation in the Schwann cell line in four out of six experiments (Figure 3.15, B).

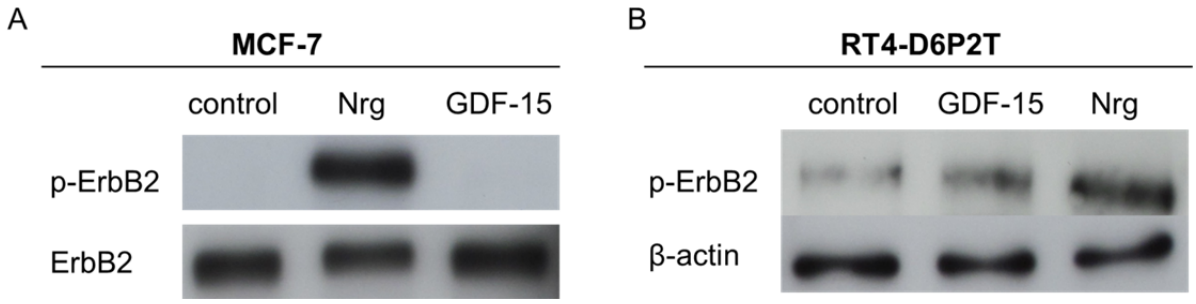


Figure 3.15: **A** MCF-7 cells were treated with 100ng/mL recombinant Neuregulin or GDF-15 for 5 minutes resulting in a phosphorylation of ErbB2 in the Neuregulin treated cells. The ErbB2 western blot shows the same protein amounts in the different samples. **B** RT4-D6P2T cells were treated with 100 ng/mL recombinant Neuregulin or GDF-15 for 5 minutes, resulting in a phosphorylation of ErbB2 in the GDF-15 treated cells in four out of six experiments. The β-actin western Blot shows the same protein amounts in the different samples.

Chapter 4: Discussion

Schwann cells play a key role in nerve regeneration and are important during every stage of the regeneration process, starting with the early immune response. They are responsible for the phagocytosis of axonal and myelin debris, guidance of the newly formed axons to the distal parts of the injury and finally remyelination of the new axons. However, remyelinated axons fail to gain back the normal myelin thickness. Nerve regeneration is a complex process and limited to the severity of injury (Rodrigues, Rodrigues et al. 2012). Elucidation of the regeneration mechanism has just begun. It was shown that overexpression of Neuregulin, an important factor during Schwann cell development and myelination, restores normal myelin thickness after remyelination (Fricker, Brelstaff et al. 2011). Interestingly, Neuregulin expression is increased by the neurotrophic factors NGF and BDNF (Taveggia, Zanazzi et al. 2005). GDF-15 is another promising factor. It was not only shown that GDF-15 is a neurotrophic factor *in vitro* and *in vivo* (Strelau, Sullivan et al. 2000; Subramaniam, Strelau et al. 2003; Strelau, Strzelczyk et al. 2009); it was also shown that GDF-15 expression is increased after nerve crushes (Charalambous, Wang et al. 2013) and that submission of recombinant GDF-15 enhances nerve regeneration (Mensching, Borger et al. 2012). Notably, further investigation of the neurotrophic effects of GDF-15, also showed that GDF-15 deficiency does not only induce a progressive motoneuron loss in adulthood, it furthermore induces an axonal loss (Strelau, Strzelczyk et al. 2009) and hypermyelination of peripheral nerves (Dr. Jens Strelau, unpublished data) suggesting regulatory effects of GDF-15 on Schwann cells. The here presented work focuses on the effect of GDF-15 deficiency on Schwann cells *in vivo* and *in vitro*.

4.1 GDF-15 deficiency reduces Schwann cell numbers in adult mice

Myelination of developing nerves starts at birth when myelin inducing factors as axonally derived Neuregulin triggers immature Schwann cells to differentiate into myelinating mature Schwann cells. Schwann cell numbers are now regulated by survival and proliferation of immature Schwann cells controlled by Schwann cell

mitogens as Neuregulin and TGF-βs (Jessen and Mirsky 2005). To further elucidate the effects of GDF-15 deficiency on Schwann cells, total cell numbers in peripheral nerves at different ages (P5-12 months) of wild type and GDF-15 deficient mice were quantified and compared in this work. As expected, five day old mice possess more Schwann cells than compared to adult mice. At that age, the myelinogenesis is still at its beginning. Most Schwann cells are still non-myelinating and only differentiation into myelinating Schwann cells leads them to exit the cell cycle (Jessen and Mirsky 2005). An *in vitro* study of the colonization of distal parts of sympathetic axons showed the importance of high Schwann cell numbers. Reduced Schwann cells numbers inhibited Schwann cell migration towards the distal parts of the axons (Heermann, Schmucker et al. 2011). Another study showed that at the age of 6 days, apoptotic Schwann cells can be seen which occur only occasionally at the age of three weeks and none are seen at the age of three months (Grinspan, Marchionni et al. 1996). This correlates with the total Schwann cell numbers in peripheral nerves quantified here. In 3.5 months old mice, Schwann cell numbers were highly reduced compared to those of the newborn animals, whereas there is no difference between the different adult stages in the wild type mice. However, by comparing cell numbers in wild type and GDF-15 mutant mice, mutant mice show a Schwann cell loss detectable at 3.5 months of age and reaching significance at six months. At the age of 12 months, Schwann cell loss is even more severe. Mutant mice only possess about 50% Schwann cells compared to wild type mice. Prior studies in our laboratory showed that only about 13% of axons are missing in six months old GDF-15 mutant mice and that a motoneuron loss starts at 4.5 months, getting more severe till the age of 12 months (Strelau, Strzelczyk et al. 2009). It seems that the axonal loss just started and will continue with progressing age as the motoneuron loss and the here showed Schwann cell loss. This hypothesis is supported by the progression of the neuropathy Charcot-Marie Tooth disease in which Schwann cell dysfunctions lead to axonal loss. This disease is induced by a nonsense mutation in the *Ndrg-1* gene (Kalaydjieva, Gresham et al. 2000). Interestingly, a correlation between GDF-15 and NDRG1 was shown in a study from Tsui and colleagues. Overexpression of GDF-15 induced an upregulation of NDRG1 in a cancer cell line (Tsui, Chang et al. 2012). A possible regulation of NDRG1 by GDF-15 deficiency in peripheral nerves is discussed in the following section.

4.2 GDF-15 regulates Interleukin-6 expression in peripheral nerves

As discussed in the previous section, the here presented work shows that GDF-15 deficiency reduces Schwann cell numbers in adults. A publication from 2012 by Mensching et al. showed that substitution of recombinant GDF-15 supports nerve regeneration by enhancing the quality of repaired axons (Mensching, Borger et al. 2012). As Schwann cells play an important role in nerve regeneration, it is of interest to analyze possible correlations of GDF-15 deficiency and factors known to be regulating Schwann cell functions and myelin especially during the regeneration process. Today several factors are known which regulate Schwann cell development (e.g. the growth factor Neuregulin and members of the TGF-β superfamily (Jessen and Mirsky 2005)), myelination (e.g. Neuregulin (Jessen and Mirsky 2005)) and some factors involved in nerve regeneration (e.g. GDF-15 (Mensching, Borger et al. 2012), NDRG1(Hirata, Masuda et al. 2004)).

Strelau and coworkers showed the neurotrophic role of GDF-15 on motoneurons and the loss of DRG neurons in GDF-15 deficient mice (Strelau, Sullivan et al. 2000). In this coherence it is of interest that the neurotrophic factors NGF and BDNF upregulate Neuregulin 1 type I and type II in DRG neurons (Esper and Loeb 2004). Neuregulin regulates the cross talk between the axon and the Schwann cells, defining the myelin sheath thickness (Taveggia, Zanazzi et al. 2005) and it is suggested that Neuregulin and its receptor ErbB2 regulate myelination by controlling the cholesterol synthesis on a transcriptional level (see 1.1.3.1 for more details). (Pertusa, Morenilla-Palao et al. 2007). Notably, as shown in this work that GDF-15 deficiency induces a Schwann cell loss in adulthood, Berciano and colleagues showed that cholesterol synthesis inhibition leads to an autophagic cell death in mature Schwann cells (Berciano, Calle et al. 1998). ErbB2, the receptor activated by Neuregulin and involved in the control of cholesterol synthesis is also activated by treatment with recombinant GDF-15 in some cancer cell lines (Kim, Lee et al. 2008; Park, Lee et al. 2010). Thus, it is possible that ErbB2 is activated by GDF-15 in the nervous system as well. Interestingly, it was not only shown that Neuregulin regulates myelinogenesis, it was further more shown by Stassart et al. that mice, overexpressing the Neuregulin 1 isoform type III were able to restore myelin thickness after nerve crushes whereas myelin repair in wild type mice results in reduced myelin thickness (Stassart, Fledrich et al. 2013). Notably, a similar effect

was shown for GDF-15. Its submission after nerve injuries induced a better quality of regenerated axons (Mensching, Borger et al. 2012). These data, the effects of Neuregulin on nerve regeneration, ErbB2 activation by Neuregulin and GDF-15 as well as transcriptional control of cholesterol synthesis and that inhibition of cholesterol synthesis leads to a Schwann cell death suggest that GDF-15 deficiency involves a similar pathway as the Neuregulin-ErbB2 signaling. In this relation, I analyzed the expression of Neuregulin 1 type III, ErbB2 and Hmgcr (encoding the enzyme which is the rate limiting step in cholesterol synthesis) in adult wild type and GDF-15 mutant peripheral nerves. Although the above discussed data strongly suggest a correlation between GDF-15 and Neuregulin or its myelin regulating pathway, neither Neuregulin 1 type III, ErbB2 nor Hmgcr expression were altered by GDF-15 deficiency in adult nerves. Thus it seems that the pathway leading to the here showed reduced Schwann cell number in mutant nerves is different than the Neuregulin-ErbB2-Hmgcr pathway. Notably, another member of the TGF-β family, GDNF upregulates Neuregulin expression in DRG axons and this effect is rapidly occurring within minutes after treatment (Esper and Loeb 2004). As the effect of GDNF is very rapid but the Schwann cell loss in GDF-15 mutant mice is a very slow, continuous process taking several months, it is unlikely that expression differences of Neuregulin, ErbB2 or Hmgcr are responsible for the Schwann cell loss. However, ErbB2 signaling and the effect of GDNF in correlation with GDF-15 deficiency will be discussed in more detail later on.

Besides Neuregulin, another interesting and promising factor is NDRG1. As already mentioned, a nonsense mutation in the NDRG1 gene causes the Charcot-Marie Tooth disease, a peripheral neuropathy in which Schwann cell dysfunction leads to axonal loss (Kalaydjieva, Gresham et al. 2000). NDRG1 is also involved in the terminal differentiation of Schwann cells during nerve regeneration, showing an increased expression of NDRG1 at the stage of remyelination after nerve crushes (Hirata, Masuda et al. 2004). Similar to NDRG1, Charalambus et al. showed an increased GDF-15 expression after nerve crushes (Charalambous, Wang et al. 2013). Notably, Tsui and colleagues showed a regulation of NDRG1 and Interleukin-6 expression by GDF-15. GDF-15 overexpression increases the NDRG1 expression in cancer cells (Tsui, Chang et al. 2012). But as shown in the here presented work, in mature GDF-15 wild type and mutant nerves, no difference of NDRG1 expression in correlation with the different genotypes was observed. These data show that GDF-15

deficiency has now effect on the NDRG1 expression in uninjuried nerves. However, future studies should address the question if GDF-15 deficiency affects the increased NDRG1 expression after nerve injury.

As mentioned is the passage above, Tsui and coworkers did not only show the effect of GDF-15 overexpression on the NDRG1 expression. They also showed that GDF-15 regulates Interleukin-6, the key regulator of inflammatory response, expression in cancer cells (Tsui, Chang et al. 2012) similar as it was shown by Bonaterra and colleagues in macrophages (Bonaterra, Zugel et al. 2012). In the here presented study, by comparing IL-6 expression in GDF-15 wild type and mutant adult peripheral nerves, an increase in IL-6 expression is seen in the mutant nerves. This demonstrates an altered immune response induced by GDF-15 deficiency in peripheral nerves. A publication focusing on IL-6 involvement in the nervous system showed that increased IL-6 expression kills frataxin-deficient Schwann cells. Frataxin mutation causes the neurodegenerative disease Friedreich´s ataxia (Lu, Schoenfeld et al. 2009). In light of my observations that Schwann cell numbers are reduced and that IL-6 expression is increased in GDF-15 deficient peripheral nerves and that IL-6 kills frataxin-deficient Schwann cells, one important question arises: Is the Schwann cell loss in GDF-15 deficient mice similarly induced as the death of frataxin-deficient Schwann cells? Does the increased IL-6 expression kills GDF-15 deficient Schwann cells? A question, that needs to be addressed in further studies.

To further elucidate the effects of the here observed increased IL-6 expression it is interesting if the Schwann cells are responsible for the increased IL-6 expression. Since IL-6 expression was investigated in whole peripheral nerve lysates, it is not known in which cell type, including Schwann cells, macrophages, fibroblasts and neurons, IL-6 expression is increased. Nevertheless, Schwann cells express and secrete GDF-15 and are suggested to be an important source of GDF-15 in the peripheral nerve (Strelau, Strzelczyk et al. 2009). Thus, in this work IL-6 expression after an induced GDF-15 knockdown was investigated to further elucidate the direct effect of GDF-15 deficiency in cells used to threshold levels of GDF-15. Wild type cells were transfected with siRNAs, inducing *gdf15* knockdown. siRNA studies require a high cell number therefore, because the cell number is limited in primary Schwann cell cultures, the Schwann cell line RT4-D6P2T was used. Due to the stressful process of transfection an increased expression of GDF-15 could not be avoided. Nevertheless, a significant *gdf15* knockdown was achieved compared to the

control situation. However, IL-6 expression was unaltered and did not show a correlation with GDF-15 knockdown in Schwann cells. This suggests that the increased IL-6 expression in peripheral nerve lysates is not Schwann cell derived; whether other neuronal cell types express IL-6 under the influence of GDF-15 must be further investigated. Moreover, a siRNA induced knockdown only reduced the GDF-15 expression to 25%. As Schwann cells are suggested to be an important source of GDF-15 (Strelau, Strzelczyk et al. 2009), it is possible that the residual level of GDF-15 expression is high enough to maintain the normal threshold level of IL-6. This test system should be further optimized in future studies, for example, by the blockage of the residual secreted GDF-15 by the submission of a monoclonal antibody against GDF-15 as described by Johnen and coworkers (Johnen, Lin et al. 2007).

4.3 Efficient enrichment of adult mouse Schwann cell cultures for use in functional studies

In the siRNA studies, the limiting factor for the use of primary Schwann cells was the amount of required cells. For functional analysis like proliferation, apoptosis and migration a lower cell number is needed and primary Schwann cells can be used. Several publications focus on preparation of primary Schwann cell cultures (Lopez and De Vries 1999; Haastert, Mauritz et al. 2007; Kaewkhaw, Scutt et al. 2012; Tao 2013). However, mostly neonatal animals are used as tissue source; of these most are rats. Only a few publications are based on the use of adult tissue (Haastert, Mauritz et al. 2007). The challenging part in adult Schwann cell culture preparation is a low cell yield and fibroblasts which overgrow Schwann cell numbers caused by a higher proliferation rate. This work presents a Schwann cell preparation and cultivation method leading to high cell numbers and efficient and easy enrichment of Schwann cells and reduction of fibroblasts. It leads to ~95% pure Schwann cell cultures within 4 weeks.

Preparation methods, established for neonatal material, result in a low Schwann cell yield by the use of adult tissue, as neonatal nerves possess a higher number of Schwann cells than adult nerves (see above). The here established method enhances the outcome by inducing the Wallerian degeneration. The Wallerian

degeneration is induced by extraction of the nerve from the mice and culturing *in vitro*. During the Wallerian degeneration Schwann cells re-enter the cell cycle and start to proliferate again (Johnson, Zoubos et al. 2005), leading to a high number of Schwann cells in the nerves. This step is also used in the method established for adult human and rat Schwann cell cultures by Haastert and coworkers (Haastert, Mauritz et al. 2007). While extracting the nerves, one other step is important: the thorough removal of connective tissue, the source of fibroblasts. However, a complete removal of the connective tissue is not possible and some fibroblasts will still remain in the cultures.

Several purification methods for Schwann cell cultures are known but they are established for neonatal or human and rat cultures. Schwann cells from neonatal cultures are purified by the use of cytosine arabinoside, an antimitotic reagent (Yuan, Zhang et al. 2013) or by treatment with an anti-Thy1 antibody and complement serum (Ji, Shen et al. 2012). These methods involve a more often medium change, and this is the limiting stage for the here needed adult mouse cultures. Adult mouse Schwann cells detach very easily, even by changing the medium. Thus, these methods lead to a high Schwann cell loss and are unsuitable for adult cultures. Haastert et al. used another purification method for adult rat and human cultures the so called “cold jet” method. As already mentioned adult Schwann cells detach easily. When they are rinsed with ice cold PBS, Schwann cells detach and can be transferred to new plates. Fibroblasts are still adherent and remain in the old plate (Haastert, Mauritz et al. 2007). Even so, the “cold jet” method is suitable for rat and human adult Schwann cells; it is unsuitable for mouse adult cultures as they do not survive the stress induced by the “cold jet” method. Without stress induced death of Schwann cells, a short trypsin-EDTA treatment leads to the same result as the “cold jet” method. The fibroblasts which grow flat at the bottom stay attached to the plates whereas the Schwann cells, growing on top of the fibroblasts detach within less than a minute of trypsin-EDTA treatment. This fast and easy purification method results in ~95% pure Schwann cell cultures which are suitable as an *in vitro* model for functional analysis.

4.4 GDF-15 protects Schwann cell survival and migration

The established method, discussed in the passage above is an efficient method to produce enough primary Schwann cell material for functional studies. To further elucidate the importance of GDF-15 on Schwann cells, proliferation, survival and migration were investigated in wild type and mutant Schwann cell cultures.

Even though it was shown by Ridley and coworkers that other members of the TGF- β family, TGF-β 1 and 2, stimulate rat Schwann cell proliferation *in vitro* (Ridley, Davis et al. 1989), the proliferation studies in the here presented work demonstrated that GDF-15 deficiency has no proliferative effect on cultured Schwann cells. Interestingly, another publication showed that TGF-β has dual effects on Schwann cell proliferation depending on the duration of the cultures. Short-term cultures showed increased proliferation whereas long-term cultures showed reduced proliferation after TGF-β submission. This publication showed the multifunctional effects of TGF-β family members on Schwann cells (Eccleston, Jessen et al. 1989). It would be of interest if there is also a correlation on proliferation in short- and long-term cultures regarding GDF-15 deficiency. This should be part of future studies.

Schwann cell survival is regulated amongst others by Neuregulin. Syroid and colleagues showed that serum withdrawal induced cell death is counteracted by the application of Neuregulin on postnatal Schwann cells *in vitro* (Syroid, Maycox et al. 1996; Li, Tennekoon et al. 2001). The here presented work shows a similar effect regarding GDF-15. GDF-15 deficient cultures possessed a higher number of apoptotic Schwann cells than the wild type cultures, and more importantly, they regained a lower apoptotic cell number after GDF-15 submission, showing the survival protective effect of GDF-15 on Schwann cells. Although, the survival protecting effect of GDF-15 here shown *in vitro* strongly suggests that the Schwann cell loss in mutant nerves observed *in vivo* is due to an increased apoptosis, further *in vivo* studies are needed to support these *in vitro* data. An approach is a TUNEL assay and histochemical analysis of peripheral nerves in wild type and mutant mice as described by Grinspan and coworkers (1996). They showed that Schwann cells in developmental nerves undergo apotosis after axotomy which is prevented by the submission of Neuregulin, verifying the Schwann cell protecting effect of Neuregulin *in vivo*, which was already shown *in vitro* (Grinspan, Marchionni et al. 1996). Analysis

of apoptotic Schwann cells in GDF-15 wild type and mutant nerves after axotomy is also of interest considering the nerve regeneration supporting effect of GDF-15 as shown by Mensching and coworkers. They showed that treatment with recombinant GDF-15 enhanced the quality of repaired axons but which mechanism supports nerve regeneration after GDF-15 treatment is not yet clear (Mensching, Borger et al. 2012).

During nerve regeneration Schwann cell migration is essential. They have to migrate towards myelin and axonal debris to clean the injury site (David and Lacroix 2003) and afterwards adequate migration is essential for axon guidance (Evans 2001). Chang and colleagues showed that rats treated with Neuregulin after an end-to-side neurorrhaphy had a better functional recovery than the control group. Neuregulin enhanced the Schwann cell migration and spreading by the activation of the ErbB2 receptor (Chang, Shyu et al. 2013). As discussed under 4. 3, the here established Schwann cell culture method enhances the outcome by inducing an *in vitro* predegeneration and Schwann cells undergo similar changes as induced by nerve injury. Interestingly, GDF-15 regulates Schwann cell migration in those cultures. GDF-15 deficient cells only migrated around 50% compared to wild type cells and submission of recombinant GDF-15 partially rescued Schwann cell migration. GDF-15 is not the only member of the TGF-β family regulating Schwann cell migration GDNF also supports Schwann cell migration (Cornejo, Nambi et al. 2010; Deng, Deng et al. 2013) and nerve regeneration (Deng, Deng et al. 2013). Deng and coworkers showed the facilitated regeneration of Spinal cord injuries by the use of GDNF-overexpressing Schwann cells. GDNF supports the propriospinal axon regeneration and synapse formation (Deng, Deng et al. 2013). Even though, I showed that GDF-15 controls Schwann cell migration and it was shown by Cornejo and colleagues that GDNF also supports Schwann cell migration and it was shown that both factors support nerve regeneration (Mensching, Borger et al. 2012; Deng, Deng et al. 2013), a correlation of controlled Schwann cell migration by GDF-15 or GDNF and the nerve regeneration supporting effect has still to be shown. But migration investigations *in vivo* in mammals are hindered by methodological limitations such as the inaccessibility of time-lapse imaging of peripheral nerves. Heerman and coworkers established an *in vitro* assay simulating *in vivo* conditions by investigating the migration of Schwann cells along axons of sympathetic ganglion explants. They dissected superior cervical ganglia from mice at embryonic day 16.5

or 18.5 and placed them on a collagen matrix. By treatment with NGF neurite outgrowth was induced and Schwann cell migration along the axons was documented by time-lapse imaging (Heermann, Schmucker et al. 2011). As the onset of the GDF-15 deficient phenotype starts in adulthood and the neuron loss is specific on motoneurons, dorsal root ganglia (DRG) dissected from adult mice should be used in this assay. DRGs in the adult organism are integrated and completely surrounded by the spinal cord. To dissect uninjured adult DRGs is therefore a difficult procedure. Nevertheless, this is an *in vitro* assay close to *in vivo* conditions and should be established and investigated in future studies.

4.5 ErbB2 is a promising receptor for GDF-15 signaling in Schwann cells

To further investigate GDF-15 mediated mechanisms controlling Schwann cell migration, a promising receptor, the ErbB2 receptor was inhibited in a migration assay. As mentioned above, it was shown that Schwann cell migration is regulated by Neuregulin and furthermore, it was shown that the signaling pathway of Neuregulin is essential. In zebrafish, a deletion of the ErbB2 receptor reduces Schwann cell migration similar as Neuregulin deletion (Perlin, Lush et al. 2011) and a study from Chang and colleagues showed that ErbB2 is activated by Neuregulin, facilitating nerve regeneration by speeding up Schwann cell migration (Chang, Shyu et al. 2013). The signaling pathway of GDF-15 is not unequivocally identified. There is proof that GDF-15 signaling involves the ErbB2 receptor in cancer cell lines as GDF-15 treatment leads to phosphorylation of ErbB2 (Kim, Lee et al. 2008; Park, Lee et al. 2010). However, on neural tissue, there was no evidence so far that GDF-15 activates ErbB2. In this work, the ErbB2 receptor was blocked pharmacologically in a migration assay to show that wild type Schwann cell motility is altered similarly to that of GDF-15 mutant Schwann cells but wild type cell motility was not reduced by ErbB2 inhibition. Notably, the effect of altered ErbB2 function on Schwann cell motility concerning Neuregulin signaling is already controversially discussed. In contrary to the above mentioned publications, Neuregulin/ErbB2 signaling only indirectly affects Schwann cell colonization of sympathetic axons by controlling Schwann cell numbers (Heermann, Schmucker et al. 2011), and furthermore, ErbB2 signaling is dispensable

after nerve injury (Atanasoski, Scherer et al. 2006). As GDF-15 enhances the quality of repaired axons after nerve injury (Mensching, Borger et al. 2012) it also seems that the ErbB2 receptor is not essential for the enhanced nerve regeneration by GDF-15. If other receptors or pathways compensate for the loss of the ErbB2 receptor is not known. However, ErbB2 signaling is still of interest. Previous studies showed that only low levels of ErbB2 signaling are needed for Schwann cell motility (Meintanis, Thomaidou et al. 2001) and the pharmacological inhibition of ErbB2 signaling may still allow residual activity as already discussed by Heermann et al. (Heermann, Schmucker et al. 2011). To ensure that there is no ErbB2 activity during the migration assay, the use of ErbB2 deficient Schwann cells would be the best choice. However, other factors, for example GDNF (Cornejo, Nambi et al. 2010), NGF (Anton, Weskamp et al. 1994) or IGF-1(Cheng, Steinway et al. 2000) could compensate for the inhibited GDF-15/ErbB2 signaling as a slight increase of migration is seen in the inhibitor treated wild type cells. Mutant cells treated with the ErbB2 inhibitor did not show any effect, suggesting that GDF-15 influences Schwann cell migration by a complex coregulation of different factors and/or signaling pathways. Whether different factors and/or other receptors compensate the ErbB2 inhibition needs to be addressed in future studies.

Next to the functional study, the migration assay, ErbB2 activation by recombinant GDF-15 in Schwann cells was investigated on the protein level. Due to the lack of antibodies reactive to mouse derived tissue, the rat derived Schwann cell line RT4-D6P2T (the same Schwann cell line used for the siRNA studies) was used. The here presented work shows the activation of ErbB2 in Schwann cells by treatment with recombinant GDF-15 for the first time. Thus, further investigation of the functional influence of ErbB2 on GDF-15 controlled Schwann cell migration gains an even higher importance. A side from the functional study, further elucidation of the ErbB2 activation of GDF-15 has to be part of future studies. Even though, there are no available antibodies against ErbB2 and activated ErbB2 reactive to mouse tissue, future studies should establish an assay to measure ErbB2 activity in primary Schwann cells; for example by the use of an antibody detecting tyrosine phosphorylation of membrane proteins. A similar approach was done by Chang et al. (Chang, Shyu et al. 2013).

4.6 Conclusion

This is the first study showing the physiological and significant role of GDF-15 on mature Schwann cells. In the adult organism, GDF-15 deficiency induces a progressive Schwann cell loss and an altered immune response in peripheral nerves, showed by increased IL-6 expression. Functional studies by the use of the here established adult Schwann cell cultures showed that GDF-15 deficiency induces apoptosis and that migration is strongly reduced in deficient cells. Both deficits could be rescued by a treatment with recombinant GDF-15. Furthermore, this work shows the activation of the ErbB2 receptor by GDF-15 in Schwann cells for the first time.

Further studies should address the question, if the increased IL-6 expression is responsible for the *in vivo* Schwann cell loss induced by GDF-15 deficiency as similarly seen in frataxin-deficient Schwann cells.

Furthermore, future studies should determine whether the nerve regeneration enhancing effect of GDF-15 submission as shown by Mensching and colleagues (Mensching, Borger et al. 2012) is Schwann cell dependent as the here presented work shows the regulation of Schwann cell survival and migration by GDF-15. Both, survival and adequate migration is essential for nerve regeneration.

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