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Original Paper

The microRNA miR-375-3p and the Tumor Suppressor NDRG2 are Involved in Sporadic Amyotrophic Lateral Sclerosis

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Key Words
Wobbler mouse • p53 • miRNA • Neurodegeneration • Motor neuron

Abstract
Background/Aims: Amyotrophic lateral sclerosis (ALS) is the most common degenerative motor neuron disease in humans. However, the pathogenesis of ALS is not yet understood. The wobbler mouse is considered as an animal model for the sporadic form of ALS due to its spontaneous mutation in the Vps54 gene. Due to transactivation of NDRG2 by p53, this tumor suppressor might play a functional role in stress induced cell death in wobbler mice as well as ALS patients. Furthermore, deregulated microRNAs are often related to neurodegenerative diseases. Thus, the NDRG2 linked miR-375-3p was of interest for this study. ***Methods:*** Here, we investigated the relevance of NDRG2 and miR-375-3p for the pathomechanism of the motor neuronal degeneration in wobbler mice by investigating expression level via qPCR and Western Blot as well as localization of these molecules in the cervical spinal cord by in situ hybridization, immunostaining and mass spectrometric analysis. ***Results:*** We were able to show a differential regulation of the expression of NDRG2 as well as miR-375-3p in the cervical part of the spinal cord of wobbler mice. In addition, for the first time we were able to demonstrate an expression of NDRG2 in motor neurons using different techniques. ***Conclusion:*** The present study has shown NDRG2 and miR-375-3p to be promising targets for further research of the pathogenesis of sporadic ALS in the wobbler mouse model. Based on these results and in combination with previous published data we could develop a putative pro-apoptotic mechanism in the spinal cord of the wobbler mouse.

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Introduction

The wobbler mouse, first described in 1956, is an important model for the sporadic form of amyotrophic lateral sclerosis (ALS), the most common motor neuron disease in adults [1]. 95% of ALS patients suffer from the sporadic form without any known hereditary component. Degeneration of the upper motor neurons in the cerebral cortex and the lower motor neurons in the brainstem and spinal cord are characteristic hallmarks for ALS. This degeneration leads to spasticity, generalized muscle weakness, atrophy and therefore progressive paralysis [2]. ALS patients die within 2-5 years after the appearance of the first symptoms due to a missing beneficial treatment [3].

The symptoms displayed by the homozygous wobbler mouse correspond to the human ALS phenotype [4, 5]. Therefore, the wobbler mouse is used to reveal the pathological mechanisms underlying the amyotrophic lateral sclerosis. A missense point mutation (L967Q) in the Vps54 gene of the wobbler mouse, a component of the Golgi-associated retrograde protein (GARP) complex that is involved in retrograde transport from early and late endosomes to the *trans*-Golgi network, was already identified, but there is still no link between the occurring symptoms and the mutation [6]. However, recently mutations in the Vps54 gene of ALS patients with moderate or even high impact were detected within the project MinE (http://databrowser.projectmine.com/) . The symptom progression of the wobbler mouse can be monitored and divided into three distinct stages [7]. From postnatal 0 (p0) to p20 there is no phenotypic difference between the wobbler mouse and their littermates. Therefore, this phase is called the presymptomatic phase. During the preclinical phase from p20 until p40 the symptoms begin to develop. The wobbler mouse stays visibly smaller than their healthy littermates, shows typical muscle weakness of the front feet and develops a head tremor [8]. From p40 the stable clinical phase starts, where the symptoms are fully developed and stagnate [9].

The N-myc downstream-regulated gene 2 (NDRG2) protein is involved in cell proliferation, differentiation, transmembrane transport but also in stress response and depression [10–12]. NDRG2 is also involved in cell death by different pathways. Within one possible signaling cascade, NDRG2 is transactivated directly by the transcription factor p53 and therefore might play a role in p53-mediated apoptosis [13, 14]. Interestingly, p53 levels are known to be elevated in the spinal cord of wobbler mice as well as ALS patients [15]. This indicates, that p53 as well as NDRG2 might play a functional role in stress induced cell death in wobbler mice as well as ALS patients. While in the past it was assumed that NDRG2 is only expressed in astrocytes and oligodendrocytes, NDRG2 was detected in pyramidal neurons of patients with Alzheimer's disease [16, 17]. A recent study found the Death-associated protein kinase 1 (DAPK1) to be a critical regulator of NDRG2 by phosphorylating it and subsequently mediate neuronal cell death during the progression of Alzheimer's disease [18, 19]. Another reason for NDRG2 to be an interesting target is its connection to FUS, a protein that is connected to the familial form of ALS (fALS) [20, 21]. Even though the consequence is not yet understood, it is known that depletion of FUS leads to alternative splicing of NDRG2 and ultimately cell death [20].

Neurodegenerative diseases are often related to deregulation of microRNA (miRNA, miR). These small molecules are able to prevent translation of proteins by binding to their specific target mRNA and either block the translation or degrade them [22]. The miRNA miR-375-3p is directly linked to NDRG2: microglia secret microvesicles containing miR-375-3p after overexpression of NDRG2 leading to a damaging effect on neuronal cells *in vitro* [23]. Contrary to this work, a neuro protective function of miR-375-3p by inhibiting p53 and therefore preventing apoptosis could be shown by Bhinge et al. [24]. They showed miR-375-3p to be essential for spinal cord motor neuron development. Downregulation of the miR-375-3p in patients with spinal muscular atrophy leads to an increase of the p53 protein level and thus to apoptosis [24]. In line with that, De Santis and colleagues have shown a decreased expression of miRNA miR-375-3p in human iPSC-derived motor neurons with a

fALS typical FUS mutation [25]. They also showed an increase of proapoptotic factors, like p53, to be due to the downregulation of miR-375-3p [25].

NDRG2 overexpression leading to neuronal degeneration through increased secretion of miR-375-3p by microglia and reduction of miR-375-3p leading to elevated level of p53 and therefore apoptosis, are basic pathological mechanisms, making miR-375-3p an interesting candidate for further research in a mouse model for ALS.

In this study we investigate the relevance of NDRG2 and miR-375-3p for the pathomechanism of the motor neuronal degeneration in wobbler mice by investigating the expression level and localization of these molecules in the cervical spinal cord.

Materials and Methods

Animals

All experiments were conducted under established standards of the German federal state of North Rhine Westphalia, in accordance with the European Communities Council Directive 2010/63/EU on the protection of animal used for scientific purposes. Breeding, handling and genotyping of the mice, which carried the wobbler mutation, was performed as previously described [8]. For each experiment the cervical part of the spinal cords of three to five animals of each group [wild type (WT) and wobbler (WR), at three different developmental stages (p0, p20, and p40)] were collected.

qPCR

Total RNA was extracted from 10-15mg cervical spinal cord tissue with the aid of the NucleoSpin miRNA Kit (#740971, Macherey-Nagel, Düren, Germany) according to manufacturer's protocol. Afterwards the tRNA was transcribed into cDNA. For analysis of NDRG2 mRNA expression, qScript cDNA Synthesis Kit (Quanta) with 500ng tRNA was used. For miRNA expression analysis, 10ng tRNA was reverse transcribed with the miRCURY LNA RT Kit (#339340, Qiagen, Exiqon, Denmark). The quantitative real-time PCR (qPCR) was performed using GoTag qPCR Master Mix (#A6001, Promega, Madison, WI, USA) on a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The housekeeping gene *GAPDH* was used for mRNA normalization and *U6* for miRNA normalization. Following primer were used: *NDRG2*-fw 5'-AGGGCGGACCTAAGTCAAAG-3' and *NDRG2*-rev 5'- ATCTCCCTGCACAATCTGCC-3', *GAPDH*-fw 5'- GGA GAA ACC TGC CAA GTA TGA -3' and *GAPDH*-rev 5'- TCC TCA GTG TAG CCC AAG A -3'. Primer used for expression analysis of miRNA were custom designs from Exiqon (Denmark): *hsa-miR-375-3p* 5'- UUUGUUCGUUCGGCUCGCGUGA and *hsa-miR-125b-5p* 5'- UCCCUGAGACCCUAACUUGUGA-3'. Expression levels were analyzed in three independent qPCR runs in triplicates and normalized with the appropriate housekeeper. Furthermore the obtained Ct-values were analyzed using the $2^{-\Delta\Delta Ct}$ method [26].

SDS-Page and Western Blotting

Protein from 10-15 mg cervical spinal cord tissue was extracted with cell lysis buffer (#9803, Cell Signaling Technology, Germany) supplemented with protease inhibitor (#11697498001, Sigma Aldrich, Germany). After determination of protein concentrations by using Pierce BCA Protein Assay Kit (#23225, Thermo Fisher Scientific, USA), 30µg of protein was applied per lane on an SDS gel. The samples were transferred with the Trans-Blot Turbo Transfer System (BioRad, USA) onto a nitrocellulose membrane and blocked with 1% RotiBlock (#A151, Roth, Germany) in 1x TBS for 1h at RT. Primary antibodies were incubated over night at 4°C. For detection of NDRG2, an polyclonal IgG antibody was used (rabbit polyclonal IgG anti-NDRG2 antibody 1:750, #HPA002896, Atlas Antibody, Sweden). Calnexin was used as housekeeper (rabbit polyclonal IgG anti-calnexin antibody 1:200, #sc-11397, Santa Cruz, USA). After washing with TBS-T, the membrane was incubated for one hour at RT with a HRP-coupled secondary antibody(goat anti-rabbit antibody 1:5000; #sc-2054, Santa Cruz, USA). Luminol reagent (#sc-2048, Santa Cruz, USA) was used for signal detection with an imaging system (ChemiDoc XRS+, Bio-Rad, USA).

To verify the Western Blot results, an SPL Western Blot was additionally performed (data not shown).

Cryosectioning

For immunostaining, *in situ* hybridization and laser micro dissection fresh/frozen cervical spinal cord tissue from p20 and p40 wildtype and wobbler mice was used. Therefore, the tissue was isolated and frozen in isopentane at –45°C. For immunostaining and *in situ* hybridization, samples were embedded in tissue freezing medium (Cryoglue #30001100, Slee, Germany) and cut on a cryostat (Leica CM 3050 S; chamber and stage temperature at -22 °C), whereas for laser micro dissection no embedding medium was used (object and chamber temperature at -15°C). Sections for *in situ* hybridization had a thickness of 16µm, while for immunostaining and laser micro dissection the samples were cut to a thickness of 10µm. Sections were collected on super frost glass slides (#J1800AMNZ, Menzel-Gläser, Germany). For laser micro dissection the sections were placed on 1.0 PEN membrane (#415190-9041-000, Carl Zeiss Microscopy GmbH, Germany). All slides were stored at -80°C until further use.

In situ hybridization

In situ hybridization was performed according to the one-day-protocol of the manual FFPE *in situ* hybridization using double-labeled Fluorescein or DIG miRCURY LNA microRNA Detection probes (Exiqon, Denmark). In brief, 16µm fresh/frozen sections of the cervical spinal cord (p20 and p40) were fixed with 4% PFA for 30min at RT and permeabilized with 1µg/ml Proteinase K for 10min at 37°C. Each probe (60nM mNDRG2_2, /5DigN/ACACACACACACTCTCTCTCCT/3Dig_N/, #651992-2, Exiqon, Denmark; 80nM mmu-miR-375-3p, /5DigN/TCACGCGAGCCGAACGAACAAA/3Dig_N/, #616554-360, Exiqon, Denmark) was denaturated at 90°C for 4min and supplemented afterwards with 200 µl *in situ* hybridization buffer (#ENZ-33808, Enzo Life Sciences, USA). A scrambled mRNA probe (#300514-15, Qiagen, Exiqon, Denmark) and a scrambled miRNA probe (#90-001, Exiqon, Denmark) were used as negative controls. This mixture was applied to the sections and incubated for 2h at 54°C in a humidified chamber. Afterwards, the tissue was washed once with 5×SSC for 5min and twice with 1×SSC and with 0.2×SSC for 5min at hybridization temperature and finally once with 0.2×SSC for 5min at RT. Blocking was performed with PBS-T supplemented with 2% sheep serum and 1% BSA for 15min at RT. For detection, incubation with an antibody against digoxigenin (Anti-Digoxigenin-AP # 11093274910, 1:800, Roche, Swiss) was performed for 1h at RT. NBT-BCIP (NBT/BCIP Ready-to-Use Tablets #11697471001, Roche, Swiss) was supplemented with levamisole (#31742, Fluka, Germany). This substrate was applied to the tissue sections and incubated for 2h at RT. The reaction was stopped with KTBT-Buffer and washed with water. Nuclear Fast Red (#N3020 Sigma-Aldrich, Germany) was used for counterstaining. The sections were washed and drained with immersing ethanol series. The samples were embedded with Eukitt® Quick-hardening mounting medium (#03989 Sigma-Aldrich, Germany) and imaged with light microscopy (Olympus microscope BX61VS, Japan; UPlanSApo 10x/0.4, Olympus, Japan).

Immunofluorescence

To co-localize NDRG2 with specific cell types of the spinal cord, immunofluorescence was used. Therefore fresh/frozen 10µm slices of the cervical part of the spinal cord (p40) were post fixated with 4% PFA for 10min at RT. Permeabilization, blocking and incubating was performed according to Table 1. NDRG2 antibody was always used for duplex staining after staining of the first structural protein, as second primary antibody. For this reason, no additional permeabilization or blocking was performed. DAPI (#D9542,

Table 1. Used conditions for duplex-immunofluorescence staining on spinal cord tissue

Protein of Interest	Permeabilization	Blocking	Primary antibody	Secondary antibody
GFAP	1% Triton/1x PBS 10min	10% goat serum/1xPBS 30min	chicken-anti-GFAP #ab4674, abcam, UK 1:500 over night at RT	anti-chicken-Alexa568 #A11041, Thermo Fisher Scientific, USA 1:1000 2h at RT
ChAT	0,3% Triton/1x PBS 10min	2% goat serum/PBS 30 min	sheep-anti-ChAT #ab18736, abcam, UK 1:1000 over night at 4°C	anti-sheep-Alexa594 #A11016, Thermo Fisher Scientific, USA 1:1000 2h at RT
CD68	0,3% Triton/1x PBS 10min	2% goat serum/PBS 30 min	rat-anti-CD68 #MCA1957T, BioRad 1:200 over night at 4 °C	anti-rat-Alexa594, #A11007, Thermo Fisher Scientific, USA 1:500 2h at RT
NDRG2	-	-	rabbit-anti-NDRG2 #HPA002896, Atlas antibodies, Sweden 1:300 over night at 4 °C	anti-rabbit-Alexa488 #A11008, Thermo Fisher Scientific, USA 1:200 2h at RT

Sigma-Aldrich, USA; 1:1000 30 min at room temperature) was used for nuclei staining and the samples were embedded with fluoromount (#F4680, Sigma-Aldrich, USA). Finally, the samples were imaged with an inverted confocal laser scanning microscope (LSM 800, Zeiss, Germany; PlanApo 20x/0.8 DICII and PlanApo 40x/1.4 Oil DICII, Nikon instruments). Secondary antibodies were tested for specificity and showed no unspecific binding.

Laser micro dissection

Laser micro dissection (PALM Micro Beam, P.A.L.M.-System, Carl Zeiss Microscopy GmbH, Germany) was used to obtain pure motor neurons as well as different regions of spinal cord sections of p40 mice. To detect motor neurons, the sections were stained with cresyl violet for 30s and then briefly washed with ethanol. Motor neurons could be detected by their specific morphology in the ventral horn of the spinal cord. Using a 40x objective and the supplied software by the manufacturer (PALMRobo 4.6 pro, Carl Zeiss Microscopy GmbH) motor neurons were marked. A non-adhesive microtube cap (MicroTube 500, Carl Zeiss Microscopy GmbH) was filled with 47µl of RNase-free-water and placed over the tissue slices. Then the marked spots were cut with a laser and catapulted into the sample cup. The samples were stored at -80°C until further use.

Mass spectrometric analysis

The mass spectrometric analysis for identification of NDRG2 in motor neuronal cells was performed by DIA analysis.

Generating of a mice specific spinal cord spectral library

First a complete spinal cord of a wild type mouse was dissected. Then the spinal cord tissue was lysed and fractionated by 1D gel electrophoresis. Afterwards in-gel tryptic digestion was performed, followed by peptide extraction. Following, a DDA analysis was performed on a QExactive HF and the spectral library was generated using Spectronaut Pulsar. For a comprehensive protocol, see preparation of human *substantia nigra* tissue for generating a spectral library described by Barkovits et al. [27].

Identification of NDRG2 in motor neuronal cells y DIA

After laser microdissection, the motor neuronal cells were prepared for mass spectrometric analysis as described in detail by Aring et al. [28]. The mass spectrometric analysis itself was performed in DIA mode on a QExactive HF. For a comprehensive description of DIA measurements and data analysis see Barkovits et al. [27].

Statistical Analysis

For statistical analysis Prism 6 (GraphPad Inc., La Jolla, CA, USA) was used. Data represent mean values of at least three independent experiments ± standard error of the mean (SEM). Data were tested for significance using *Student's t-test* and results with p-value <0.05 were considered statistically significant.

Results

Differential expression of NDRG2 in the cervical spinal cord of the wobbler mouse

To analyze the importance of NDRG2 for the pathomechanism of ALS in wobbler mice, we analyzed the mRNA and protein level in the cervical part of the spinal cord during all three developmental stages (p0, p20, p40) of the ALS within the wobbler mouse.

Gene expression of *NDRG2* was highly significant deregulated in the cervical spinal cord of the wobbler mouse, compared to wild type mouse. Interestingly, the expression level changes during disease development from a significant downregulation at p0 to a significant upregulation at p20 and p40 (Fig. 1A). In order to specify in which region of the spinal cord the deregulation of NDRG2 mRNA at the clinical stage p40 is to be found, different areas and cells were separated with the aid of laser micro dissection and examined for mRNA expression differences by means of quantitative PCR. Significantly increased expression of *NDRG2* mRNA was detected in the anterior horn, the posterior horn, and motor neurons

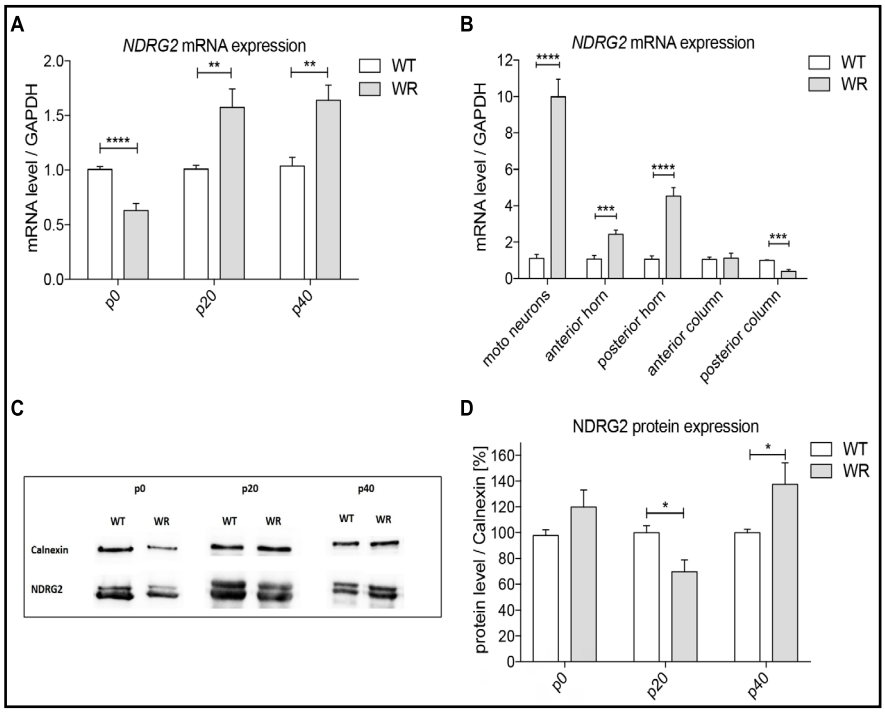

Fig. 1. Differential expression of NDRG2 gene and protein level in the cervical spinal cord of the wobbler mouse. (A) Expression levels of NDRG2 mRNA from presymptomatic to stationary phase of wild type (WT) and wobbler (WR) spinal cord were investigated by qPCR. Significant deregulation of NDRG2 was detected at all developmental stages. (B) Quantification of NDRG2 mRNA level in different regions of the spinal cord after laser micro dissection at p40. Significant deregulation was observed in all regions except the anterior column. For relative quantification of NDRG2 expression, the $2^{-\Delta\Delta Ct}$ method was conducted using the housekeeping gene GAPDH for normalization. Data are provided as means ± SEM. Data were tested for significance using Student's t-test. Significant differences are indicated by *p<0.1, **p<0.01, ****p<0.0001. n= 5-7. (C) Representative Western Blot image of NDRG2 expression levels in WT and WR mouse spinal cord at three developmental stages of the wobbler motor neuron disease. Two bands indicate two isoforms of NDRG2. Calnexin was used as control protein. (D) Quantitative analysis of NDRG2 protein expression level in the cervical spinal cord from presymptomatic to stationary phase of WT and WR spinal cord. A significant deregulation was found in developmental stages p20 and p40, while there is no significant deregulation at time point p0. Data are provided as means ± SEM. Data were tested for significance using Student's t-test. Significant differences are indicated by *p<0.1. n = 3-5.

(Fig. 1B). In contrast, the anterior column showed nearly no difference, the posterior column showed a significantly decreased *NDRG2* mRNA expression (Fig. 1B). Here we could show for the first time, that *NDRG2* mRNA is detectible in motor neuronal cells of the spinal cord in mice. Protein level of NDRG2 was also found to be differentially expressed in the cervical spinal cord of the wobbler mouse. The banding pattern in the Western Blot indicates the existence of both isoforms of NDRG2 in the spinal cord of the mice (Fig. 1C [29]). At the presymptomatic phase no significant difference of NDRG2 protein expression was found. Only a slight increase could be shown. A significantly down regulated expression of NDRG2 protein could be observed in p20, whereas a significantly up regulated expression was found in p40 (Fig. 1D). The Western Blot results were validated by SPL-Western Blot.

Noticeable is the negative correlation between the gene and protein expression of NDRG2 in the cervical spinal cord at the developmental stages p0 and p20. At the final clinical stage, where the symptoms are fully developed and stagnate, gene as well as protein levels are significantly increased.

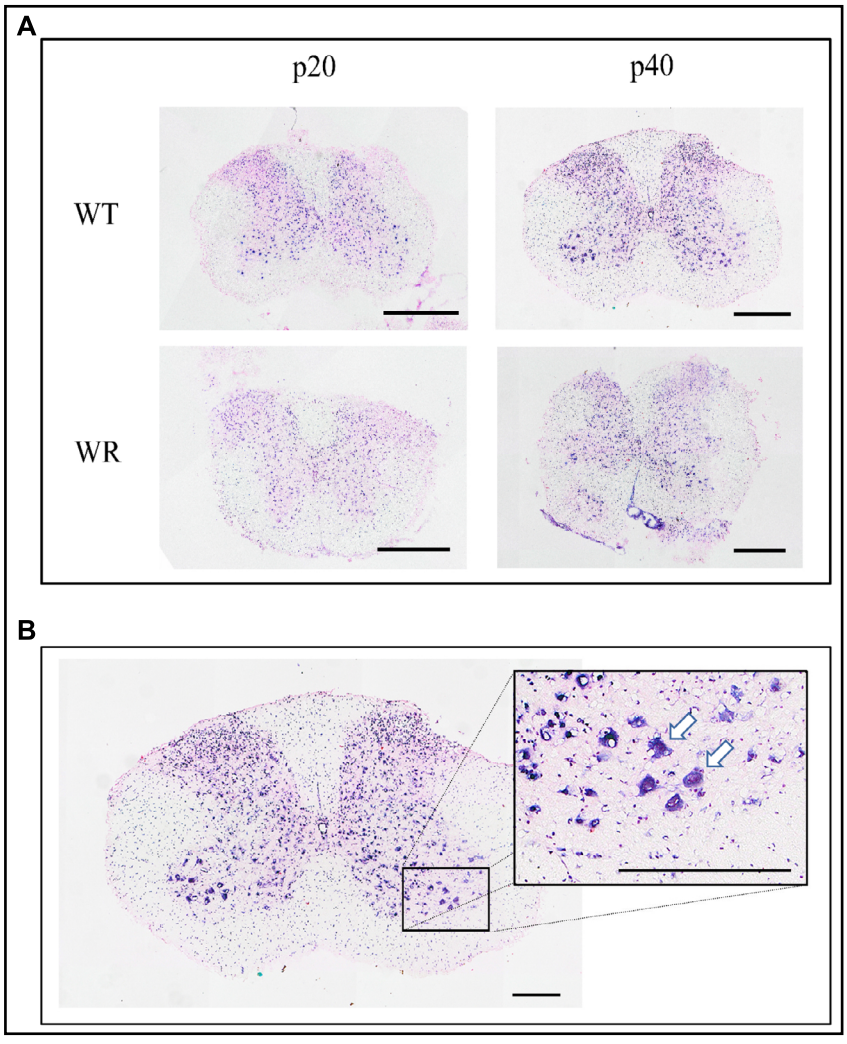

Fig. 2. Expression pattern of NDRG2 mRNA in the cervical spinal cord of wild type and wobbler mice. (A) Exemplary overview of in situ hybridization with NDRG2-probe in a cross section of the cervical spinal cord of wild type (WT) and wobbler (WR) mice at the developmental stage p20 and p40. There are no visible staining pattern differences between WT and WR. Scale bar = 1 mm. (B) Cross section of the cervical spinal cord of a wild type mouse at p40. Exemplary large-scale picture of the anterior horn after in situ hybridization with NDRG2-probe. Arrows indicate motor neurons with a clearly visible prominent staining for NDRG2. Scale bar = 500 µm. All pictures were taken with a light microscope (Olympus microscope BX61VS, Japan) and a 20x objective (UPlanSApo 20x/0.4, Olympus, Japan). For counterstaining Nuclear Fast Red was used.

Localization of NDRG2 in motor neurons

NDRG2 was always discussed as a marker for astrocytes, since it was only found to be expressed in astrocytes and oligodendrocytes but not in neurons in the brain [17, 30]. Furthermore NDRG2 was found to be present in pyramidal neurons under pathological conditions of patients with Alzheimer's disease [16]. To locate NDRG2 in the spinal cord of wild type and wobbler mice, we performed *in situ* hybridization, immunofluorescence and laser micro dissection followed by mass spectrometry.

In situ hybridization was performed with a labeled probe detecting the *NDRG2* mRNA. Staining was carried out in the cervical spinal cord of wobbler and wild type mice at the age of p20 and p40. A prominent staining of single cells can be found in the grey matter, whereas the white matter only revealed scattered light signals (Fig. 2A). At higher magnification of the anterior horn, a clear signal can be detected in large cells with a large nucleus that can be assigned to motor neuronal cells (Fig. 2B). Comparing wild type and wobbler sections during two different stages p20 and p40, no differences in *NDRG2* mRNA localization can be found. Negative controls with a scrambled mRNA probe showed no staining (Supplementary Fig. 1 - for all supplemental material see www.cellphysiolbiochem.com).

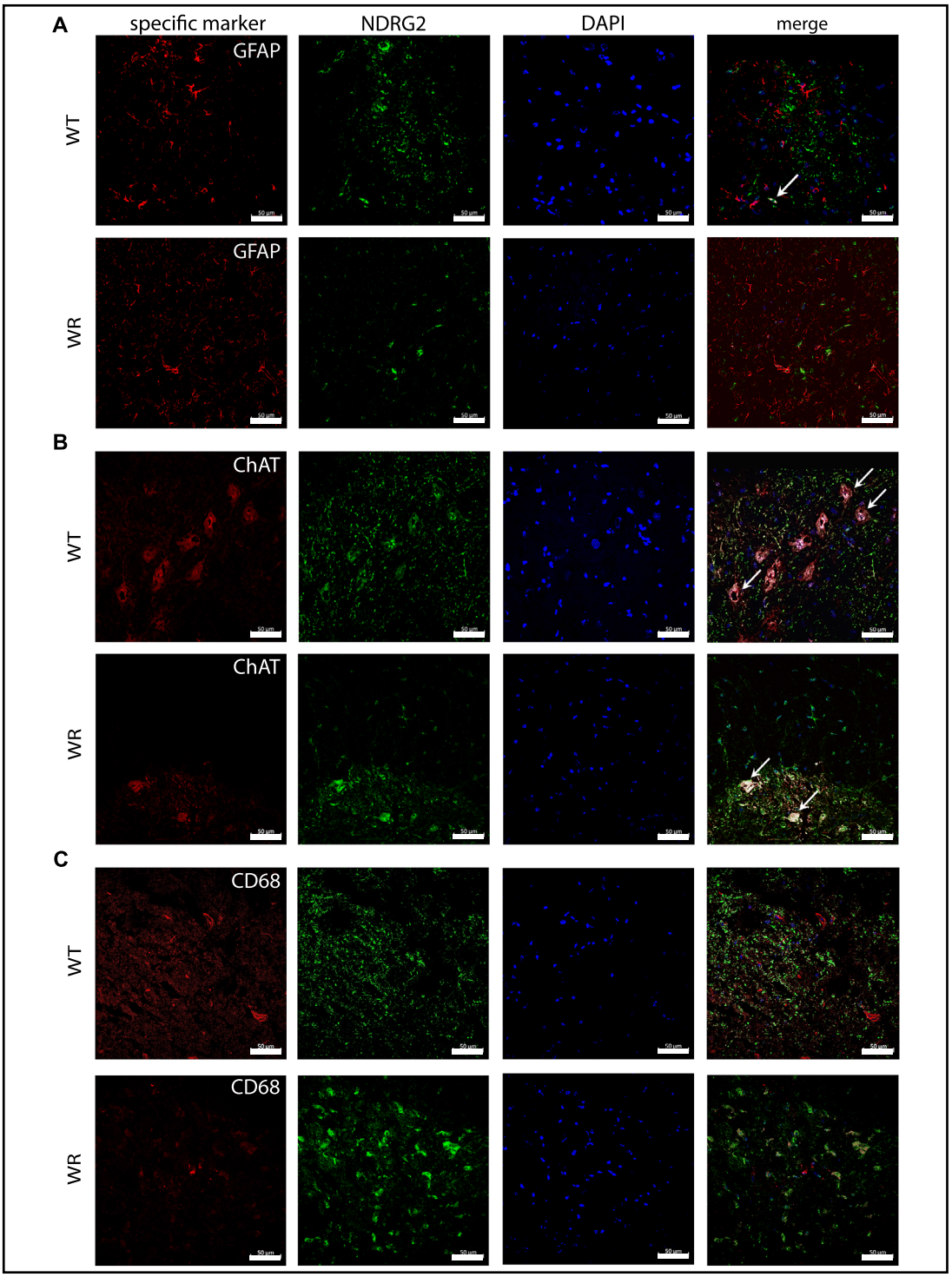

Fig. 3. Co-localization of NDRG2 with specific cell marker in immunofluorescence staining. Exemplary immunofluorescence staining of the following marker to define specific cell types: GFAP as marker for astrocytes (A), ChAT as marker for motor neurons (B) and CD68 as marker for microglial cells (C). A clear co-localization is visible for ChAT and NDRG2, whereas the other marker show nearly no co-localization. White areas in the merge channel represent a co-localization of both markers used. Nuclei were stained with DAPI (blue). Pictures were taken using a confocal laser scanning microscope (LSM 800, Zeiss, Germany with a 20x and a 40x objective (PlanApo 20x/0.8 DICII and PlanApo 40x/1.4 Oil DICII, Nikon instruments). White arrows indicate co-localized signals.

To verify the localization of NDRG2 on protein level, immunofluorescence was carried out to co-localize NDRG2 with different markers: GFAP representing astrocytes (Fig. 3A), ChAT for motor neurons (Fig. 3B) and CD68 as a marker for microglial cells (Fig. 3C). While the immunofluorescence showed nearly no co-localization of NDRG2 with GFAP and CD68, there is a clear visible overlap in the signals for ChAT and NDRG2 (Fig. 3A-C). Therefore, an expression of NDRG2 in motor neurons could be considered.

Since expression of NDRG2 in neuronal cells has always been negated, mass spectrometric analysis after laser microdissection should verify the expression of NDRG2 protein in mouse motor neuronal cells. Using DIA mass spectrometric analysis, NDRG2 could be detected with a sequence coverage of 70%. Two of 14 identified peptides had a Q-value ≤ 0.01 (ILLDQGQTHSVETPYGSVTFTVYGTPKPK; LDPTQTSFLK) and one had a Q-value of ≤ 0.05 (CPVMLVVGDQAPHEDAVVECNSKLDPTQTSFLK). This indicates an NDRG2 expression in motor neurons of the spinal cord in wobbler and wild type mice.

miR-375-3p deregulation and expression pattern in spinal cord of mice

Overexpression of NDRG2 in a microglial cell line leads to an increase of miR-375-3p expression and secretion followed by neuronal degeneration [23]. This observations, and the fact that miR-375-3p suppresses the expression of the apoptosis initiating transcription factor p53, makes miR-375-3p an interesting miRNA in regard to motor neuron degeneration [24]. We also analyzed miR-375-3p by using the LNA technology by Exiqon (Denmark) to carry out a qPCR. Gene expression of *miR-375-3p* was significantly deregulated in the cervical spinal cord of the wobbler mouse, compared to wild type mouse. The expression level changes during disease development from a significant upregulation at p0 and p20 to a significant downregulation at p40 (Fig. 4A). A prominent staining of single cells can be found in the grey matter, whereas the white matter revealed no light signals (Fig. 4B). At higher magnification of the anterior horn, a clear signal can be detected in large cells with a large nucleus that can be assigned to motor neuronal cells (Fig. 4C). Looking at the posterior horn in a larger magnification, a clear signal can also be detected in smaller cells. Comparing wild type and wobbler sections during two different stages p20 and p40, no differences in *NDRG2* mRNA localization can be found. Negative controls with a scrambled miRNA probe showed no staining (Supplementary Fig. 1).

Discussion

The protein NDRG2 is mentioned to have many different functions. Among others, NDRG2 is involved in stress response [10, 12]. It was shown, that *NDRG2* has a direct binding side for p53, playing a role in p53 mediated apoptosis [13, 14]. A possible mechanism for neurodegeneration was shown to be caused by an interplay between NDRG2 and a miRNA, miR-375-3p. miR-375-3p is expressed in dependence of NDRG2 expression level in N9 microglial cells causing damage to neuronal cells after secretion [23]. In contrast to that, miR-375-3p was shown to prevent apoptosis by inhibiting p53 [24]. Both molecules were already mentioned in context of familial ALS and therefore present interesting targets for further investigations [20, 25].

The aim of this study was to investigate the relevance of these two molecules for the pathomechanism of the motor neuronal degeneration in wobbler mice.

Using molecular techniques, we were able to show a differential expression of NDRG2 and miR-375-3p in the spinal cord of the wobbler mouse. Already immediately after birth, without pathological symptoms, the wobbler mouse exhibit significant deregulation of *NDRG2* mRNA and miR-375-3p expression. Furthermore, we were able to demonstrate for the first time the expression of NDRG2 on mRNA as well as on protein level in motor neuronal cells of the spinal cord under physiological and pathological conditions. In previous publications NDRG2 was discussed exclusively as an astrocyte marker [17, 30]. The only exceptional

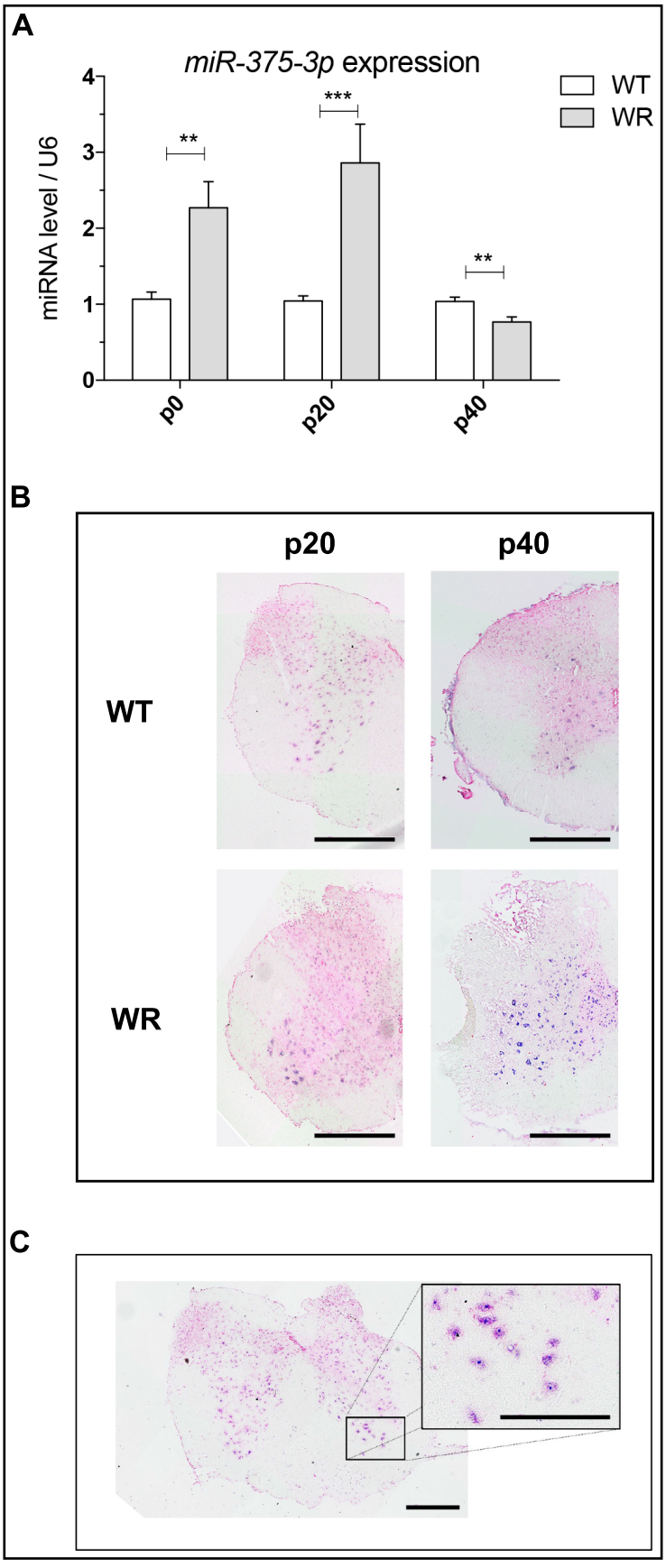

Fig. 4. Qualitative and quantitative analysis of miR-375-3p expression level in the cervical spinal cord of the wobbler (WR) in comparison to wild type (WT) mouse. (A) Expression levels of miR-375-3p from presymptomatic to stationary phase of wild type (WT) and wobbler (WR) spinal cord were investigated by qPCR. Significant deregulation of miR-375-3p was detected at all developmental stages. For relative quantification of miR-375-3p expression, the $2^{-\Delta\Delta Ct}$ method was conducted using the housekeeping gene U6 for normalization. Data are provided as means ± SEM. Data were tested for significance using Student's t-test. Significant differences are indicated by **p<0.01, ***p<0.001. n = 6-10. (B) Exemplary overview of in situ hybridization with miR-375-3p-probe in a cross section of the cervical spinal cord of wild type (WT) and wobbler (WR) mice at the developmental stage p20 and p40. There are no visible staining pattern differences between WT and WR. Scale bar = 1mm. (C) Cross section of the cervical spinal cord of a wild type mouse at p40. Exemplary large-scale picture of the anterior horn after in situ hybridization with miR-375-3p-probe. Arrows indicate motor neurons with a clearly visible prominent staining for miR-375-3p. Scale bar = 500 µm. All pictures were taken with a light microscope (Olympus microscope BX61VS, Japan) and a 20x objective (UPlanSApo 20x/0.4, Olympus, Japan). For counterstaining Nuclear Fast Red was used.

case showed Mitchelmore and colleagues in 2004. In this study, NDRG2 expression could be shown in cortical pyramidal neurons under pathological conditions of Alzheimer's disease [16].

We observed a negative correlation of NDRG2 mRNA and protein expression at p0 and p20. Several publication show, that a correlation of mRNA expression and protein abundance is not always given. In Crohn's disease, for instance, an increase of a specific gene does not correlate with the protein level [31]. Thus, analyzing both molecule levels is even more important. Possible reasons for absence of correlation are plenty, but can be resumed into two main causes: Post-transcriptional mechanisms and variations in *in vivo* half-lives [32]. Regulatory proteins as well as non-coding RNA, e.g. miRNA, take influence on translation by either promoting the binding of RNA and ribosome or blocking it [33]. Additionally, the translation efficiency depends on the density of ribosomes as well as the secondary structure of the RNA [33, 34]. Secondly, the turnover of protein and RNA can vary significantly and depend on different conditions [35].

Recently, the role of NDRG2 in the central nervous system has attracted a lot of attention. Pan et al. showed NDRG2 overexpressed leading to decreased intracellular ATP and NADPH generation as well as an elevated ROS-level [36]. Thus, leading to significantly decreased proliferation and increased apoptosis of hepatoma cells [36]. A contrary effect can be observed, if NDRG2 expression is inhibited [37]. Ma and colleagues showed a significantly decreased ROS level after reduced NDRG2 expression in astrocytes [37]. Therefore, a direct correlation between the expression level of NDRG2, oxidative stress and the associated apoptosis of cells can be hypothesized.

A recent publication showed an elevated ROS level in the cervical spinal cord of wobbler mice at p40 [38]. Deregulation of NAD^+ metabolism through a deregulated Nmnat2 expression was shown and discussed as a reason for the elevated ROS levels. The up-regulation of NDRG2 expression in the spinal cord of the wobbler mice at p40 shown in the current study, could be another reason for increased ROS and degeneration in the tissue of this ALS model. This confirms the assumption that ALS is a multifactorial disease [39].

In several publications NDRG2 was shown to be involved in the p53 mediated apoptosis [13, 14]. Liu and colleagues also found a direct p53-binding site in intron 1 of the *NDRG2* gene. This leads to a p53-dependent transactivation of NDRG2 [13]. Interestingly, they also indicated NDRG2 to be required for p53 mediated apoptosis [13]. The actual role in this process is not yet understood. In various tumor cell lines, NDRG2 was proposed to play a crucial role by inhibiting cell proliferation when overexpressed [40, 41]. Since motor neurons do not proliferate but show overexpression of NDRG2 in wobbler mouse, a different approach concerning the involvement of NDRG2 in the p53 mediated apoptosis should be considered. An overexpression of NDRG2 is known to increase the Bax/Bcl-2 ratio, which is determining cell death while both Bax and Bcl-2 are regulated by p53 [42–44]. In astrocytes, Li et al. showed a pro-apoptotic effect of Bax after overexpression of NDRG2, whereas Bcl-2 was uninfluenced [43]. This indicates, that NDRG2 might influence downstream targets of p53 and therefore contribute to the p53 mediated apoptosis.

In accordance with the here performed *in situ* hybridization, Bhinge et al. showed miR-375-3p to be expressed in motor neurons. Additionally different studies showed that miR-375-3p is essential for motor neuron development [24, 25]. Furthermore, both Binghe et al. and De Santis et al. showed in different disease models that down-regulation of miR-375-3p leads to an increase in proapoptotic factors, such as p53 [20, 24, 25]. Farther, Wang et al. demonstrated that miR-375-3p suppresses epithelial apoptosis by inhibiting JAK2/Stat3-signaling pathway [46]. Thus, miR-375-3p appears to regulate various target structures that intervene at different sites in an apoptotic pathway. In this study, we could show a significant decrease of miR-375-3p within the spinal cord of wobbler mice at the stable clinical phase (p40), whereas at p0 and p20 a significant increase of miR-375-3p was observed. According to Bhinge et al., De Santis et al., and Wang et al., miR-375-3p is able to protect cells from apoptosis, which might explain the late start of symptoms in the wobbler mouse. However, a significant down regulation of miR-375-3p could lead to an increase of p53 expression and

thus apoptosis [21, 22, 25]. It is also known that p53 is increased in both ALS patients and the wobbler mouse [15, 47]. Here, our results complement the interaction of the two molecules by elucidating the expression levels of miR-375-3p. Therefore, miR-375-3p seem to have an anti-apoptotic function in motor neurons of wobbler mice. Otherwise, it was shown that this miRNA can have a negative effect on neuronal cells. Tang and colleagues have revealed that overexpression of NDRG2 in N9 microglia leads to increased secretion of miR-375-3p. These miRNA-filled vesicles have a negative influence on neuronal cells *in vitro* in form of increased degeneration [23]. This suggests an apoptotic effect of miR-375-3p.

Some miRNAs are known to exert opposing effects depending on their expression locus. miRNA-204, for example, shows contrary effects in form of tumor suppressor or oncomiR in different prostate cancer cell lines [48, 49].

Since both molecules, NDRG2 and miR-375-3p, have a connection to p53, we used published data and our results to establish a model for a putative pro-apoptotic mechanism in the spinal cord of the wobbler mice (Fig. 5). Responding to stress signals like elevated ROS level [38], p53 is activated, and triggers apoptosis by initiating the expression of pro-apoptotic genes like Bax and Apaf-1 [50, 51]. P53 is known to be highly increased in the spinal cord of the wobbler mouse as well as ALS patients [15, 47]. miR-375-3p inhibits the expression of p53, thereby affecting *NDRG2* transcription [13, 24]. In addition to that, Pan et al. showed that an increased NDRG2 level lead to an elevated ROS level [36]. Fig. 5 shows a simplified mechanism that might occur in the spinal cord of wobbler mice at the clinical stage leading to increased apoptosis. miR-375-3p is decreased at this stage. Deregulation of miR-375-3p leads to an inefficient inhibition of translation of p53 compared to wildtype resulting in increased p53. This leads to an increased protein level of p53 and thus to an increased *NDRG2* transcription in the spinal cord of the wobbler mouse. Finally, the overexpression of NDRG2 protein leads to elevated ROS, and thus maintains the vicious circle.

Based on this model we can discuss the results for the other stages p0 and p20. At p0 the phenotype of the wobbler mouse is unobtrusive. Here we could show a decreased *NDRG2* mRNA expression without differences on protein level. Furthermore, we could show an increased miR-375-3p expression, that could lead to decreased p53 protein expression. Thus, pathologic mechanisms would not overweight. At the onset of disease at p20, we could show an increased *NDRG2* mRNA and miR375-3p expression, whereas protein expression of NDRG2 was decreased. This results in a still efficient inhibition of p53 translation as well as normal ROS level. The increased miR-375-3p could be a protective mechanism, preventing the cell to induce apoptosis by decreasing p53 and therefore maintaining the level of NDRG2.

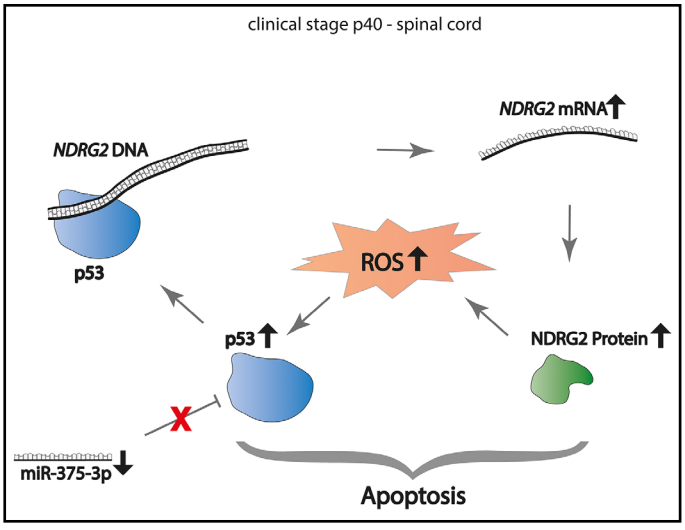

Fig. 5. Putative pro-apoptotic mechanism in the spinal cord of the wobbler mouse at the clinical stage p40. P53 is known to bind to NDRG2 and induce its transcription. Therefore NDRG is associated with the apoptotic pathway of p53 [13]. MiR-375-3p inhibits the translation of p53 by directly targeting its mRNA [24]. Additionally, p53 expression is known to be increased in the spinal cord of the wobbler mice [15]. NDRG2 mRNA and protein expression was shown to be upregulated in the spinal cord of wobbler mice at p40. Since the expression of miR-375-3p is decreased, translation of p53 is not effectively inhibited, which affects NDRG2 transcription. High level of NDRG2 can lead to elevated ROS level, which in turn can activate p53. The increased p53 and NDRG2 level lead to increased apoptosis in the spinal cord of the wobbler mice.

Additionally, a negative correlation between NDRG2 mRNA and protein level is obvious. This could raise the question of NDRG2 specific miRNAs in the protective mechanism.

Conclusion

The present study has revealed NDRG2 and miR-375-3p to be promising targets for research in regard to the pathogenesis of sALS in the wobbler mouse model. Here a deregulation of the tumor suppressor NDRG2 as well as miR-375-3p during the progression of disease in the spinal cord could be shown. Elevated NDRG2 levels lead to increased ROS formation, which serve as a stress signal for the activation of p53. Due to downregulation of miR-375-3p, inefficient inhibition of p53 leads to overexpression of NDRG2, increasing ROS production and maintaining a vicious cycle.

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Disclosure Statement

The authors state that there are no personal or institutional conflicts of interest.

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