MicroRNA miR-204 regulates proliferation and differentiation of oligodendroglia in culture

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Abstract
Oligodendrocytes wrap and physically shield axons of the central nervous system with myelin sheaths, resulting in rapid signal transduction and accurate neuronal function. The complex oligodendroglial development from immature oligodendrocyte precursor cells (OPCs) to myelinating oligodendrocytes (OLs) is profoundly dependent on the activity of transcription factors of the Sox protein family. Target genes of the crucial regulator Sox10 have recently been expanded to microRNAs. Here, we report miR-204 as a novel transcriptional target of Sox10. Regulatory regions of miR-204 show responsiveness to and binding of Sox10 in reporter gene assays and electromobility shift assays. Once expressed, miR-204 inhibits OPC proliferation and facilitates differentiation into OLs in the presence of Sox10 as evident from over-expression in primary rat and mouse oligodendroglial cultures. Phenotypes are at least in part caused by miR-204-dependent repression of the pro-proliferative Ccnd2 and the differentiation inhibiting Sox4. These findings argue that the transcriptional activator Sox10 forces oligodendroglial cells to exit the cell cycle and start differentiation by gene inhibition via miR-204 induction.

KEYWORDS
glia, microRNA, myelination, proliferation, regulatory network, Sox proteins

1 | INTRODUCTION

MicroRNAs (miRs) are increasingly recognized as important regulatory factors in oligodendroglia in development and disease. They are necessary when myelinating glia of the central nervous system (CNS) develop from oligodendrocyte precursor cells (OPCs) either during embryonic myelination or during remyelination in de- and dysmyelinating diseases such as multiple sclerosis and leukodystrophies (for review, see Galloway & Moore, 2016). For instance, measurement of miR levels in different subtypes of multiple sclerosis lesions showed a significant dysregulation in the expression of at least 50 miRs (Junker et al., 2009; Noorbakhsh et al., 2011). The importance of miRs is even more obvious from diverse mouse mutants with deletion of components of the miR processing machinery such as the endoribonuclease Dicer. In the absence of Dicer, mature miRs are not cleaved from pre-miR precursors so that mature miRs are completely missing. Early Olig1-Cre or Olig2-Cre driven deletion of Dicer results in strongly reduced differentiation into mature myelinating cells during development. Deletion by CNP-Cre at a slightly later stage, before OPCs start to differentiate, impairs myelination as well and causes tremor and motor deficits typical for dysmyelination (Dugas et al., 2010; Zhao et al., 2010). Inducible Dicer knockout in already mature oligodendrocytes (OLs) by PLP-Cre-ERT2 induces CNS demyelination showing that miRs also help to maintain myelin in the adult (Shin, Shin, McManus, Ptacek, & Fu, 2009). Since deletion of Dicer affects all miRs, these mouse mutants show that the presence of miRs is crucial...
for maturation and maintenance of myelinating OLs at embryonic and postnatal stages.

Nonetheless, specific functions of single miRs still have to be identified. Only few miRs are characterized in their role of fine-tuning the complex network of transcription factors that controls differentiation from the OPC to the mature OL. The small inhibitory RNAs recognize complementary binding sites in target mRNAs and lead to their degradation or inhibition of translation. For example, miR-338, miR-219, and miR-138 support differentiation of oligodendroglia by targeting mRNAs of factors that keep the cells in a precursor stage (Dugas et al., 2010; Zhao et al., 2010). On the other hand, repression of the pro-myelinating transcription factor Myrf by miR-145 keeps cells in an undifferentiated stage and inhibits precocious maturation (Hoffmann et al., 2014). The transient expression of Tcf7l2, another transcription factor involved in OL differentiation, is under control of Sox10 (Cantone et al., 2019; Reiprich et al., 2017). Some additional miRs show changes in expression when Sox10 is missing. Their role needs to be identified. Only few miRs are characterized in their role of fine-tuning of Sox10 are also miRs with relevance in OL development. Sox10 binds evolutionary conserved regions (ECRs) from different miR-338, miR-335, and miR-155 transcription factor involved in OL differentiation, is under control of (Cantone et al., 2019).

Transcription factors are not only targets of miRs, but also regulators of miR expression. The HMG-box protein Sox10 is indispensable for proper differentiation of OLs. In the absence of Sox10, OPCs are specified, but do not differentiate (Stolt et al., 2002). Among the target genes of Sox10 are also miRs with relevance in OL development. Sox10 binds and activates regulatory regions of miR-338, miR-335, and miR-155 (Cantone et al., 2019; Reiprich et al., 2017). Some additional miRs show changes in expression when Sox10 is missing. Their role needs to be determined.

Here, we identify miR-204 as a regulator of OL proliferation and differentiation. We report that miR-204 expression depends on the oligodendroglial differentiation stage and on the presence of Sox10. Sox10 binds and activates evolutionary conserved regions (ECRs) from the miR-204 genetic locus. We show that miR-204 reduces proliferation and induces differentiation of primary OLs, partially by regulating Ccnd2 and Sox4. Therefore, miR-204 fulfills an important function in the progression from OPCs to OLs downstream of Sox10.

2 METHODS

2.1 Plasmids and viruses

To analyze the translational capability by Sox10, ECRs upstream of the miR-204 genetic locus were amplified from rat genomic DNA by PCR using primers 5′-AGCGTACCCACATGACACACACAGAGACGAC-3′ and 5′-AGCTCGAGGCTGCTGCTGCTGCCAATA-3′ for ECR1 (chr1:246898114-246898533; Rn5), 5′-AGGGTATCGAAGCTTTCCGGGTAGGATTTCCTG-3′ and 5′-AGCTCGAGGCTGCTGCTGCTGCCAATA-3′ for ECR2 (chr1:247022526-247023064; Rn5), 5′-AGCGGATCCACGCAGCTTTCCGAGCTGCTGCTGCTGCCAATA-3′ for ECR3 (chr1:247636913-247637432; Rn5) and 5′-AGCGGATCCACGCAGCTTTCCGAGCTGCTGCTGCTGCCAATA-3′ and 5′-AGCTCGAGGCTGCTGCTGCTGCCAATA-3′ for ECR4 (chr1:247646153-247646200; Rn5). They were placed in front of a beta globin minimal promoter and the luciferase open reading frame. Site directed mutagenesis of Sox10 binding sites (Figure 3c,d) were again performed with the Q5 Site-Directed Mutagenesis Kit (New England Biolabs) using primers 5′-GGTCATTGAGCAGTTAAAAAC-3′ and 5′-AGAGAGAGAGAGAGGAGGTCTACCGAGCTGCTGCTGCTG-3′ for Ccnd2 and as well as 5′-AGCTCGAGGCTGCTGCTGCTGCCAATA-3′ and 5′-AGCGGATCCACGCAGCTTTCCGAGCTGCTGCTGCTGCCAATA-3′ for Sox4. Site-directed mutations of miR-204 binding sites within the analyzed UTRs (see Figure 7b) were again performed with the Q5 Site-Directed Mutagenesis Kit using primers 5′-AGCTCGAGGCTGCTGCTGCTGCCAATA-3′ and 5′-AGCTCGAGGCTGCTGCTGCTGCCAATA-3′ for Ccnd2 and as well as 5′-AGCTCGAGGCTGCTGCTGCTGCCAATA-3′ and 5′-AGCGGATCCACGCAGCTTTCCGAGCTGCTGCTGCTGCCAATA-3′ for Sox4. For transfection of shRNA against Ccnd2, oligonucleotides 5′-GATCCCCCTCTCCGGCAATGTTCATTGACATGGAGAAGCAGAGTCGCCACCTCGAGCAGCTGCTGCTGCTGCCAATA-3′ and 5′-CCAGCTGCGCGCGGGG-3′ were annealed, enzymatically cut and inserted into the pSuper.neo+gfp (Oligoenine) plasmid. For the corresponding scrambled probe, oligonucleotides 5′-GATCCCCCATCGTACGCACCTTCGAGCTGCTGCTGCTGCCAATA-3′ and 5′-AGCTCGAGGCTGCTGCTGCTGCCAATA-3′ were inserted in pSuper.neo+gfp for miR-204 overexpression in Oln93 cells. For overexpression of rat Ccnd2, the open reading frame was amplified using 5′-AGCGGATCCACGCAGCTTTCCGAGCTGCTGCTGCTGCCAATA-3′ sequence with up- and downstream flanking sites from mouse genomic DNA and insertion in pCAG-ires-GFP by using primers 5′-CCGTTATGACAAAGGGAAGGG-3′ and 5′-CCGTTATGACAAAGGGAAGGG-3′ for miR-204 overexpression in Oln93 cells. For overexpression of rat Sox4 was from pCMV5-Sox4 expression plasmid (Kuhlbrodt, Herbarth, Sock, Enderich, et al., 1998). To analyze the effect of miR-204 on the translation of putative target genes, 3′-UTR fragments of those targets containing predicted or reported miR-204 binding sites were amplified from mouse genomic DNA by PCR with primers 5′-AGCTCGAGGCTGCTGCTGCTGCCAATA-3′ and 5′-AGCGGATCCACGCAGCTTTCCGAGCTGCTGCTGCTGCCAATA-3′ for ECR3 and 5′-AGCTCGAGGCTGCTGCTGCTGCCAATA-3′ and 5′-AGCTCGAGGCTGCTGCTGCTGCCAATA-3′ for ECR4.
2.2 | Cell culture, transduction, BrdU administration and transfection

Rat Oln93 oligodendroglial cells, mouse Neuro2a neuroblastoma cells and human embryonic kidney (HEK) 293 cells were kept in Dulbecco’s Modified Eagle’s Medium with 10% fetal calf serum (FCS).

To obtain primary rat oligodendroglia, mixed glial cultures were prepared from brains of newborn animals. Following shake-off (McCarthy & de Vellis, 1980) cells were grown on poly-ornithine and either kept in N2-supplemented medium containing 10 ng/ml PDGF-AA and 10 ng/ml Fgf2 to retain proliferative properties or treated with SATO medium containing 0.5% FCS to ensure differentiation. For analysis of miR overexpression by immunocytochemistry, rat OPCs were transduced in proliferation medium with miR-204 containing retrovirus and medium was changed 24 hr posttransduction. Analysis of proliferation rates was performed in 12-well plates with 200,000 cells per well by administration of 3 μg/ml BrdU 72 hr posttransduction. Cells underwent fixation 4 hr after BrdU treatment for consecutive immunocytochemical stainings. Transfection of siRNAs in primary oligodendroglia was performed using the siGenome system. Cells were transfected with DharmaFECT Duo transfection reagent and siRNA against Sox4 (siGenome Rat Sox4 [364712] siRNA) or a nontargeting siRNA control (siGenome Non-Targeting siRNA #3), each in combination with siGLO Green Transfection Indicator according to the manufacturer’s instruction. All products for siRNA experiments were obtained from Dharmacon. Analysis of oligodendroglial differentiation was performed in 12-well plates with 150,000 primary OPCs seeded per well.

To retrieve primary mouse oligodendroglia, mixed glial cultures from brains of newborn mice carrying floxed Sox10 alleles were prepared (Finzsch et al., 2010). After shake-off, cells were kept on PDL/Laminin-coated coverslips in medium supplemented with B27, N2, and ITS either with 10 ng/ml PDGF-AA and 10 ng/ml Fgf2 to keep cells in proliferation or with 1% FCS to initiate differentiation. For retroviral transduction, 250,000 primary mouse oligodendroglia per well were grown in proliferation conditions before medium was changed to differentiation conditions 24 hr posttransduction. In parallel to transduction, cells were treated with 0.2 pmol/ml Tat-Cre protein to ensure in vitro deletion of Sox10 (Peitz, Pfannkuche, Rajewsky, & Edenhofner, 2002).

Sox10-deficient Oln93 cells carrying a doxycycline-inducible full length Sox10 construct were generated by transfection of the Oln93 Sox10 KO line with a Sox10 and GFP expressing pSBtet plasmid (pSBtet-GN was a gift from Eric Kowarz; Addgene plasmid #60501, Kowarz et al., 2015) followed by enrichment for transfected cells by fluorescence activated cell-sorting and single cell seeding in 96-well plates. Expression of Sox10 was induced by treating cells with DMEM containing 10% FCS and doxycycline (1 μg/ml) for 3 days.

Sox10-deficient Oln93 cell lines carrying either pCAG-IRE5-GFP, pCAG-IRE5-GFP-mir-204, pEF1α-IRE5-GFP, or pEF1α-IRE5-GFP-Sox10 were generated by transfection of the Oln93 Sox10 KO line with corresponding viral constructs, followed by fluorescence activated cell-sorting and bulk seeding in 10 cm plates.

2.3 | Luciferase assays, electromobility shift assays, and Western blotting

In luciferase reporter assays Oln93 and Neuro2a cells were treated with SuperFect Reagent (Qiagen) or polyethylenimine for transfection of 0.25 μg reporter plasmid together with 0.15 μg pCMV5 or CAG-IRE5-GFP-based plasmids. Samples were transfected as triplicates in 24-well plates and whole cell extracts were obtained for measuring luciferase activity 48 hr (pCMV5-based experiments) or 72 hr (CAG-IRE5-GFP-based experiments) after transfection. Oln93 transfection for immunocytochemical stainings was performed using polyethylenimine and 2 μg of plasmid DNA.

Extracts used as protein source for electromobility shift assays (EMSA) were prepared as previously described (Kuhlbrodt, Herbarth, Sock, Enderich, et al., 1998). In brief, HEK293 cells were transected with 16 μg pCMV-Sox10 expression plasmid per 100-mm dish by polyethylenimine treatment and harvested 48 hr posttransfection, followed by preparation of whole cell extracts. Double-stranded oligonucleotides S1–S7 of ECR2-4, which contained putative Sox10 binding sites, and an oligonucleotide containing Site B of the Mpz gene were labeled with radioisotope 32P in order to perform EMSAs. In the presence of HEK293 extracts and poly-dGdC as unspecific competitor, labeled oligonucleotides of 28 bp length were analyzed. Site B served as control for monomeric Sox10 binding (Peirano, Goerich, Riethmacher, & Wegner, 2000).

Cell extracts for SDS-PAGE and Western blotting were prepared as described (Kuhlbrodt, Herbarth, Sock, Enderich, et al., 1998). In short, HEK293 cells were transfected in 3.5 cm plates with 0.5 μg Ccnd2 or Sox4 expression plasmid together with either 1.5 μg pSuper.neo+gfp-Ccnd2-shRNA, an siRNA against Sox4 or a corresponding control construct using DharmaFECT Duo transfection reagent. Cells were harvested 72 hr after transfection and whole cell extracts were prepared. Western blotting was performed as previously reported, using guinea pig anti-Sox4 antiserum (1:3,000 dilution, Hoser et al., 2008), rabbit anti-Ccnd2 antiserum (1:500 dilution, Proteintech), rabbit anti-GAPDH antiserum (1:3,000 dilution, Santa Cruz Biotechnology), protein A coupled to HRP (Bio-Rad) and Luminol reagent for detection (Bischof, Weider, Küspert, Nave, & Wegner, 2015).

2.4 | Immunocytochemistry

Cells were fixed in 3% paraformaldehyde. For immunocytochemical stainings, guinea pig anti-Sox4 antiserum (1:1,000 dilution, Maka, Stolt, & Wegner, 2005), rabbit anti-Gfp antiserum (1:10,000 dilution, Invitrogen), rabbit anti-Myrf antiserum (1:3,000 dilution, Horning et al., 2013), rabbit anti-Ki67 antiserum (1:500 dilution, Lab Vision), rat anti-BrdU antiserum (1:250 dilution, Abcam), rat anti-Gfp antiserum (1:10,000 dilution, Nacalai Tesque Inc.), and rat anti-Mbp antiserum (1:750 dilution, Serotec) were used as primary antibodies. Secondary antibodies coupled to Alexa488 (Molecular Probes), Cy3 (Dianova), or Cy5 (Dianova) were used for detection. For assessment of cell
numbers, nuclei were counterstained with 4',6-diamidino-2-phenylindole. Micrographs of samples were taken with a Leica DMI 6000B inverted microscope (Leica) combined with a DFC 360FX camera (Leica).

2.5 | RNA preparation from mice and cultured cells

Mice were kept at 12/12 hr light–dark cycles with permanent access to drinking water and food in accordance with animal welfare laws. All mice used for this publication were on C3H background. Tissue was prepared at postnatal days (P) 7, 14, and 21 of both males and females. Animals were killed by decapitation and brains were isolated. Callosal areas were excised and RNA was isolated using TRIZOL reagent (Invitrogen). All experiments were approved by the responsible local committees and government bodies. RNA isolation from Oln93 cells was performed according to the manufacturer’s instructions either using the miRvana miRNA isolation kit (Ambion, Thermo-Fisher) for the analysis of miR expression or the RNeasy Micro Kit (Qiagen) for the analysis of mRNA expression. For analysis of miR expression in primary rat OPCs, cells were harvested with TRIZOL reagent (Invitrogen) and RNA was isolated.

2.6 | Quantitative RT-PCR

RNA samples prepared from Oln93 cells were reverse transcribed and subjected to quantitative PCR (Bio-Rad CFX96 Real-Time PCR System) using the primers 5'-CGATGTGGATTGTCTCAAAGCC-3' and 5'-TGAGGGCTCTCCTGTAAGCA-3' for Ccnd2. Transcript levels were normalized to Rpl8. Target-specific cDNA synthesis with TaqMan MicroRNA Reverse Transcription kit was performed on RNA samples prepared from Oln93 cells, mouse brain and primary rat OPCs to address miR-204 expression levels. miR-204-5p (hsa-miR-204, Assay ID 000508) expression was measured by quantitative PCR (Bio-Rad CFX96 Real-Time PCR System) using the TaqMan Universal Master Mix II. Samples were normalized to U6 (U6 snRNA, Assay ID 001973).

2.7 | Bioinformatics and statistical analysis

TargetScan 7.1 was used for the prediction of target genes of murine miR-204-5p (Lewis, Burge, & Bartel, 2005) and the analysis of target 3'-UTR profiles (Nam et al., 2014). Gene ontology terms-subcategory biological processes (GO-BP) for predicted miR-204-5p targets were determined by the use of DAVID 6.8 (Huang Da, Sherman, & Lempicki, 2009a; Huang Da, Sherman, & Lempicki, 2009b). miR-204 ECRs were checked for potential transcription factor binding sites using P-Match (Kel et al., 2003). Quantification of Western blotting was performed using Fiji ImageJ (Schindelin et al., 2012). Experimental results from independent animals, as well as from independent transfection and transduction experiments were treated as biological replicates (n ≥ 3). Differences in cell numbers, transcript expression, protein levels, or luciferase activities were analyzed by two-tailed Student’s t test for statistical significance (*, p ≤ .05; **, p ≤ .01; ***, p ≤ .001).

3 | RESULTS

3.1 | miR-204-5p is differentially expressed in oligodendroglia

In a quantitative RT-PCR (qrt-PCR)-based high-throughput screening of miR expression in the oligodendroglial cell line Oln93, we identified a number of miRs that were strongly downregulated in the absence of Sox10 (Reiprich et al., 2017). We analyzed predicted target genes of the downregulated miRs for associated biological processes (BP) by GO term analysis and identified miR-204-5p as miR with the strongest relation of putative targets to CNS development, OL myelination and cell proliferation (Figure 1). Individual qrt-PCR experiments for miR-204-5p in wild-type versus Sox10-deficient Oln93 cells confirmed the reduction of miR-204-5p expression in the absence of Sox10 as seen by a near complete loss of miR-204-5p expression in Sox10-deficient Oln93 cells (Figure 1b). To check for differential expression during differentiation, we isolated RNA from primary rat oligodendroglia in the OPC stage (d0) and after 6 days in differentiation medium (d6). In the differentiated stage, miR-204-5p expression was increased 3.5-fold compared to the precursor stage (Figure 1c).

Next, we wanted to see, whether miR-204-5p expression also increases in vivo during developmental myelination. As miR-204-5p expression has been reported in neurons (Conte et al., 2014; Mohamed et al., 2016), we isolated tissue enriched for mouse corpus callosum as the region with the highest density of oligodendroglia at P7, P14, and P21, to specifically assess oligodendroglial miR-204-5p expression during the active phase of developmental myelination. In corpus callosum cDNA, we determined a 3.4-fold increase in miR-204-5p expression by qrt-PCRs from P7 to P21 coincident with maximum induction of myelin gene expression (Figure 1d). We conclude from expression analyses and GO-BP studies that miR-204-5p is involved in oligodendroglial development, and that its expression depends on the presence of Sox10 and increases during differentiation of oligodendroglia.

3.2 | Sox10 binds and activates regulatory regions of the miR-204 genetic locus

To analyze whether reduction of miR-204-5p expression in the Sox10-deficient Oln93 cell line is a direct effect, we reintroduced Sox10 in Sox10-KO Oln93 cells by transfection with an inducible Sox10 expression vector and subsequent FACS of transfected cells. Indeed, re-expression of Sox10 led to a reactivation of miR-204-5p expression as shown by qrt-PCR (Figure 2a). We made use of publicly available ChIP-seq data for genomic occupancy by Sox10 in
oligodendroglia (Lopez-Anido et al., 2015) and overlapped Sox10-binding with evolutionary conservation. Upstream of the genetic locus of \( \text{miR-204} \) four such ECRs were identified (Figure 2b). \( \text{miR-204} \) is encoded in intron VI of the \( \text{Trpm3} \) gene in the mouse. ECR1 and ECR2 are located upstream of the transcriptional start site of \( \text{Trpm3} \), ECR3 lies in intron I and ECR4 in intron III. In reporter gene assays in oligodendrogial Oln93 cells and Neuro2a neuroblastoma cells, all ECRs showed responsiveness to Sox10 (Figure 2c,d). While luciferase expression was highly increased for ECR2-containing reporter constructs (48-fold in Oln93 cells and 398-fold in Neuro2a cells), ECR3 and ECR4 showed a medium activation in response to Sox10 (ninefold in Oln93 cells and 50-fold in Neuro2a cells for ECR3, 13-fold in Oln93 cells and 40-fold in Neuro2a cells for ECR4). ECR1 in contrast was only weakly induced and therefore not considered in further analysis.

A more detailed sequence analysis yielded three potential Sox protein binding sites in ECR2 (S1, S2, S3), two potential binding sites in ECR3 (S4, S5) and two in ECR4 (S6, S7) (Figure 3a). Potential binding sites were defined as completely matching the Sox consensus sequence \( 5'-\text{A/T A/T CAA A/T G-3'} \). Sites closely spaced in the typical arrangement for dimeric binding were not present in the ECRs so that all potential sites are predicted to be monomeric binding sites (Peirano & Wegner, 2000). In EMSAs, we observed binding of full-length Sox10 to oligonucleotides containing site S2 or S3 of ECR2,
but not to S1 (Figure 3b). Site S5 in ECR3 showed the strongest binding of Sox10, while S4 was not bound. In ECR4, the oligonucleotide containing S7 showed complex formation with Sox10, whereas S6 was negative for Sox10 binding (Figure 3b,c). In further analyses, no Sox10 binding was detected to sites with one mismatch compared to the consensus site (data not shown). Having identified binding sites S2, S3, S5, and S7, we wanted to validate their functional relevance for Sox10-dependent ECR activation and mutated the sites in the context of the complete ECRs. Reporter gene plasmids carrying the mutated ECRs were again analyzed for responsiveness to Sox10 (Figure 3d). In transient transfections in Neuro2a cells, both ECR2-S2m and ECR2-S3m showed a reduced activation by Sox10 as compared to wild-type ECR2. The stronger reduction of reporter gene expression for ECR2-S3m argues that S3 might be more important for activation by Sox10 than S2. Simultaneous mutation of S2 and S3 reduced induction by Sox10 from 137-fold in the wild-type ECR2 to a residual 16-fold in ECR2-S2+3m. Mutation of sites S5 or S7, which had shown binding within ECR3 or ECR4, also significantly reduced activation by Sox10 to 25% or less compared to the corresponding wild-type ECRs. Considering the strong reduction in Sox10 responsiveness in the mutant ECRs, we conclude that we identified the major Sox10 binding sites within the ECRs. We show that three of four ECRs upstream of miR-204 are activated by Sox10 and that binding of Sox10 occurs to specific and functionally relevant sites.

3.3 | miR-204 reduces proliferation of primary oligodendroglia

Differential expression of miR-204-5p in progenitor and differentiated state in oligodendroglia hints to a possible impact during specific phases. We therefore asked, whether proliferation of primary oligodendroglia is changed after overexpression of miR-204. miR-204 was introduced in primary rat OPCs by transduction with a miR-204 encoding retrovirus that can be visualized by GFP expression. Average transduction rates were determined as 22 ± 2%. After 3 days in proliferation medium, BrdU was added to the medium for 4 hr. Cells transduced with a control retrovirus were used as control. Among transduced GFP-positive cells, 52% were positive for BrdU and had therefore actively started DNA synthesis and entered S-phase (Figure 4a–g). The fraction of BrdU-positive cells was reduced to 35% when miR-204 was overexpressed. To label cells in the complete interphase instead of S-phase only, we stained for the Ki67 marker and counted transduced GFP-expressing cells that were positive for Ki67 (Figure 4h–m). After control
transduction, the fraction of proliferative cells among all transduced cells amounted to 89% (Figure 4n) and was comparable to the percentage of Ki67-positive cells among nontransduced cells (data not shown). In contrast, proliferative cells among miR-transduced cells were reduced to 75% (Figure 4n) corresponding to a 2.4-fold increase in nonproliferative cells. We conclude that significantly less cells undergo proliferation when miR-204 is present in OPCs at high levels.

3.4 | miR-204 induces differentiation of primary oligodendroglia

Next, we wanted to know whether increased exit from the cell cycle coincides with higher rates of differentiation. Therefore, we transduced primary rat OPCs with control or miR-204 encoding retrovirus and incubated the cells in differentiation medium for 6 days. Immunocytochemical stainings for the myelin protein Mbp (myelin basic protein) confirmed an increased rate of differentiation upon miR-204 overexpression (Figure 5a–f). Among transduced GFP-positive cells, 53% expressed Mbp in the control and 69% after miR-204 overexpression (Figure 5g). A comparable increase in Mbp-positive cells in miR-204 overexpressing cells was observed after 4 days in differentiation medium (data not shown). Myrf (myelin regulatory factor), a transcription factor that is expressed from the onset of differentiation into the mature OL state, was expressed in 49% of nontransduced cells (data not shown) and in 47% of oligodendroglial cells transduced with control retrovirus, while 63% of cells transduced with miR-204 encoding retrovirus were Myrf-positive (Figure 5h–n). Thus, primary oligodendroglia differentiate more efficiently in the presence of elevated levels of miR-204.

Considering the stimulating effect of miR-204 on differentiation, we asked if reintroduction of miR-204 in a Sox10-negative background can at least in part rescue the previously reported differentiation failure of Sox10-mutant oligodendroglia (Stolt et al., 2002). Primary mouse OPCs with both Sox10 alleles floxed were treated with Tat-Cre protein to achieve in vitro deletion. In parallel, cells were transduced with either control or miR-204 encoding retrovirus. As evident from immunocytochemical staining, Sox10 deletion with Tat-Cre protein occurred only in approximately half the cells. This allowed us to compare the effects of miR-204 overexpression in

**FIGURE 3** Binding analysis of Sox10 to evolutionary conserved regions (ECRs) and mutation of binding sites show direct transcriptional regulation of miR-204 by Sox10. (a) Schematic representation of positions of Sox10 consensus motifs in ECR2-4 as black circles (S1–S7). (b) Electromobility shift assays (EMSAs) were performed with oligonucleotides containing potential binding sites S1–S7. S1–S7 were incubated without cell extract (−) or with extract from human embryonic kidney (HEK) 293 cells transfected with empty pCMV vector (C) or full length Sox10 expression vector (S10). Site B of the Mpz promoter served as positive control for monomeric binding (Peirano et al., 2000). (c) Sequence of the Sox10 consensus motif and putative binding sites S1–S7 analyzed in EMSAs. Sites bound by Sox10 in EMSAs are indicated by black letters, sites showing no binding of Sox10 by gray letters. (d) Luciferase assays were performed in Neuro2a cells using reporter plasmids containing ECR2 (black bars), ECR3 (gray bars), or ECR4 (white bars) either in the wild-type (WT) version or with single (S2m, S3m, S5m, S7m) or combined (S2+3m) mutations of Sox10 binding sites. Luciferase activities were measured 48 hr after cotransfection with empty pCMV plasmid as control or Sox10-expression plasmid. Luciferase activities in the control were arbitrarily set to 1 (bars not shown) and activities in the presence of Sox10 are shown relative to the control (mean + SD; n = 3). Statistically significant differences between WT and mutant ECRs are indicated (Student’s t test; **, p ≤ .01; ***, p ≤ .001)
Sox10-positive and Sox10-negative cells of the same cultures (Figure 6a–d). Similar to experiments in primary rat oligodendroglia, miR-204 overexpression in Sox10-positive mouse OPCs led to a significant increase of Myrf-positive, differentiating OLs from 65% in the control to 80% (Figure 6e). Deletion of Sox10 on the other hand decreased the number of Myrf-expressing cells to approximately 10–11% confirming the essential function of Sox10 in terminal differentiation (Figure 6e). This dramatic decrease was comparable for control-transduced and miR-204-transduced cells. Overexpression of miR-204 in the Sox10-negative background was therefore not sufficient to overcome the differentiation deficit. We thus conclude that miR-204 can only facilitate differentiation when Sox10 is present and activates myelin gene expression in addition.

### 3.5 miR-204 regulates oligodendroglial proliferation and differentiation partially by targeting Ccnd2 and Sox4

As we have observed reduced proliferation and increased differentiation, we asked which target genes of miR-204 might contribute to these phenotypes. Based on previous studies (Wu, Pan, et al., 2015; Wu, Zeng, et al., 2015) and target gene prediction programs, we hypothesized that miR-204-5p may target Ccnd2 and Sox4 mRNAs in oligodendroglia. To study the impact of miR-204 on the putative targets, fragments of the 3’-UTRs of the corresponding mRNAs with predicted miR binding sites were inserted downstream of the luciferase coding sequence into reporter plasmids in wild-type or mutated versions (Figure 7a,b). A reporter with the wild-type fragment of the Ccnd2-3’-UTR behind the luciferase coding sequence showed responsiveness to miR-204 in Neuro2a cells (Figure 7c). In the presence of
miR-204, reporter gene activity was reduced to 38% compared to samples transfected with an empty control plasmid. When the corresponding mutated version of the 3′-UTR lacking the predicted miR-204-5p binding site was used, the effect was largely absent with reporter gene activity amounting to 81% compared to controls. The residual reduction in reporter activity was attributed to putative additional imperfect miR-204 binding sites in the Ccnd2-3′-UTR fragment. Analogous experiments for the Sox4-3′-UTR with wild-type miR-204-5p binding site (Figure 7b) revealed comparable effects of miR-204 on reporter gene activity (Figure 7d). Luciferase expression was reduced to 33% in the presence of the wild-type 3′-UTR upon treatment with miR-204. Mutation of the miR-204 binding site allowed expression at 81% of the level in control samples. We conclude from these findings that miR-204-5p is able to bind fragments of the Ccnd2-3′-UTR and the Sox4-3′-UTR, thereby restricting translation of the corresponding mRNAs. To assess whether the reduction of miR-204 in Oln93 cells upon loss of Sox10 (Figure 1b) results in a derepression of the newly identified miR-204 target genes Ccnd2 and Sox4, we compared expression of these genes in Sox10-deficient and wild-type Oln93 cells by qrt-PCR. Expression of Sox4 was undetectable in Oln93 cells under any condition, but expression of Ccnd2 was significantly increased by 1.9-fold in Sox10-deficient Oln93 cells (Figure 7e). Reintroduction of Sox10 in Sox10-deficient Oln93 cells halved the amount of Ccnd2 again (Figure 7f). To check whether miR-204 was the main mediator of the repressive effect of Sox10 on Ccnd2 expression, we introduced a control or a miR-204 expression plasmid in Sox10-deficient Oln93 cells and measured again Ccnd2 expression. Transcript levels of Ccnd2 were reduced to 0.7-fold of control after miR-204 overexpression (Figure 7g).

Havening seen that miR-204-5p can regulate Ccnd2 and Sox4 expression, we wanted to verify that these target genes also contribute to the phenotypes that we observed during oligodendrogenesis. Therefore, we inhibited Ccnd2 or Sox4 by RNAi and analyzed if this would mimic the effects of miR-204 overexpression in cultured oligodendroglia. Knockdown efficiencies were 58% for Ccnd2 and 57% for Sox4 as determined by Western blots of HEK293 extracts after cotransfection of expression plasmids for proteins with antisense RNA or scrambled control (Figure 7h,i).

For analysis of the functional effects of knockdown of Ccnd2, Oln93 cells were transfected with a vector expressing GFP for visualization and either a shRNA construct targeting Ccnd2, a miR-204 short-hairpin expression construct or a scrambled shRNA. To assess the influence on cell proliferation, cells were kept in proliferative conditions for 3 days and stained for the proliferation marker Ki67. Compared to the scrambled control (Figure 7j–l), proliferation rates of cells treated with miR-204 (Figure 7m–o) or Ccnd2-shRNA (Figure 7p–r) were diminished to a similar extent. While 47% of cells transfected with the scrambled construct expressed Ki67, marker expression was reduced to 26% for cells overexpressing miR-204 and 31% for cells carrying the Ccnd2-shRNA (Figure 7s). Thus, we see similar effects on cell proliferation for inhibition of Ccnd2 and overexpression of miR-204 and conclude that Ccnd2 is a target of miR-204 with impact on proliferation.

To analyze the function of Sox4 on differentiation, we transfected rat primary OPCs with an siRNA against Sox4 or a nonbinding negative control together with the siGLO Green transfection indicator for visualization and compared it with miR-204 overexpression (Figure 5a–g). Cells were kept for 6 days in differentiation medium and stained for the differentiation marker Mbp (Figure 7t–y). While 44% of the cells treated with siRNA control were positive for Mbp and had started differentiation the percentage was increased to 58%, when the cells were transfected with Sox4-siRNA (Figure 7z). After miR-204 overexpression, we had observed 63% of Mbp-positive cells (Figure 5a–g). Thus, we conclude that knockdown of Sox4 mimics the effect of miR-204 overexpression in promoting oligodendroglial cell differentiation.

4 | DISCUSSION

The lineage determining transcription factor Sox10 plays an essential role in oligodendroglia at all stages from the OPC to the mature OL. Sox10 is important for such diverse biological processes as specification, terminal differentiation and myelin maintenance. This is achieved by stage-specific interactions of Sox10 with distinct partner proteins resulting in the activation of different target genes and specific effects (for review see Weider & Wegner, 2017). Additionally, miRs downstream of Sox10 enlarge its functional diversity by targeting a variety of mRNAs with the respective seed sequences in the 3′-UTRs. While several of Sox10’s coregulators and downstream factors are known, the picture is far from complete with regard to the whole spectrum of Sox10-dependent effects as we know them from...
different Sox10-mutant mouse models. With miR-204, we identified a novel Sox10 target gene, which impacts oligodendroglial differentiation at the posttranscriptional level. We present for the first time a miR that regulates mRNAs of proliferation- and differentiation-relevant genes in parallel and therefore explains part of the diversity in the functions of Sox10.

Sox10 interacts with many other transcription factors. After specification of OPCs, Sox10 is in a cross-regulatory relation with Olig2.

**FIGURE 7** Analysis of the putative miR-204-5p targets Ccnd2 and Sox4. (a) Schematic representation of predicted miR-204-5p binding sites in 3'-UTRs of Ccnd2 and Sox4. Boxes represent fragments of the 3'-UTRs used in reporter gene assays, arrows point to positions of putative miR-204 binding sites. (b) Sequences of wild-type (WT) and mutated (suffix "m") miR-204-5p binding sites (black letters) and adjacent bases (gray letters) in Ccnd2- and Sox4-3'-UTR fragments. (c,d) Luciferase assays were performed in Neuro2a cells using reporter plasmids containing WT or mutated (mut) 3'-UTR fragments of Ccnd2 or Sox4. Luciferase activities were measured 72 hr after cotransfection with empty CAG-IRES-GFP vector (−) as control or miR-204-expressing vector (+). Luciferase activities in the control were arbitrarily set to 1 and activities in the presence of miR-204 were determined relative to the control (mean ± SD; n ≥ 3). Statistically significant differences between control and miR-204-transfected cells are indicated for WT 3'-UTRs, while differences for mutant 3'-UTRs were not statistically significant (Student's t test; **, p ≤ .01; ns, p > .05).

(e) Transcript levels of Ccnd2 in WT and Sox10-deficient (KO) Oln93 cells were compared (Aprato et al., 2019). (f,g) Transcript levels of Ccnd2 in Sox10-deficient Oln93 cells carrying a control (KO + Ctrl) or a Sox10 (KO + Sox10) expression construct or in (g) Sox10-deficient Oln93 cells carrying a control (KO + Ctrl) or a miR-204 (KO + miR-204) expression construct were compared by qRT-PCR. (e–g) Ccnd2 levels of WT or controls were arbitrarily set to 1, while levels of KO, Sox10-expressing or miR-204-expressing Oln93 cells are shown relative to WT or control (mean ± SD; n ≥ 3). Statistically significant differences are indicated (Student's t test; *, p ≤ .05; ***, p ≤ .001). (h,i) Knockdown efficiency of Ccnd2 or Sox4 as determined in human embryonic kidney (HEK) 293 cells. Quantification of protein levels from Western blotting with specific antibodies after overexpression of Ccnd2 (h) or Sox4 (i) together with corresponding control (Ctrl), shRNA construct (Ccdn2-sh) or siRNA (Sox4-si). Protein levels in control samples were arbitrarily set to 1, while levels of shRNA or siRNA treated cells are shown relative to controls (mean ± SD; n = 3). Statistically significant differences are indicated (Student's t test; *, p ≤ .05; **, p ≤ .01). (j–r) Immunocytochemical stainings on Oln93 cells with antibodies against GFP (green) and Ki67 (magenta). Cells were transfected with pSuper-gfp-based expression plasmids for scrambled control (Ctrl), miR-204 (miR-204), or Ccnd2-shRNA (Ccnd2-sh). (s) Quantification of Ki67 and GFP double-positive cells relative to all GFP-positive cells. (t–y) Immunocytochemical stainings on primary oligodendroglia kept in differentiation medium for 6 days with antibodies against Mbp (magenta). Cells were treated with a nontargeting siRNA control (Ctrl) or a Sox4-siRNA (Sox4-si) in combination with siGLO Green Transfection Indicator (green). (z) Quantification of Mbp and siGLO double-positive cells relative to all siGLO-positive cells. (s–z) Stainings were quantified from at least 24 images from three biological replicates for each condition (mean ± SD for biological replicates; n = 3). Statistically significant differences are indicated (Student's t test; *, p ≤ .05; **, p ≤ .01) [Color figure can be viewed at wileyonlinelibrary.com]
the other lineage determining transcription factor of oligodendroglia (Küspert, Hammer, Bösì, & Wegner, 2011; Weider et al., 2015; Weider et al., 2018). With Sox6 and Sox9, Sox10 modulates other Sox proteins that are involved in oligodendrogial development (Stolt et al., 2003; Stolt et al., 2006). At the initiation of terminal differentiation, Sox10 then induces myelin gene expression by activating expression of the pro-differentiation factors Nkx2.2 and Myrf and of the myelin gene Mbp itself, in part with other transcription factors such as Nfatc2 (Hornig et al., 2013; Liu et al., 2007; Stolt et al., 2002; Weider et al., 2018). Among miRs, miR-338, miR-335, and miR-155 are known to be activated by Sox10 (Cantone et al., 2019; Reiprich et al., 2017). We now show that Sox10 binds ECRs upstream of the miR-204 gene and that these ECRs are highly responsive to Sox10. Sequence analyses of the ECRs disclosed potential binding sites for further transcription factors that are involved in oligodendrogial development such as Olig1, Olig2, Ngn3, E47, Foxb1, Nfat, Nkx2.2, Nkx6.2, Sp1, and Yy1. At least some of these factors may interact with Sox10 in the regulation of miR-204 expression. The miR-204 sequence is located in intron VI of the Trpm3 gene in the mouse, which encodes a member of the melastatin-like subfamily of TRP channels (Grimm, Kraft, Sauerbruch, Schultz, & Harteneck, 2003). Trpm3 has previously been shown to be involved in the regulation of sphingosine-induced cellular Ca2+ flux during oligodendrogial differentiation (Hoffmann et al., 2010). Whether activation of the ECRs upstream of the Trpm3/miR-204 genetic locus only affects expression of miR-204 or also expression of the host gene Trpm3 will be subject of further studies. Sox10 is known as a transcriptional activator, but through the activation of miRs, which exert repressive functions, it can also indirectly inhibit expression of target genes. Activation and repression of gene expression are both necessary to proceed through development. While proliferation and progenitor characteristics need to be shut off, differentiation has to be activated for the onset of myelination. miR-204 inhibits translation of the Ccnd2 mRNA, which encodes the pro-proliferative CyclinD2 protein, and at the same time translation of the Sox4 mRNA, which has differentiation preventing effects. Both proteins are subject to rapid turnover at least in nonglial cell types, which makes them prone to dynamic posttranscriptional regulation (Beekman et al., 2012; Kida, Kakihana, Kotani, Kurosou, & Miura, 2007). Thus, miR-204 influences two decisive steps towards myelination, namely exit from the cell cycle and entry into differentiation. Despite its positive effect on differentiation, overexpression of miR-204 was not able to revert the differentiation failure caused by Sox10 deletion in primary mouse oligodendroglia. This argues that induction of differentiation and myelin genes by Sox10 is distinct from the differentiation promoting effects of Sox10-induced miR-204 expression. Supporting the direct and active role of Sox10 in the induction of myelin gene expression, repression of differentiation inhibitors by miR-204 is by itself not sufficient to permit OL differentiation. This is in line with a predominantly fine-tuning function of miRs. miR-204 has not been described in oligodendroglia before. It is mildly expressed in OPCs and increases during differentiation correlating to the observed role in progression of maturation. An antiproliferative effect of miR-204 as described here in oligodendroglia has also been observed in other studies. miR dysregulation is seen in many types of cancer where miRs often are involved in the regulation of proliferation. Expression of miR-204 is downregulated in highly proliferative human glioblastoma tissue compared to healthy tissue (Song, Fajol, Tu, Ren, & Shi, 2016). Vice versa, ectopic expression of miR-204 reduces cell proliferation. The phenotype was mostly ascribed to inhibition of the transcription factor ATF2 as a miR-204 target gene. Similarly, miR-204 downregulation is seen in retinoblastoma tissue and cell lines, where restoration of miR-204 levels can inhibit tumor growth (Wu, Zeng, et al., 2015). In this cellular context, Ccnd2 and MMP-9 are identified as target genes of miR-204. In the present study, we confirm that miR-204 regulates Ccnd2 mRNA via the same recognition seed sequence in its 3'-UTR in the mouse as described for human retinoblastoma. Whether ATF2 and MMP-9 are also targets in oligodendroglia could be a question of further studies, although expression databases argue for low amounts or even absence of these proteins in oligodendroglial cells. A single-base mutation in human MRI-204 is associated with inherited retinal dystrophy (Conte et al., 2015). The mutation within the seed sequence leads to a shift in the set of target genes, some target sites are not recognized anymore, but many more new targets arise as a result of the mutated seed sequence. Phenotypically, this results in an increase in apoptosis and reduced differentiation of functional photoreceptors. In the eye, proliferation is not affected by the loss of wild-type miR-204. This may be consequence of the new targets that are generated by the mutation. This complication makes a target comparison between this and other studies impossible.

Sox4, the other miR-204 target that we identified, is expressed in OPCs, and needs to be downregulated before the onset of myelination. Transgenic mice with prolonged Sox4 expression beyond the OPC stage present a severe hypomyelination although oligodendroglia are present in normal numbers and proliferation is not significantly changed (Potzner et al., 2007). At early stages, Sox4 seems to be important to keep the cells in a precursor stage and to inhibit premature differentiation. Recently, the effect of Sox4 on differentiation has also been studied in primary OPCs by shRNA-mediated knockdown (Braccioli, Vervoort, Puma, Nijboer, & Coffer, 2018). Stainings showed an increase of mature OLs in the absence of Sox4. Here we argue, that miR-204-5p comes into play at the onset of differentiation and downregulates Sox4 by recognizing a binding site in its 3'-UTR. This binding site is also targeted in human renal cell carcinoma cells, where overexpression of miR-204 not only leads to reduced levels of Sox4, but also to decreased proliferation, migration and invasion (Wu, Pan, et al., 2015). Although Sox4 is the only described target of miR-204 in this study, further targets are likely to exist. Thus, reduced proliferation in renal cell carcinoma may also be a consequence of Ccnd2 targeting by miR-204. Addition of miR-204 to the regulatory network may help to explain the effects of Sox10 during OL development. Sox10 activates expression of miR-204 concomitant with its own increase during differentiation of oligodendroglia. miR-204 then supports exit from the
cell cycle by targeting Ccnd2 and promotes differentiation by inhibiting the suppressor of maturation Sox4.

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DATA AVAILABILITY STATEMENT

All data generated in this study are contained within the manuscript and available.

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