miR-124-9-9* Potentiates Ascl1-Induced Reprogramming of Cultured Müller Glia

Stefanie Gabriele Wohl and Thomas Andrew Reh

The Müller glia of fish provide a source for neuronal regeneration after injury, but they do not do so in mammals. We previously showed that lentiviral gene transfer of the transcription factor Achaete-scute homolog 1 (Ascl1/Mash1) in murine Müller glia cultures resulted in partial reprogramming of the cells to retinal progenitors. The microRNAs (miRNAs) miR-124-9-9* facilitate neuronal reprogramming of fibroblasts, but their role in glia reprogramming has not been reported. The aim of this study was to test whether (1) lentiviral gene transfer of miR-124-9-9* can reprogram Müller glia into retinal neurons and (2) miR-124-9-9* can improve Ascl1-induced reprogramming. Primary Müller glia cultures were generated from postnatal day (P) 11/12 mice, transduced with lentiviral particles, i.e., miR-124-9-9*-RFP, nonsense-RFP, Ascl1-GFP, or GFP-control. Gene expression and immunofluorescence analyses were performed within 3 weeks after infection.

1. Overexpression of miR-124-9-9* induced the expression of the proneural factor Ascl1 and additional markers of neurons, including TUJ1 and MAP2.
2. When Ascl1 and miR-124-9-9* were combined, 50 to 60% of Müller glia underwent neuronal reprogramming, whereas Ascl1 alone results in a 30 to 35% reprogramming rate.
3. Analysis of the miR-124-9-9* treated glial cells showed a reduction in the level of Ctdsp1 and Ptbp1, indicating a critical role for the REST pathway in the repression of neuronal genes in Müller glia.

Our data further suggest that miR-124-9-9* and the REST complex may play a role in regulating the reprogramming of Müller glia to progenitors that underlies retinal regeneration in zebrafish.

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Key words: retina, neuronal differentiation, microRNA, REST, Ctdsp1/SCP1, Ptbp1/PTB

Introduction

The adult mammalian central nervous system (CNS) largely lacks regenerative capacity. However, recent reports show that glial cells can be reprogrammed and can potentially serve as a source for new neurons to replace those lost by injury or disease (Goldman, 2014; Guo et al., 2014; Heinrich et al., 2012, 2014; Niu et al., 2013; Robel et al., 2011). This approach has advantages, since the cells are produced *in situ* and transplantation is not required. A variety of factors have been used to successfully reprogram fibroblasts, P19 carcinoma cells, and glia into neurons. Several groups have shown that overexpression of key proneural transcription factors such as Sox2, NeuroD1, and Ascl1 causes a significant percentage of astrocytes (Corti et al., 2012; Guo et al., 2014; Heinrich et al., 2012; Niu et al., 2013) or NG2 glia (Heinrich et al., 2014) to differentiate into neurons.

In the retina, Müller glia are the predominant glia (for review see (Bringmann et al., 2006; Reichenbach and Bringmann, 2013)) and express some genes that are also expressed by neural progenitor cells (NPCs) (Bernardos et al., 2007; Das et al., 2006; Jadhav et al., 2009; Ooto et al., 2004; Roesch et al., 2008). In non-mammalian vertebrates such as fish, retinal injury induces Müller glia to re-enter the cell cycle, de-differentiate and generate new neurons. (Goldman 2014; Lenkowski and Raymond, 2014; Ramachandran et al., 2010). In mammals however, this potential is very limited (Löffler et al., 2015); the ability to proliferate decreases with age (Close et al., 2006; Ueki and Reh, 2013) and very few neurons are generated after injury in adult mammalian retina, even after multiple injections of mitogens (Karl et al., 2008) for review see (Fischer and Reh, 2003; Goldman, 2014; Karl

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and Reh, 2010; Wohl et al., 2012). We recently reported that lentiviral-mediated expression of Ascl1 was sufficient to reprogram ~30% of dissociated Müller glia to a neurogenic state. However, many of the cells failed to generate neurons, even though they expressed Ascl1 (Pollak et al., 2013). Thus, additional factors are likely necessary for full reprogramming of Müller glia and complete retinal regeneration in mice.

Many studies have highlighted the importance of miRNAs in the regulation of cell fate and stable patterns of gene expression (for review see (Filipowicz et al., 2008; Sundermeier and Palczewski, 2012)). One miRNA that plays an important role during neurogenesis is miR-124, a brain enriched miRNA, expressed in differentiating and mature neurons, but not in non-neuronal cells, including glia (Cheng et al., 2009; Conaco et al., 2006; Krichevsky et al., 2006; Makeyev et al., 2007; Papagiannakopoulos and Kosik, 2009; Smirnova et al., 2005). miR-124 (also known as miR-124a) is encoded by three genes (miR-124a-1, miR-124a-2, miR-124a-3) (Conaco et al., 2006; Deo et al., 2006) and the mature sequence is highly conserved from *C. elegans* to humans (Lagos-Quintana et al., 2002). Another brain enriched miRNA involved in neurogenesis is miR-9. miR-9 is also encoded by three genes (miR-9-1, miR-9-2, miR-9-3), but in contrast to miR-124, where only one mature miRNA exists, each of the miR-9 genes gives rise to two mature miRNAs, miR-9 (which is the 5' strand, miR-9-5p) and miR-9* (which is the 3' strand, miR-9-3p) (Conaco et al., 2006).

Gain of function experiments with miR-124 alone or in combination with miR-9/9* in embryonic stem cells (ESCs) (Krichevsky et al., 2006; Maiorano and Mallamaci, 2009) and NPCs (Cheng et al., 2009; Maiorano and Mallamaci, 2009), show that miR-124 promotes neuronal differentiation, while inhibiting glial genes. Moreover, miR-124-9-9*, has been shown to reprogram fibroblasts (Yoo et al., 2011), and miR-124 alone can increase retinoic acid-induced neurogenesis in P19 stem cells (Makeyev et al., 2007). miR-124 and miR-9/9* target components of the REST complex, known to repress neuronal gene expression in non-neuronal cells (Nesti et al., 2014; Wu and Xie, 2006; Yeo et al., 2005). The REST complex is composed of the RE1-silencing transcription factor, REST, also known as neuron-restrictive silencer factor NRSF (Chong et al., 1995; Schoenherr and Anderson, 1995); the REST corepressor 1, also known as CoREST, encoded by *Rcor1* (Andres et al., 1999); and the carboxy-terminal domain RNA polymerase II polypeptide A small phosphatase 1 (Ctdsp1), also known as small c-terminal domain phosphatase 1, SCP1 (Nesti et al., 2014; Visvanathan et al., 2007; Yeo et al., 2005). In light of these previous studies, we tested whether the REST pathway was active in Müller glia, repressing neuronal gene expression and stabilizing their phenotype. Specifically, we tested whether miR-124-9-9* overexpression would reprogram Müller glia into neuronal progenitors and neurons, either alone or in combination with Ascl1, and if the reprogramming mechanism involves RNA downregulation of members of the REST complex.

Here we report that miRNAs miR-124, miR-9, and miR-9* can promote Müller glia reprogramming. Lentiviral gene transfer of miR-124-9-9*-induced cells to express genes normally present in retinal progenitor cells and neurons. Furthermore, the combination of Ascl1 and miR-124-9-9* yielded in ~60% newly generated neurons from Müller glia primary cultures. Two of the known targets in the REST pathway, Ctdsp1/SCP1 and the polypyrimidine tract binding protein 1 (Ptbp1/PTB), were reduced in the miR-124-9-9* infected glia, while the neuronal Ptbp2 was increased. These results support a role for the REST pathway in neuronal gene silencing in Müller glia cells, and show that targeting this pathway with miRNAs is an effective method to promote reprogramming of these cells.

Materials and Methods

Animals

All mice were used in accordance with University of Washington Institutional Animal Care and Use Committee approved protocols (UW-IACUC). All experiments were carried out on C57BL/6J mice (Jackson). Experiments to test Ascl1 induction were performed on mAscl1-creER:flox-Stop-flox tdTomato (gift from Dr. Jane Johnson).

Plasmids and Viral Production

pLemir-9-124, encoding for miR-9/9* and miR-124 pri-transcripts together with turbo-RFP reporter [(Yoo et al., 2011), plasmid #31779], and the control turbo-RFP plasmid pLemir-NS encoding for non-specific hairpins (here referred as nonsense/ns, plasmid #32809) were purchased from Addgene and inserted into lentiviral vectors using the Lenti-X^{TM} HTX packaging system protocol (Clontech). PLOC-hASCL1-IRES-turboGFP(nuc) and control PLOC-IRES-turboGFP(nuc) viral vectors were generated as described previously (Pollak et al., 2013). Dominant-negative (dn)Ctdsp1, wildtype (wt)Ctdsp1 plasmids were obtained from Dr. Soo-Kyung Lee, dnREST and wtREST plasmid from Dr. Gail Mandel, and used for DNA transfection.

Müller Glia Primary Culture

Müller glia were dissociated from postnatal day (P) 11/12 mice and grown in Neurobasal medium with N2 (supplemental factor), tetracycline-free 10% fetal bovine serum (FBS, Clontech), and epidermal growth factor (EGF, R&D Systems, 100 ng/mL) as described before (Ueki et al., 2012). The *in vitro* experiments are limited to the age of P12. Although Müller glia from mice older than P12 can be cultured, they do not grow and do not form confluent monolayers, which are required for successful reprogramming experiments. The thymidine analog 5-ethynyl-2'deoxyuridine (EdU, Invitrogen, 1 μM) was added to follow progeny of proliferating Müller glia. To activate Cre recombinase in the Ascl1-creER:flox-Stop-flox tdTomato

Müller glia cultures, 4-hydroxytamoxifen (Sigma, 1 nM) was added to the medium after passage.

Transfection

For RNA transfection, we used mimics (artificial miRNAs, 20 nM–1 μM) and antagomiRs (antisense miRNAs, 20 nM) purchased from Thermo Scientific in combination with mCherry RNA and/or miRNA-GFP sensors (1 μg/μL each). To generate mRNA for transfection, we used the pSLU plasmid as described previously (Hayashi et al., 2010) and the Ambion mMessage mMachine (AM1345; Ambion) according to manufacturer's instructions. We also used dnCtdsp1, wtCtdsp1, dnREST, wtREST and mCherry or ns-RFP control plasmids (500 ng/well of a 24-well plate) for DNA transfection. Müller glia from either C57/BL6J or mAscl-creER:flox-Stop-flox tdTomato mice were transfected using Dharmafect transfection reagent in Optimem medium, in accordance with manufacturer's instructions, one day post passage. After transfection, the media was changed to neuronal media (1% FBS with B27 supplemental factor).

For reference and to test functionality, both, miR-GFP sensors and miR-124-9-9*-RFP lentiviral vectors have been tested on human embryonic kidney cell line HEK293. Cultures were analyzed 6 h to 6 days post-transfection/transduction.

Lentiviral Gene Transfer

Lentiviral particles were added in neuronal medium (see above) together with brain derived neurotrophic factor (hBDNF, R&D Systems 10 ng/ml), fibroblast growth factor (bFGF, R&D Systems, 100 ng/mL), glia derived neurotrophic factor (rGDNF, R&D Systems, 10 ng/mL), and the Rho-associated kinase (ROCK) inhibitor Thiazovinin (Sigma Aldrich, 10 nM) (Watanabe et al., 2007). Müller glia were transduced with lentiviral vectors expressing miR-124-9-9*-RFP and ns-RFP at 5-7DIV, and analyzed 2, 5, 8, and 12 days post-transduction. In other experiments, Müller glia were first transduced with lentiviral vectors expressing Ascl1-GFP or control GFP at 5-7DIV, then with either miR-124-9-9*-RFP or ns-RFP lentiviral vectors at 9-10DIV, and analyzed at 15-21DIV.

Reverse Transcriptase Quantitative PCR (RT-qPCR) for mRNA and miRNA

Cells were lysed in Qiazol and RNA was extracted and purified with a miRNeasy Mini Kit in accordance to manufacturer's instructions (Qiagen). For conventional mRNA analysis, cDNA was synthetized using the iScript cDNA Synthesis Kit (Bio-Rad; primers are listed in Table 1). For miRNAs, stem-loop RT primers were used to produce cDNA for specific miRNAs (Fiedler et al., 2010). RT-qPCR was performed using SsoFast Eva Green Supermix (Bio-Rad) on a Bio-Rad Thermocycler. Primers used are shown in Table 2. At least six biological replicates (*n*) were used; every *n* consisted of 6 to 10 mice of one litter. Reactions were run in triplicates or duplicates for RT or –RT samples. Values were normalized to beta actin and/or 5sRNA. Delta ΔC_t between treatment (miR-124-9-9*, Ascl1-GFP, and Ascl1-GFP/miR-124-9-9*) and corresponding controls (ns-RFP or GFP) were calculated and are expressed together with standard error of the mean (SEM).

Immunofluorescent Labelings

Müller glia were cultured on Poly-D-lysine (PDL) and Matrigel (Corning) covered coverslips in 24-well plates. Cells were fixed with 2% paraformaldehyde (PFA). EdU labeling was carried out using Click-iT EdU Kit (Invitrogen). For immunofluorescent staining, cells were incubated in blocking solution (5% milk block: 2.5 g nonfat milk powder in 50 mL PBS; with 0.5% Triton-X100) for 1 h at RT. Primary antibodies (Table 3) were incubated in 5% milk block overnight, secondary antibodies (Invitrogen/Molecular Probes, and Jackson ImmunoResearch, 1:500–1,000) for 1 h at RT and counterstained with 4′,6-diamidino-2-phenylindole (DAPI, Sigma, 1:1,000).

Microscopy, Cell Counts, and Statistical Analysis

Live imaging was performed using Zeiss Observer D1 with AxioCam. Fixed cells were analyzed by Olympus FV1000 confocal microscope. Five to 10 random fields per coverslip, one to two coverslips per condition, at 200× magnification were counted for every condition, of at least five independent experiments. Values are expressed as mean ± SEM. Statistical analysis were performed by Student's *t*-test for independent samples combined with Levene's test for equality of variances. Holm-Bonferroni method was used to correct for multiple comparisons.

Results

miR-124 is Absent in Most Müller Glia

Previous studies have shown that miR-124 is not highly expressed in glia (Cheng et al., 2009; Smirnova et al., 2005), including adult Müller glia (Sanuki et al., 2011). In order to analyze the expression levels of miR-124 together with other miRNAs in P11/12 Müller glia cultures, we used either RT-qPCR or miR-GFP sensors (Fig. 1A). By RT-qPCR we found low levels of miR-124 and miR-9* (Fig. 1B) in dissociated cultures of Müller glial cells (*naive*). By contrast, miR-9 displayed high levels, similar to those of miR-24 and several let-7 family members known to be present in glia (La Torre et al., 2013; Patterson et al., 2014; Shenoy et al., 2015; Smirnova et al., 2005). Next, we compared the expression levels of miRNAs from dissociated *naive* Müller glia with those from newborn (P0) mouse retinas, which contain a mix of immature neurons and progenitor cells. Normalization to P0 retinas showed that Müller glia have much less miR-124, less miR-9, let-7a, and let-7f, but show comparable levels of miR-24 and let-7b (Fig. 1C).

To confirm the findings of the RT-qPCR analysis, we used miRNA-sensors. miR-GFP sensors contain mRNA for GFP with a specific 3' UTR that have consensus binding sites for a particular miRNA. Therefore, if the particular miRNA is absent, GFP mRNA is stable and translated (Fig. 1D). In the presence of the miRNA, the sensor mRNA will be degraded or its translation will be blocked (Fig. 1D'). First, Müller glia cells were transfected with miR-124-GFP sensor and mCherry transfection control. mCherry RNA transfection resulted in expression in almost every Müller glia after 2 to 3

TABLE 1: RT-qPCR Primers

Gene name	Forward sequence (5' to 3')	Reverse sequence (3' to 5')
Actb (beta actin)	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAACAATGCCATGT
Aqp4	CTTTCTGGAAGGCAGTCTCAG	CCACACCGAGCAAAACAAAGA T
Ascl1 (m)	GCAACCGGGTCAAGTTGGT	GTCGTTGGAGTAGTTGGGGG
Ascl1 (h)	CATCTCCCCCAACTACTCCA	CAGTTGGTGAAGTCGAGAAGC
Atoh7	ATCACCCCTACCTCCCTTTCC	CGAAGAGCCTCTGCCCATA
Ctdsp1	GAAGTCAGCAGTTTCCCAGAAG	GCGCCATTTTCCTCCACCA
Dcx (doublecortin)	GCAATGGGGACCCCTTTTTC	GGTGTAGATGTTCCTAACCGC
Gapdh	GGCATTGCTCTCAATGACAA	CTTGCTCAGTGTCCTTGCTG
Glul (glutamine synthetase)	TGAACAAAGGCATCAAGCAAATG	CAGTCCAGGGTACGGGTCTT
Hes5	AGTCCCAAGGAGAAAAACCGA	GCTGTGTTTCAGGTAGCTGAC
Isl1 (islet 1)	TATCCAGGGGATGACAGGAAC	GCTGTTGGGTGTATCTGGGAG
Lin28b	AGAATGCAGTCTACCTCCTCAG	CCTCCCACTTCTCTTGGTGC
Map2	CTCAGCACCGCTAACAGAGG	CATTGGCGCTTCGGACAAG
Nefm (neurofilament-M)	ACAGCTCGGCTATGCTCAG	CGGGACAGTTTGTAGTCGCC
Onecut2	CCACGCCATGAGTATGTCCTG	CGTCAGCGTAGTGTAGGTGTT
Otx2	TATCTAAAGCAACCGCCTTACG	AAGTCCATACCCGAAGTGGTC
Pou4F1(Brn3a)	TGGACATCGTCTCCCAGAGTA	GTGTTCATGGTGTGGTAAGTG
Ptbp1	CCTCTCCGTATGCAGGAGC	CCGTAGACGCCGAAGAGAATAA
Ptbp2	ATGGACGGAATTGTCACTGAGG	TGCCACTCATATTAGAGTTGGGG
Rcor (CoREST)	GTGCCCGACTTCGATCCTG	GATGTACTCGTCCAGTTTTGCT
Rest	CATGGCCTTAACCAACGACAT	CGACCAGGTAATCGCAGCAG
Rlbp1	GGCACTTTCCGCATGGTTC	CCGGGTCTCCTCCTTTTCAT
S100b	TGGTTGCCCTCATTGATGTCT	CCCATCCCCATCTTCGTCC
Slc1a3 (GLAST)	ACCAAAAGCAACGGAGAAGAG	GGCATTCCGAAACAGGTAACTC
Tubb3 (beta III tubulin)	TAGACCCCAGCGGCAACTAT	GTTCCAGGTTCCAAGTCCACC

days (Fig. 1E). Approximately 65% of all transfected mCherry$^+$ cells were also GFP$^+$; meaning they did not express sufficient levels of miR-124 to prevent the sensor expression (Fig. 1F-F',H). To validate miR-124-GFP sensor functionality, miR-124 mimics were added to the glial cultures along with the sensors. The miR-124 mimics caused a large reduction in the number of cells expressing the sensor (Fig. 1G-G',H). We also tested miR-124-GFP sensors on HEK293 cells, known to lack most miRNAs, to verify their functionality (Supp. Info. Fig. S1A-A").

Next, we used a sensor for miR-9 to confirm the RT-qPCR data. We first tested miR-9-GFP sensors for functionality in HEK293 cells (Supp. Info. Fig. S1B-B"). When the sensor was transfected into cultured glia, we found very few green cells (less than 10%), indicating that ~90% of Müller glia *in vitro* have sufficient levels of miR-9 to prevent sensor expression (Supp. Info. Fig. S2A'-A",C). We confirmed that the sensor was reporting correctly by co-transfection of miR-9 antagomiR, which resulted in an increase in sensor expression (Supp. Info. Fig. S2B'-B",C).

Taken together, the RT-qPCR and transfection data show that miR-124 and miR-9* are expressed at low levels in the majority of dissociated P11/12 Müller glia; however, miR-9 is expressed by glia but in lower levels than in progenitors/immature neurons.

miR-124-9-9 Overexpression Leads to Upregulation of Progenitor and Neuronal Genes*

To determine whether miR-124-9-9* can promote Müller glia reprogramming into neurons, we used a viral approach which

TABLE 2: RT-qPCR Primers for miRNAs

miRNA	Forward sequence (5′ to 3′)	Reverse sequence (3′ to 5′)	Stem loop RT primer (3′ to 5′)
let-7a	CGG CCT GAG GTA GTA GGT TG	GCT TGC GTG TTA TTT CCT GAT GG	CGC TAC ACG CTT GCG TGT TAT TTC CTG ATG GCG TGT AGC GAA CTA TAC A
let-7b	CGC CCT GAG GTA GTA GGT TG	GCA CGC TAG GGA ACA TCA GG	ATC AGG TAG CGC ACG CTA GGG AAC ATC AGG GCT ACC TGA TAA CCA CAC
let-7f	CGC CGC TGA GGT AGT AGA TTG	GCT TGC GTG TTA TTT CCT GAT GG	CGC TAC ACG CTT GCG TGT TAT TTC CTG ATG GCG TGT AGC GAA CTA TAC A
miR-1	GCG GCC TGG AAT GTA AAG AA	CAG TTC AGC AGG GTC ATA GG	TCG CGA CTG CCA GTT CAG CAG GGT CAT AGG GCA GTC GCG AAT ACA TAC
miR-9	GCC GGT CTT TGG TTA TCT AGC	TTT CCT CGA CCG CAC CAC TC	AAA TCG CAG CTT TCC TCG ACC GCA CCA CTC GCT GCG ATT TTC ATA CAG
miR-9*	GCG CCC CAT AAA GCT AGA TAA C	AGG TGG AGC TTT GGA TGG TG	AGC TAC TCG CAG GTG GAG CTT TGG ATG GTG GCG AGT AGC TAC TTT CGG
miR-124	CCG TTA AGG CAC GCG GTG A	GGA TGA CTC AAC GCG GGC TA	TGT ATC TTG CGG ATG ACT CAA CGC GGG CTA GCA AGA TAC AGG CAT TCA
miR-24	CGC CTG GCT CAG TTC AGC A	GCA GCT CTT CAT TTA CGG TCC A	CTC GAG ACG CAG CTC TTC ATT TAC GGT CCA GCG TCT CGA GCT GTT CCT
5s	GCC ATA CCA CCC TGA ACG	TGC AGG GTC CGA GGT ATT CG	ACT GCT GCG CTG CAG GGT CCG AGG TAT TCG GCG CAG CAG TAA AGC CTA

TABLE 3: Primary Antibodies

Antibody	Concentration	Company, Catalog #
rat anti RFP	1:500	Antibodies online, ABIN334653
chicken anti GFP	1:250	Abcam, ab13970
mouse anti beta III tubulin (TUJ1)	1:1,000	Covance, MMS-435P
mouse anti MAP2	1:200	Sigma, M4403
goat anti doublecortin (Dcx)	1:100	Santa Cruz, sc-8066
rabbit anti Neurofilament (NF)-M	1:250	Millipore, AB1987
rabbit anti Calbindin	1:1,000	Millipore, AB1778
rabbit anti Calretinin	1:1000	SWANT, 7697
mouse anti Ascl1	1:200	BD Pharmingen, 556604

has been previously described to reprogram fibroblasts to neurons (Yoo et al., 2011). To ensure functionality, we transfected miR-124-9-9*-transduced HEK293 cells with miR-124-GFP sensor. None of the miR-124-9-9*- transduced cells were positive for GFP implying that miR-124 was overexpressed successfully (Supp. Info. Fig. S1C-C"). As a control, we used nonsense (ns)-RFP lentiviral vectors, which encodes for a non-specific hairpin. Dissociated Müller glial cells were transduced after 6-9DIV, when they reached confluence (Fig. 2A). RT-qPCR for miRNAs revealed that miR-124-9-9* lentiviral particle-transduction resulted in increased levels of miR-124, miR-9, and miR-9*, while other miRNAs remained unaffected (Fig. 2B). Interestingly, miR-124 displayed a four-cycle increase as compared with *naive* controls. miR-9 and miR-9* were increased by about two cycles after transduction. We cannot explain why miR-9 and miR-9* levels are not increasing in a similar manner as miR-124 does. However, in case of miR-9, it is probably due to the fact that *naive* glia express this miRNA at higher levels. Next, we analyzed mRNA levels of genes expressed highly in retinal progenitors, but normally not in Müller glia. RT-qPCR data showed that there was a significant upregulation for the retinal progenitor genes Ascl1 and Hes5 (Fig. 2C). Otx2 and Islet1, two genes expressed in retinal neurons early in their differentiation (Boije et al., 2014) also increased. Interestingly, these two genes were also upregulated after Ascl1 transduction (Pollak et al., 2013). Besides progenitor genes, we found increased RNA levels of neuronal genes such as Neurofilament-M (NF-M), doublecortin (Dcx), MAP2, and TUJ1 but not Brn3a (Fig. 2D). These results are consistent with the hypothesis that miR-124-9-9* can promote retinal progenitor gene expression and reprogram the cells to a neurogenic state, possibly by reducing expression of some target gene(s). We also analyzed the expression levels of glial genes. We found that genes such as retinaldehyde binding protein 1 (Rlbp1, which encodes for the cellular retinaldehyde binding protein Cralbp), glutamine synthetase (Glul, also known as glutamate-ammonia ligase, GS), S100b calcium binding protein b, solute carrier family 1 member 3 (glial high affinity glutamate transporter, Slc1a3, also known as GLAST, Eaat1), and Aquaporin4 (Aqp4) were significantly downregulated as compared with controls, consistent with the loss of glial identity (Fig. 2E).

To further confirm Ascl1 induction after miR-124-9-9* overexpression we used cultured Müller glia from Ascl-creER:flox-Stop-flox tdTomato mice, in media containing 4-hydroxytamoxifen to allow reporter expression. We transfected them with the miR-124, miR-9, and miR-9* mimics (500 nM each, Fig. 3A). We found an increase in tdTomato$^+$ cells over time with a sixfold increase 6 days post-transfection (Fig. 3B,D).

To determine whether miR-124-9-9* transduction reprograms Müller glia to neurons, we labeled the cultures with EdU and neuronal markers, TUJ1 and MAP2. Five to 12 days after miR-124-9-9* transduction, ~15% of all proliferating RFP$^+$ cells were TUJ1$^+$ (controls <5%) and had a neuronal morphology (Fig. 4A-A",B-B",E). The increase in TUJ1 was consistent with the RT-qPCR data (Fig. 2D). We also used MAP2 as a marker for more mature neurons (Fig. 4C-C",D-D"). Five days after miR-124-9-9* transduction, no noticeable MAP2 signals were detected in RFP$^+$EdU$^+$ cells; however, after 8 to 12 days, 11 to 15% of all RFP$^+$EdU$^+$ cells were MAP2$^+$ (Fig. 4E') reflecting the MAP2 mRNA increase we observed before (Fig. 2D). The transduction efficiency for miR-124-9-9*-RFP infection was ~60%, and the control nonsense-RFP transduction was

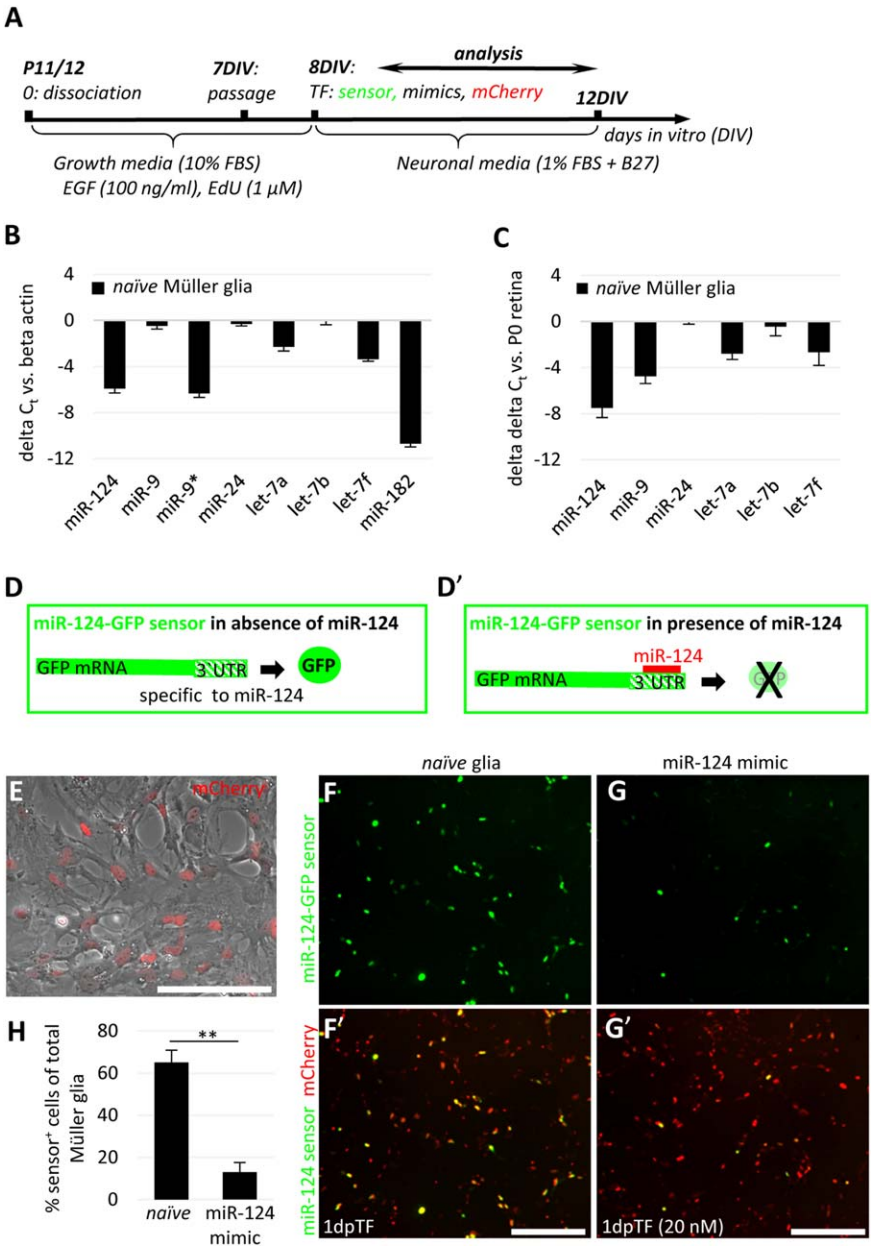

FIGURE 1: miR-124 levels are low in cultured *naive* Müller glia cells. A, Cell culture and transfection (TF) scheme. B, miRNA RT-qPCR to evaluate levels miRNAs in *naive* glia including miR-124, miR-9, and miR-9*. Levels of miR-124 are lower than the majority of other miRNAs known to be present in Müller glia (e.g., let-7). C, miRNA RT-qPCR from dissociated Müller glia normalized against miRNA levels from P0 retinae: levels of miR-124 but also miR-9 are lower in Müller glia when compared with newborn retinas than every other miRNA tested. D, Scheme of miR-124 sensor mechanism. In the absence of miR124, GFP mRNA is translated into GFP protein. In the presence of miR-124, the 3′ UTR region of the GFP mRNA is targeted by miR-124 resulting in repression of GFP protein expression (D′). E, Transfection of mCherry RNA results in expression of almost every Müller glia in culture (99%). F, G, miR-124-GFP sensor expression in cultured Müller glia together with mCherry transfection control. F, F′, Most Müller glia do not have miR-124 (GFP^+). G, G′, After transfection of miR-124 mimics (artificial miRNA), the sensor signal is knocked down in almost every cell 1d post-transfection (1dpTF). H, Number of miR-124-GFP $sensor^+$ cells in *naive* and mimic transfected Müller glia. Mean ± SEM, Student's *t*-test: *$P < 0.05$; **: $P < 0.01$; scale bar in E: 50 µm, in F′, G′: 100 µm. TF: transfection.

~80%. In miR-124-9-9*-transduced cultures we observed cell survival of ~60% over the culture period as compared with controls (not shown).

In order to investigate whether miR-124-9-9*-transduced Müller glia results in a variety of different types of neurons, we tested additional neuronal markers. Approximately 3% of all RFP^+EdU^+ cells were positive for the early neuronal marker Dcx, which has been reported to be expressed in miR-124-expressing migrating neurons ((Volvert et al., 2014); Fig. 5A-A"). Approximately 5% of all

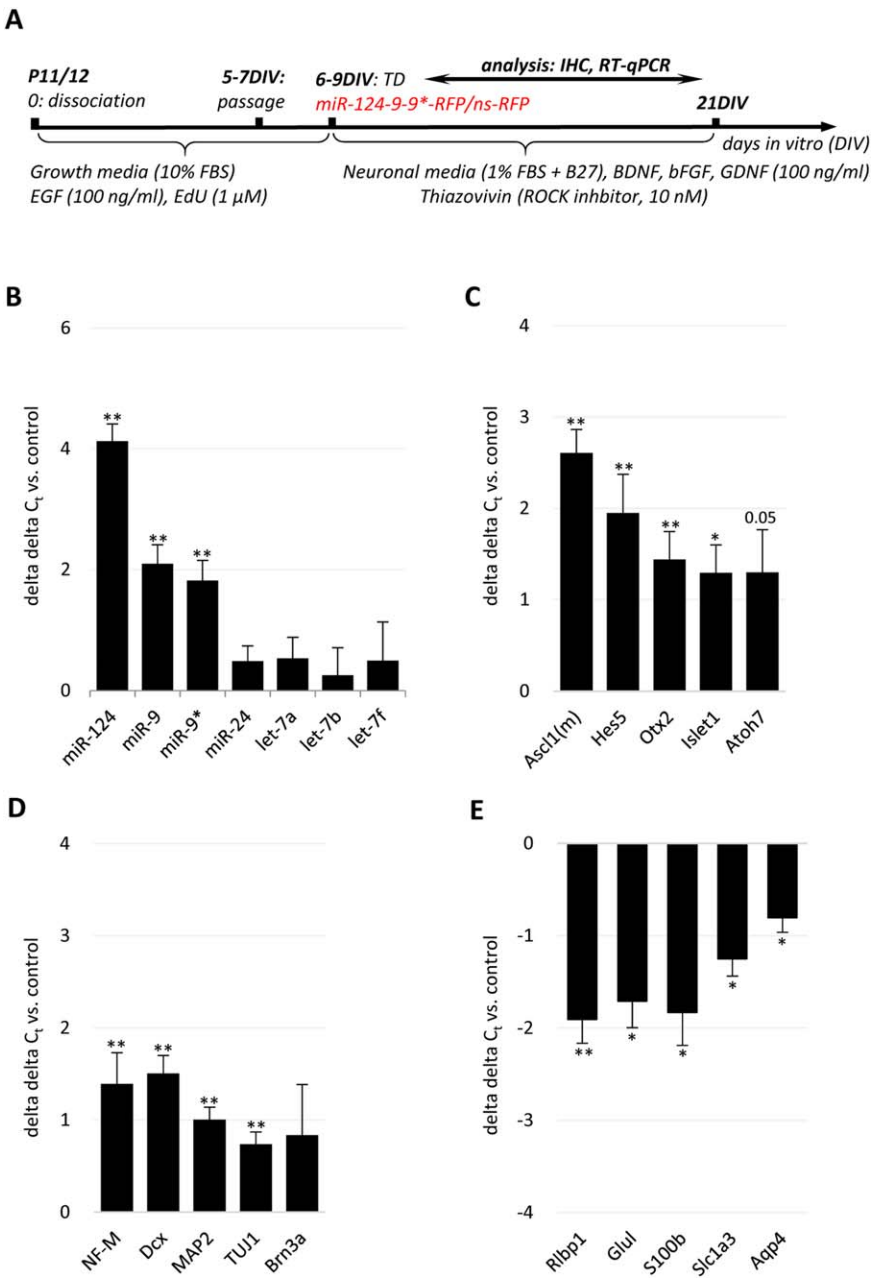

FIGURE 2: Viral expression of miR-124-9-9* in Müller glia cells results in change in upregulation of neuronal genes. A, Cell culture and transduction scheme. B, miRNA RT-qPCR analysis showed that miR-124, miR-9, and miR-9* were significantly upregulated in Müller glia after transduction (TD) while other miRNAs were unaffected. C, RT-qPCR revealed upregulation of progenitor genes Ascl1, Hes5, Otx2, and Islet1. D, In addition to progenitor genes, neuronal genes including Neurofilament (NF)-M, Dcx, MAP2, and TUJ1 were upregulated. E, Analysis of glial gene expression revealed that Rlbp1, Glul, S100b, Slc1a3, and Aqp4 were downregulated. Mean ± SEM. Student's *t*-test: *$P < 0.05$; **: $P < 0.01$.

RFP^+EdU^+ cells were labeled for Neurofilament-M (Fig. 5B-B'"). Occasional cells (less than 2%) expressed the markers Calbindin (Fig. 5C-C') and Calretinin (Fig. 5D-D'), proteins normally present in amacrine and horizontal cells (both interneurons) in the retina. Interestingly, some of these cells displayed complex morphological structures (Fig. 5C",D") consistent with retinal amacrine and ganglion cell types.

miR-124-9-9 Facilitates Müller Glia Reprogramming by Ascl1*

We previously reported that Ascl1-GFP overexpression can reprogram Müller glia to a neurogenic state, activating progenitor genes and inducing the cells to generate neurons. However, we found that only about 30% of the Müller glial cells were reprogrammed by Ascl1 (Pollak et al., 2013).

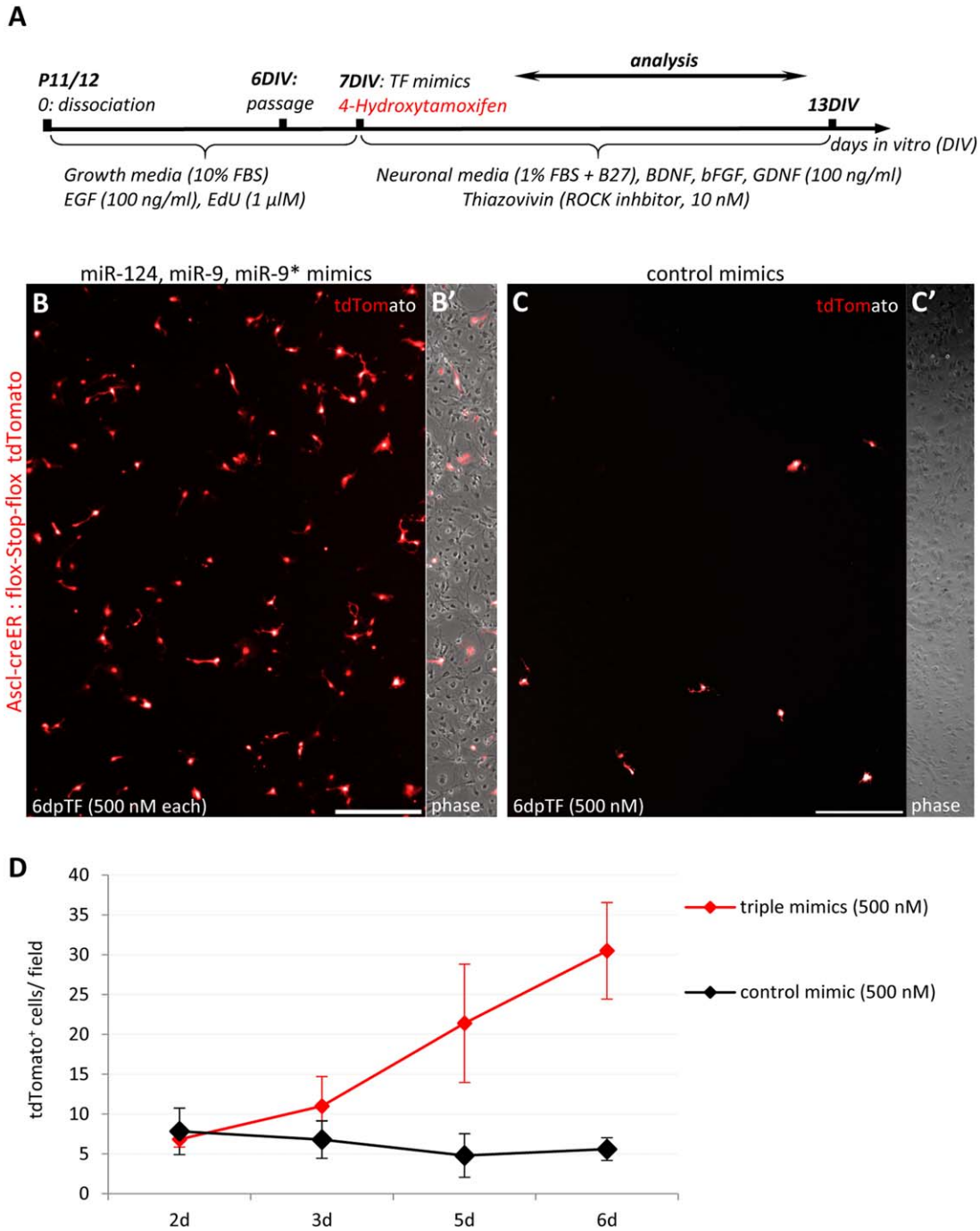

FIGURE 3: miR-124, miR-9, and miR-9* mimics induce Ascl1 expression in Müller glia. A, Cell culture and transfection scheme. 4-Hydroxytamoxifen to activate the Cre recombinase was added to medium at the time of miR-124-9-9* or control mimics transfection, 1 day post passage. B, C, Live images of Ascl-creER:flox-stop-flox tdTomato Müller glia cultures 6 days post-miR-124-9-9* mimics or control mimic transfection (500 nM). tdTomato$^+$ cells are shown as red fluorescent image merged with gray scale in order to visualize the entire cell including fine processes. B', C', phase, images. D, Time-course of the number of tdTomato$^+$ cells per field after mimic transfection. Scale bars: 100 µm. TF: transfection.

Therefore, we tested whether miR-124-9-9* can synergize with Ascl1 and improve the reprogramming process. We tested various paradigms including (a) both factors together one day post passage and (b) first miR-124-9-9* and 2 to 4 days later Ascl1. Finally decided to induce Ascl1 expression on the first day after passage, followed by miR-124-9-9* (3–4 days after Ascl1 induction, Fig. 6A,B). RT-qPCR confirmed that Ascl1 levels increased significantly and to similar levels in cells expressing Ascl1 alone, or in combination with miR-124-9-9*, as compared with GFP-controls (Fig. 6C). We also

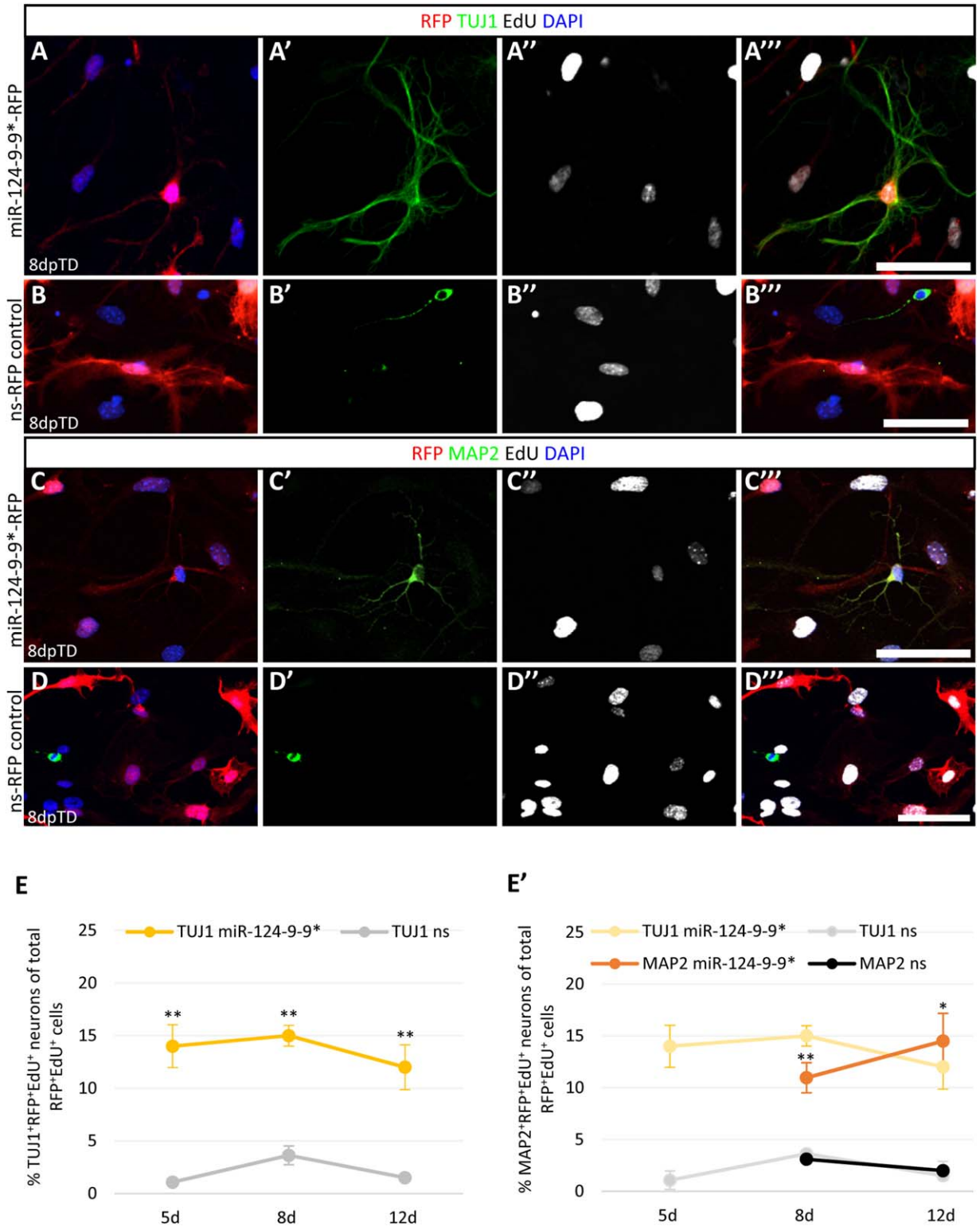

FIGURE 4: Overexpression of miR-124-9-9* results in about 15% TUJ1$^+$ glia derived neurons 8 to 12 days after transduction. **A–D,** Immunofluorescent labeling of miR-124-9-9*-transduced Müller glia with antibodies against TUJ1, MAP2, and RFP. Nuclei are stained with DAPI, proliferative cells with EdU. **E,** Relative number of newly generated TUJ1$^+$ neurons (TUJ1$^+$RFP$^+$EdU$^+$) of the total number of RFP$^+$EdU$^+$ cells 5, 8, and 12 days post-miR-124-9-9* and nonsense (ns) transduction (dpTD). **E',** Relative number of newly generated MAP2$^+$ neurons (MAP2$^+$RFP$^+$EdU$^+$) of the total number of RFP$^+$EdU$^+$ cells 8, and 12 days after miR-124-9-9* and nonsense (ns) transduction in relation to TUJ1$^+$ neurons. Mean ± SEM. Student's *t*-test: *$P < 0.05$; **$P < 0.01$. Scale bars: 50 μm.

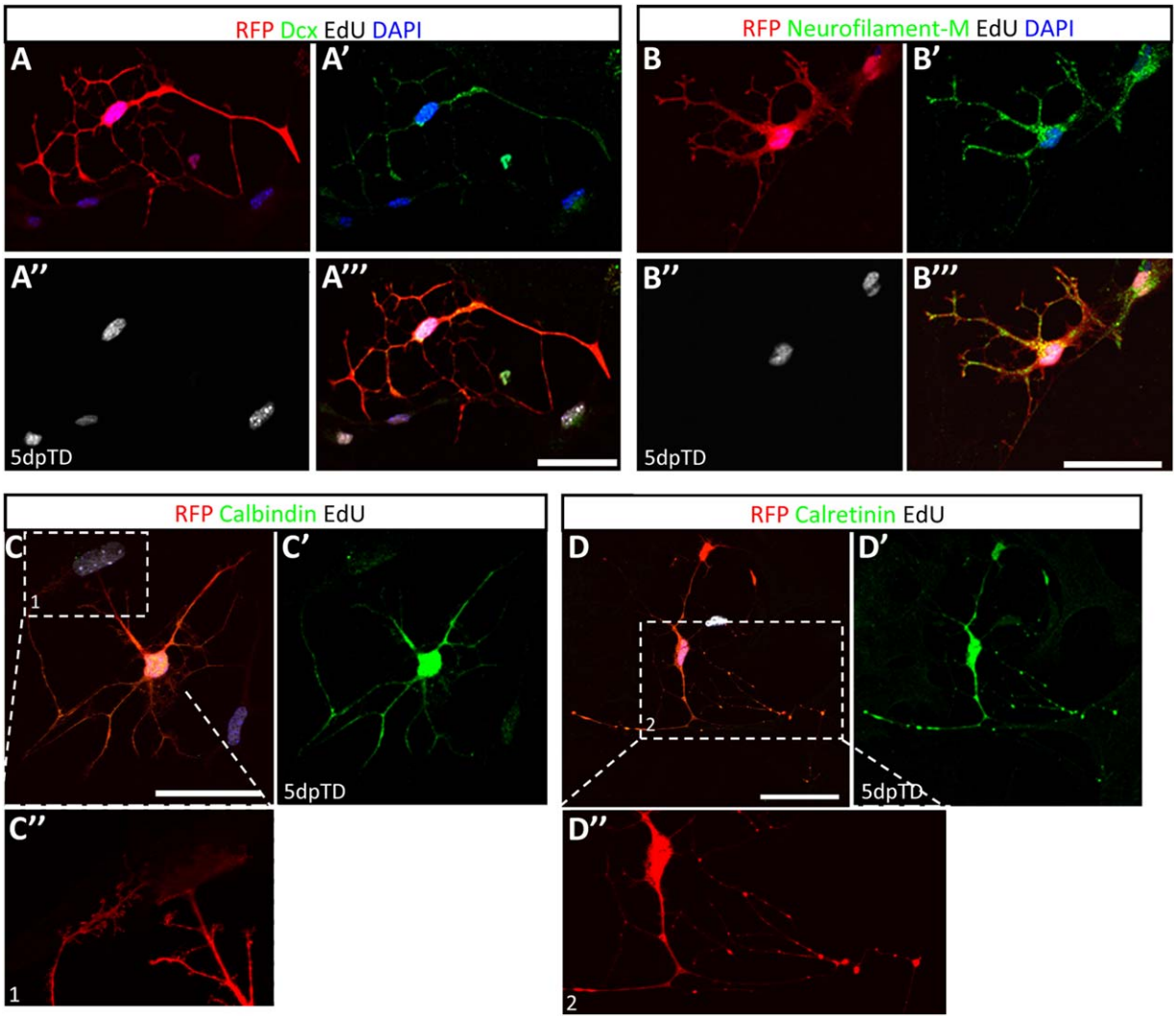

FIGURE 5: miR-124-9-9* induces expression of various neuronal markers in Müller glia including doublecortin (Dcx), Calbindin, Calretinin, and Neurofilament-M. A–D: Immunofluorescent labeling of miR-124-9-9*-transduced Müller glia with antibodies against Dcx (A-A‴), Neurofilament-M (B-B‴), Calbindin (C-C″), and Calretinin (D-D″), and RFP. Nuclei are stained with DAPI, proliferative cells with EdU. Some cells have also neuron specific structures such as complex process endings (box 1 in C, C′, in higher magnification in C″) or knot-like structures (box 2 in D, D′, in higher magnification in D″). Scale bars 50 μm.

found increased levels of miR-124, miR-9, and miR-9* in Ascl1/miR-124-9-9* co-transduced cells, confirming successful overexpression of the miRNAs in co-transduced cultures (Fig. 6D). miR-124-9-9* levels in the co-transduced cultures were similar to levels in miR-124-9-9* single transduced cultures (Fig. 2B). However, we also observed an upregulation of let-7a and let-7b after co-transduction, which was not seen after miR-124-9-9* overexpression (Figs. 2B and 6D). In addition, we observed significantly increased levels of miR-124 after Ascl1 transduction, consistent with a previous report in P19 cells (Yu et al., 2008).

Next, we tested the expression of key retinal developmental genes, specific to particular cell types. Atoh7, Islet1, and Onecut2 are expressed early in retinal neurogenesis and are important for generation of retinal ganglion cells and other early-generated cell types, whereas Otx2 is necessary for photoreceptors and bipolar cells (Boije et al., 2014). Ascl1 transduction resulted in a significant upregulation of all of these genes, in accordance to our previous study (Pollak et al., 2013). After co-transduction with Ascl1 and miR-124-9-9*, all tested progenitor genes were significantly upregulated and only Atoh7 showed an increase in expression compared with Ascl1 alone (Fig. 6E). Next, we analyzed mRNA levels of glial genes. After Ascl1 single transduction, we found a significant downregulation of Rlbp, Glul, Slc1a3, and Aqp4, consistent with the data of our previous study (Pollak et al., 2013) and that of other reports about the loss of glial properties after Ascl1 induction (Wurm et al., 2009). After transduction of Ascl1 in combination with miR-124-9-9*, similar results were found, only Slc1a3, the gene for Glast, was lower expressed as in Ascl1 transduced cultures (Fig. 6F).

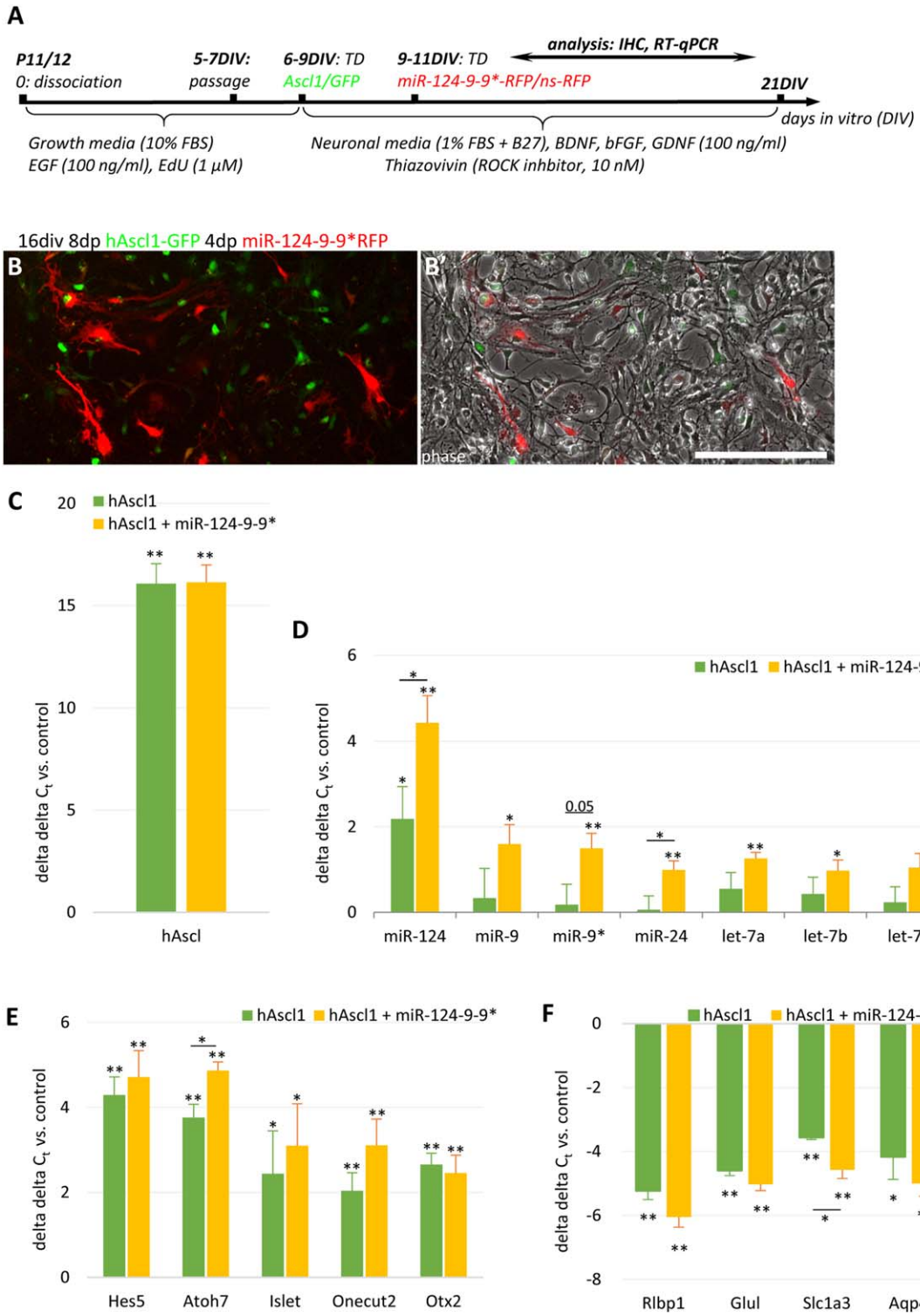

FIGURE 6: Ascl1-GFP co-transduction with miR-124-9-9* results in an altered expression of miRNAs and several neuronal and glial mRNAs. A, Cell culture and transduction scheme. B, Live images of 16DIV Müller glia infected with Ascl1-GFP/miR-124-9-9*-RFP lentiviral vectors (8 days post-Ascl1 and 4 days post-miR-124-9-9* transduction). C: RT-qPCR confirmed similar levels of upregulated Ascl1 in both, Ascl1 and Ascl1/miR-124-9-9*-transduced cells. D, miRNA RT-qPCR analysis showed that miR-124 is upregulated in Ascl1-transduced cells. Besides miR-124, miR-9/9*, miR-24, and some members of the let-7 family were also increased in Ascl1/miR-124-9-9* co-transduced Müller glia. E, RT-qPCR showed upregulation of progenitor and neuronal genes in Ascl1. Co-transduction resulted in a further increase of Atoh7, while all other genes were unaffected. F, Analysis of glial gene expression revealed that Rlbp, Glul, Slc1a3, and Aqp4 were downregulated after both, Ascl1 and Ascl1/miR-124-9-9* with lower Scl1a3 levels after Ascl1/miR-124-9-9*. Mean ± SEM. Student's *t*-test: *$P < 0.05$; **$P < 0.01$. Scale bar 100 μm.

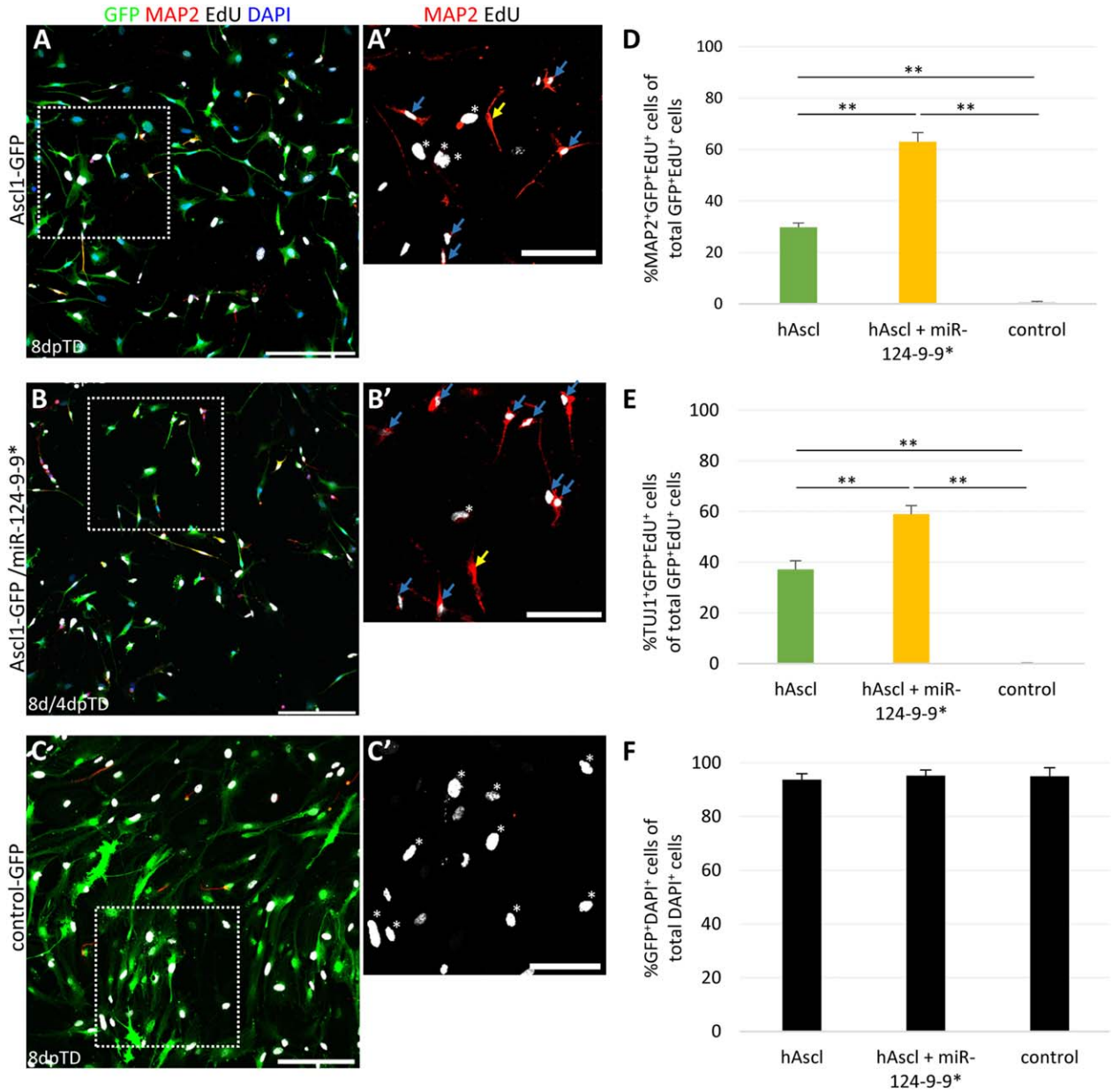

FIGURE 7: Ascl1-GFP co-transduction with miR-124-9-9* results in more MAP2$^+$ and TUJ1$^+$ neurons. A–C, Immunofluorescent staining with antibodies against MAP2 and GFP. Nuclei are counterstained with DAPI, proliferating cells with EdU. A-A', 8 days after Ascl1, B-B', 8 days after Ascl1 and 4 days after miR-124-9-9*, and C-C', 8 days after GFP control vector transduction. Boxes in A–C are shown in higher magnification in A'–C', respectively. Müller glia-derived neurons (MAP2$^+$GFP$^+$EdU$^+$) are indicated by blue arrows, EdU-negative neuronal survivors are indicated by yellow arrows. Big nuclei from non-neuronal cells are indicated by asterisks which are almost absent in the double infected cultures. D, MAP2$^+$GFP$^+$EdU$^+$ cells of the total number of GFP$^+$EdU$^+$ cells after Ascl1 alone or co-transduction. E, TUJ1$^+$GFP$^+$EdU$^+$ cells of the total number of GFP$^+$EdU$^+$ cells after Ascl1 alone or co-transduction. F, Relative number of Ascl1-GFP and control-GFP cells of total EdU$^+$ cells. Scale bars in A–C: 100 μm, A'–C': 50 μm. Mean ± SEM. Student's *t*-test: *$P < 0.05$; **$P < 0.01$.

Although RT-qPCR did not show any changes in expression levels of retinal developmental genes, except for Atoh7, we analyzed the cultures for more general neuronal markers MAP2 and TUJ1, and quantified the numbers of newly generated (EdU$^+$) neurons. We found that ~30% of the total GFP$^+$EdU$^+$ cells expressed MAP2$^+$ in Ascl1-transduced cultures (Fig. 7A-A', blue arrows, D) similar to our previous results (Pollak et al., 2013). In Ascl1/miR-124-9-9* co-transduced cultures however, the fraction of newly generated neurons was ~63% (Fig. 7B-B', blue arrows, D), which represents a further increase of the newly generated fraction by 33% as compared with Ascl1 alone. The fraction of MAP2$^+$GFP$^+$EdU$^+$ cells in control wells was <2% (Fig. 7C-C',D). In all cultures, there were a small numbers of retinal

neurons that survived the dissociation and culture period. These cells are also MAP2$^+$ but could be distinguished from newly generated neurons because of their lack of EdU (Fig. 7A',B' yellow arrows). In addition, in Ascl1-transduced cultures, we found a noticeable number of cells with big nuclei (Fig. 7A' asterisks), which are the most prevalent cell type in control cultures (Fig. 7C' asterisks), indicating incomplete reprogramming. In Ascl1/miR-124-9-9* co-transduced cultures however, these cells were rare (Fig. 7B' asterisks).

Next, we counted numbers of TUJ1$^+$GFP$^+$EdU$^+$ cells for Ascl1 alone and Ascl1/miR-124-9-9* co-transduced cultures and compared them to the GFP-controls. In controls, there were very few TUJ1$^+$ cells (<1%) that were labeled with EdU; by contrast, in Ascl1-transduced cultures, 37% of total GFP$^+$EdU$^+$ cells expressed TUJ1 (Fig. 7E), in accordance with our previous study (Pollak et al., 2013). Interestingly, similar neuronal fractions have been also reported recently after astrocyte reprogramming with Ascl1 (Masserdotti et al., 2015). However, in cultures transduced with both Ascl1 and miR-124-9-9*, 59% of the total GFP$^+$EdU$^+$ cells were labeled with TUJ1, an increase of 22% as compared with Ascl1 alone. For all three conditions, we obtained transduction efficiencies over 90% (Fig. 7F) and cell survival of between 54% (co-transduced) and 62% (Ascl1 alone) over the culture period (not shown). Taken together, the results show that the combination of Ascl1 with miR-124-9-9* results in larger fractions of glial-derived, newly generated neurons that are MAP2$^+$ and TUJ1$^+$.

Ctdsp1 and Ptbp1 of the REST Complex are Downregulated in miR-124-9-9-Transduced Müller Glia*

The mechanisms through which miR-124-9-9* promotes reprogramming may be related to those through which it regulates neurogenesis. To determine whether some of the same targets were involved, we focused on target genes of miR-124 and miR-9/9* that are down-regulated in neurogenesis, but are also expressed in Müller glia. We used Targetscan (http://www.targetscan.org/) and miRbase (http://www.mirbase.org/) to screen for these targets (Table 4). We compared expression levels of target genes in Müller glia with a previously generated cDNA microarray analysis of P12 cultured Müller glia (Pollak et al., 2013). We focused on miR-124 targets, since this miRNA showed the highest increase in RT-qPCR. We found a significant decrease in Ctdsp1 and Ptbp1 gene levels in miR-124-9-9*-transduced Müller glia as compared with controls (Fig. 8A, Table 4, blue lines). Both genes are highly expressed in cultured Müller glia and known targets of miR-124 (Makeyev et al., 2007; Visvanathan et al., 2007; Yeo et al., 2005). We also observed an increase of Ptbp2, a gene negatively regulated by Pbtb1 and known to be upregulated in neuronal cells (Boutz et al., 2007; Makeyev et al., 2007). Other potential targets, including CoREST (Volvert et al., 2014; Wu and Xie, 2006), and also REST itself, Ezh2 (Neo et al., 2014) and a variety of other genes (data not shown, see Table 4) showed no significant changes in mRNA levels in the miR-124-9-9* infected cultures. In order to confirm the repressor characteristics of Ctdsp1 and REST, we transfected Müller glia with dnCtdsp1 and dnREST plasmids (Supp. Info. Fig. S3A). Ascl-creER: flox-Stop-flox tdTomato Müller glia cell cultures were used to test for Ascl1 induction. We found increased numbers of tdTomato$^+$ cells after dnCtdsp1, but not after wtCtdsp1 plasmid transfection (Supp. Info. Fig. S3B-B',C-C', respectively), confirming that inhibition of Ctdsp1 has the same effect on Ascl1 induction as miR-124-9-9* overexpression. Moreover, 6 days after transfection with either dnCtdsp1 or dnREST, we found MAP2$^+$EdU$^+$ neurons (Supp. Info. Fig. S3D-D',E-E'), although plasmid transfections were less efficient than viral transduction (6% *vs.* 60% respectively), and so, the results are not directly comparable. Nevertheless, using this approach, we find that inhibition of the REST pathway leads to upregulation of Ascl1 and neuronal reprogramming.

Discussion

miRNAs have been reported to play important roles in glial development and maintenance (Buller et al., 2012; Georgi and Reh, 2011; Kuang et al., 2012; Zheng et al., 2010) for review see (Bian et al., 2013) and in gliosis (Mor et al., 2011; Wang et al., 2015). However, to our knowledge, this is the first report to show they can be used to reprogram glia to a neurogenic state. Here we show for the first time that the miRNAs, more precisely miR-124-9-9*, can promote the expression of neuronal progenitor genes, like Ascl1 in the glia, and ultimately reprogram the glial cells into neuronal cells. In addition, if miR-124-9-9* is combined with Ascl1, it potentiates Müller glia reprogramming, yielding more neurons than with either miR-124-9-9* or Ascl1 alone. We also found a significant downregulation of Ctdsp1 and Ptbp1 mRNA, both components of the REST complex, suggesting that this repressive pathway functions in glia to maintain their phenotype and prevent reprogramming to neurons.

A Role for miRNAs in Reprogramming Non-Neuronal Cells to Neurons

Overexpression of key proneural transcription factors such as NeuroD, Brn2, Myt1l, Sox2, and Ascl1 can cause a significant percentage of fibroblasts, P19 cells, astroglia, or NG2 glia to differentiate into neurons (Caiazzo et al., 2011; Corti et al., 2012; Heinrich et al., 2012, 2014; Pang et al., 2011; Pollak et al., 2013; Vierbuchen et al., 2010). In our previous studies, we found that overexpression of Ascl1 was sufficient

TABLE 4: miR-124, miR-9, miR-9* Target Screen and mRNA Expression Levels of Target Genes in P12 Müller Glia (Microarray). [Color table can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Gene Name	Name	P12 glia *in vitro*	miRNA sites target scan/ miRBase			
			miR-124	miR-9	miR-9*	Others
Ctdsp1	Carboxy-terminal domain, RNA polymerase II, polypeptide A	11	4	–	–	6
Ptbp1	Polypyrimidine tract binding protein 1	11	1	1	–	22
Rcor	REST corepressor 1, CoREST	9	2	1	–	>30
REST	RE1-silencing transcription factor	9	–	2	–	>30
Sox9	SRY-box containing gene 9	11	1	–	1	NA
Lhx2	LIM homeobox protein 2	11	1	–	–	1
Plekhm3	Pleckstrin homology domain containing, family M, member 3	10	4	–	–	18
Ptgn	Protogenin	7	3	3	2	>30
Vim	Vimentin	13	1	-	1	
Lin28a	LIM homeobox protein 2a	7	–	1	–	>30
Lin28b	LIM homeobox protein 2b	7	1	3	–	>30
Ezh2	Enhancer of zeste homolog 2	10	1	–	–	>30
Lhx9	LIM homeobox protein 9	6	1	1	3	NA
Cdkn1b	Cyclin-dependent kinase inhibitor 1B	11	1	1	1	NA
Kdm5a	Lysine (K)-specific demethylase 5A	9	1	6	2	NA
Gria3	Glutamate receptor, ionotropic, AMPA3 (alpha 3)	11	3	1	2	NA
Sfrp1	Secreted frizzled-related protein 1	12	1	1	1	NA
Socs5	Suppressor of cytokine signaling 5	9	1	2	1	NA
ROCK2	Rho-associated coiled-coil containing protein kinase 2	11	1	1	1	NA
Mbd2	Methyl-CpG binding domain protein 2	11	1	2	1	NA
Arnt	Aryl hydrocarbon receptor nuclear translocator	10	1	3	1	NA
Bcl6	B-cell leukemia/lymphoma 6	9	1	1	1	NA
BCl2l11	BCL2-like 11 (apoptosis facilitator)	8	4	3	2	NA

Blue lines: downregulated targets of miR-124-9-9* in the present study; gray lines: targets of all three miRNAs which did not change; white lines: reported genes of at least one miRNA from the triplet which did not change.
NA: not analyzed.

to promote partial reprogramming in mouse Müller glia (Pollak et al., 2013). Interestingly, miRNAs have been reported to reprogram fibroblasts or P19 cells into neurons, and moreover, potentiate transcription factor-induced reprogramming.

The miRNAs miR-124 and miR-9 are highly enriched in the CNS, and are known to be regulators of neurogenesis (Cheng et al., 2009; Krichevsky et al., 2006; Lim et al., 2005; Neo et al., 2014; Smirnova et al., 2005; Yu et al., 2008). Moreover, miR-124, alone or in combination with miR-9/9*, has been reported to reprogram fibroblasts (Yoo et al., 2011) and P19 cells (Visvanathan et al., 2007) into functional neurons and can substitute for proneural transcription factors. However, there are no previous reports that this approach would also apply to glial cells.

Here we show for the first time that overexpression of miR-124-9-9* can also reprogram Müller glia to produce neurons, without any additional neurogenic reprogramming factors. Overexpression of miR-124-9-9* causes an increase in progenitor genes, including Ascl1, and neuronal genes (e.g., Dcx); a significant percentage of the cells expressed neuronal markers (e.g., MAP2, TUJ1) and showed morphological characteristics of neurons. These results are consistent with

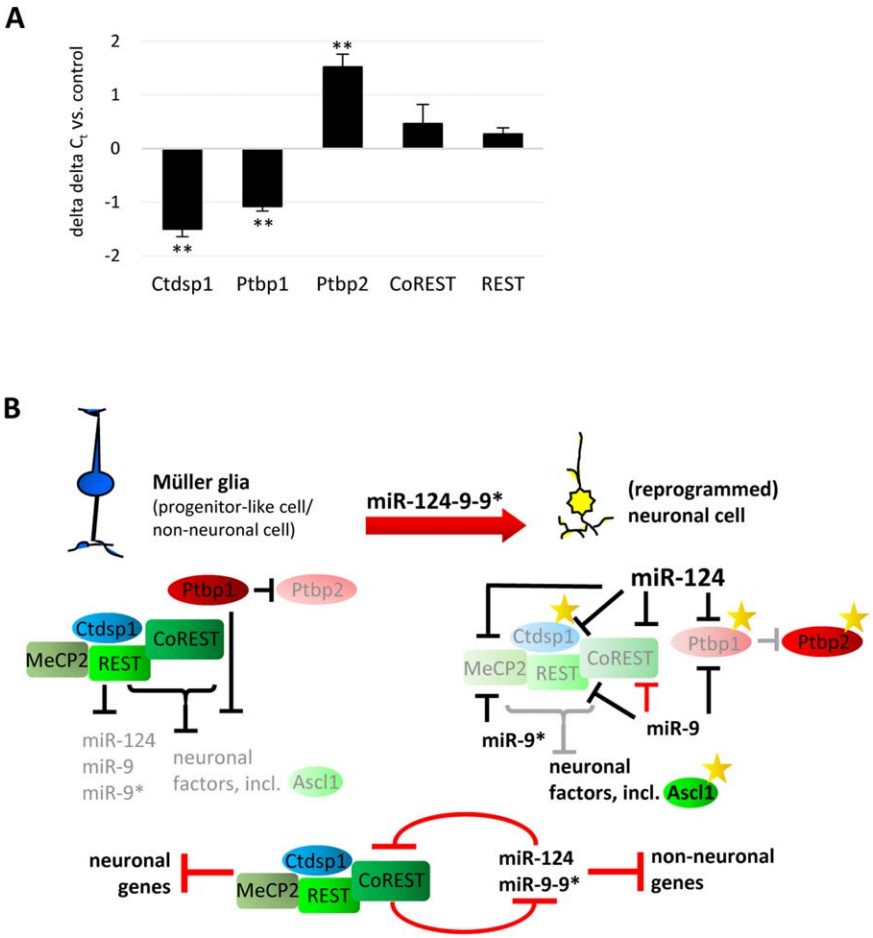

FIGURE 8: miR-124-9-9* targets Ctdsp1 and Ptbp1 and appears to act via the REST/CoREST/Ctdsp1/Ptbp1 pathway. A, RT-qPCR of miR-124-9-9*-transduced Müller glia showed downregulation of Ctdsp1 and Ptbp1 but upregulation of Ptbp2. Ctdsp1 and Ptbp1 are components of the REST complex which silences neuronal genes in non-neuronal cells. Since these components are downregulated while Ptbp2 is upregulated, Müller glia reprogramming appears to utilize the same mechanism as fibroblast reprogramming or neurogenesis. This mechanism is shown in B. For simplification, only the inhibitory interactions are shown. Inactive/silenced components are displayed in gray. In non-neuronal or progenitor cells, the REST complex, which has REST, CoREST, and MeCP2 in its core, represses the expression of neuronal genes (e.g., Ascl1) and targets brain-enriched miRNAs including miR-124, miR-9/9*. The REST complex is stabilized by Ctdsp1 and is also indirectly supported by Ptbp1. Ptbp1 also represses Ptbp2. In neuronal cells and miR-124-9-9*reprogrammed cells, the members of the REST core complex are targeted and silenced by miR-124, miR-9/9* which leads to expression of neuronal genes including Ascl1. These targets are here Ctdsp1 and Ptbp1. Modified after (Abrajano et al., 2009b; Sun et al., 2013; Visvanathan et al., 2007; Wu and Xie, 2006; Xue et al., 2013). The stars mark the factors which changed after miR-124-9-9* overexpression in converted Müller glia. *t*-test: *$P < 0.05$; **$P < 0.01$. Ctdsp1: carboxy-terminal domain, RNA polymerase II, polypeptide A; Ptbp1: polypyrimidine tract binding protein 1; Ptbp2: polypyrimidine tract binding protein 2; REST: RE1-silencing transcription factor; CoREST: Co-repressor of REST; MeCP2: methyl CpG-binding protein2; Ascl1: Achaete-scute homolog bHLH transcription factor.

previous reports in migrating neuronal progenitors (Volvert et al., 2014), P19 cells (Visvanathan et al., 2007), and fibroblasts (Yoo et al., 2011) where Dcx, Ngn2 together with NeuroD, and TUJ1 expression increased either because of increasing endogenous miR-124 expression (during neurogenesis) or vector mediated miR-124 overexpression (reprogramming). Interestingly, miR-124-9-9* overexpression alone can reprogram a small percentage of fibroblasts to neurons, but is much more effective when combined with other proneural transcription factors (Yoo et al., 2011). Moreover, miR-124 alone can also induce modest neuronal reprogramming in P19 cells (Visvanathan et al., 2007) but noticeable neuronal differentiation in neuroblastoma cells (Makeyev et al., 2007), without any other factors. These results suggest that cells in the neural lineage including glia, are more likely to be reprogrammed by miR-124 than cells from different lineages, such as fibroblasts.

We also found that the combination of Ascl1 and miR-124-9-9* resulted in a higher fraction of reprogrammed cells, when compared with that observed with Ascl1 alone. This synergistic effect has also been reported in fibroblasts (Ambasudhan et al., 2011; Yoo et al., 2011) as well as in P19 cells (Yu et al., 2008), but not for any glia cell type yet. The synergy may be in part due to the fact that Ascl1 overexpression

causes an increase of endogenous miR-124 (Yu et al., 2008), and the induction of neurogenesis induces miR-124 (Conaco et al., 2006; Makeyev et al., 2007; Yu et al., 2008), and miR-9-9* (Sempere et al., 2004).

miR-124-9-9 Act via the REST Complex by Repressing Specific Components*

One of the major mechanisms by which miR-124 regulates neuronal gene expression is via its repression of the anti-neuronal REST/CoREST/Ctdsp1/Ptbp1/Ptbp2 pathway (Chong et al., 1995; Conaco et al., 2006; Makeyev et al., 2007; Nesti et al., 2014; Schoenherr and Anderson, 1995; Visvanathan et al., 2007; Wu and Xie, 2006; Xue et al., 2013; Yeo et al., 2005), summarized in Fig. 8B. The REST complex consists of REST itself, CoREST, and the methyl CpG binding protein 2 (MeCP2) and is stabilized by Ctdsp1. In non-neuronal cells, REST targets neuronal genes as well as miR-124, miR-9, and miR-9* and represses their expression. In neurons and in their precursors, miR-124, miR-9, and miR-9* target members of the REST complex, which allows expression of neuronal genes.

Our results show that Ctdsp1 is reduced by expression of miR-124-9-9* in Müller glia, consistent with previous reports in other cell types (Visvanathan et al., 2007). Ctdsp1 is recruited by REST and is required for REST function (Nesti et al., 2014; Yeo et al., 2005). Since REST targets a variety of neuronal genes including Dcx (Mandel et al., 2011) and Ascl1 (Wu and Xie, 2006), this mechanism may explain why we see increases in these genes after miR-124-9-9* overexpression in Müller glia. We also found a decrease of Ptbp1, and an increase of Ptbp2 mRNA after miR-124-9-9* overexpression in Müller glia. In the brain, Ptbp2 (neuronal PTB/nPTB) protein has been reported to be specifically expressed in post-mitotic neurons, whereas Ptbp1 is restricted to NPCs, glia, and other non-neuronal cells (Boutz et al., 2007). Ptbp1 represses the alternative splicing of neuronal genes in non-neuronal tissue (Makeyev et al., 2007). This repression is due in part to Ptbp1-induced alternative splicing of Ptbp2 mRNA, leading to nonsense-mediated decay (Boutz et al., 2007). During neuronal differentiation, the increasing levels of miR-124 cause a reduction in Pbtp1, which leads to the accumulation of correctly spliced Ptbp2 protein (Makeyev et al., 2007). In addition, Ptbp1 blocks miR-124-mediated silencing of the REST complex, while miR-124 targets Ctdsp1 and leads to destabilization of the REST complex. Ptbp1 has been reported to compete with miR-124 on the 3' UTR of Ctdsp1 and therefore stabilize the REST complex. If Ptbp1 is antagonized, Ptbp1-mediated inhibition of miR-124 is abolished, and this leads to expression of a large number of neuronal genes, including miR-124, miR-9, and miR-9* (Xue et al., 2013). The studies using miR-124-9-9* in reprogramming of mature cells have so far used cells from non-neural tissues, where Ptbp1, Ctdsp1, and/or REST function represses neuronal gene expression (Ambasudhan et al., 2011; Makeyev et al., 2007; Visvanathan et al., 2007; Yoo et al., 2011; Yu et al., 2008). Our results now indicate that this pathway also plays a role in Müller glia conversion into neurons, since dnCtdsp1 and dnREST plasmids cause an upregulation of Ascl1 (tdTomato+ cells) and $Map2^{+}EdU^{+}$ neurons 6 days post-transfection. Since in our study miR-124-9-9* overexpression decreases Ctdsp1 and Ptbp1 mRNA and increases Ptbp2, Ascl1 and other neuronal genes in Müller glia, and moreover, inhibition of REST or Ctdsp1 resulted in Müller glia conversion into neurons, our results indicate that the REST pathway is also actively repressing neuronal genes in Müller glia, and probably also in other glia types of the brain.

The REST complex has been reported to be an evolutionary conserved transcriptional regulator that acts globally to silence genes (Yeo et al., 2005). There is increasing evidence that the REST complex is responsible for the repression of neuronal genes in glia. For example, a recent report from the laboratories of François Guillemot and Magdalena Götz demonstrated that REST blocks reprogramming in astrocytes (Masserdotti et al., 2015). Moreover, Abrajano et al. used ChIP-chip analysis to show that REST and CoREST occupy sites at promoters of many neuronal genes in astrocytes and oligodendrocytes (Abrajano et al., 2009a). REST is also upregulated after bone morphogenetic protein (BMP)-induced astrocyte differentiation and dnREST or shRNA against REST induce neuronal gene expression in astrocytes (Koyama et al., 2010). $GFAP^{+}$ astrocytes derived from Rest−/− radial glia-like NPCs co-express many neuronal genes, like NeuN, MAP2, and Dcx (Soldati et al., 2012). Our results extend these reports to Müller glia and further suggest that attempts to reprogram astrocytes in the brain may also find miR-124-9-9* as a useful additional/synergistic reprogramming factor.

Summary and Future Directions

Here we show for the first time that miR-124-9-9* can reprogram Müller glia. Overexpression of these miRNAs resulted in an upregulation of progenitor and neuronal genes; morphological changes to neuron-like cells; and the expression of the neuronal markers MAP2 and TUJ1. Moreover, miR-124-9-9* in combination with Ascl1 potentiated Müller glia reprogramming, yielding more neurons than with either miR-124-9-9* or Ascl1 alone. Furthermore, two established targets of miR-124-9-9*, Ctdsp1 and Ptbp1, were reduced in Müller glia after miR-124-9-9* expression. Since both are components of the REST complex, we propose that miR-124-9-9* reprogramming acts via blocking the repression of neuronal

genes by the REST pathway. Our results show that a combination of proneural activation and REST repression will more effectively reprogram mouse Müller glia to generate neurons *in vitro*.

It is possible that the type of reprogramming that we have described here *in vitro* might also be used to stimulate retinal regeneration *in vivo*. In zebrafish, a type of regulated reprogramming occurs after retinal injury, in which the Müller glia generate new progeny that act as neuronal progenitors. One of the early events in this natural reprogramming phenomenon is the expression of Ascl1 in the Müller glial cells. Our results show that miR-124-9-9* can induce Ascl1 expression in the Müller glia, suggesting that this may be an initiating event in fish retinal regeneration. We recently reported that transgenic expression of Ascl1 in Müller glia of young mice endows them with the capacity for regenerating new neurons after retinal injury (Ueki et al., 2015). The results of the present study indicate that combining miR-124-9-9* might potentiate this *in vivo* regeneration from mouse Müller glia, possibly allowing regeneration even in adult animals.

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