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Neurobiology: MicroRNA miR-124 Controls the Choice between Neuronal and Astrocyte Differentiation by Fine-tuning Ezh2 Expression

Wen Hao Neo, Karen Yap, Suet Hoay Lee, Liang Sheng Looi, Piyush Khandelia, Sheng Xiong Neo, Eugene V. Makeyev and I-hsin Su

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MicroRNA miR-124 Controls the Choice between Neuronal and Astrocyte Differentiation by Fine-tuning Ezh2 Expression*

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Background: Molecular mechanisms underlying reduced expression of Ezh2 during neural differentiation are poorly understood.

Results: miR-124 directly down-regulates Ezh2 expression to promote neuronal differentiation.

Conclusion: Ezh2 is an important target of miR-124 in the context of neuronal differentiation.

Significance: Our report represents a significant advance in understanding the contribution of a microRNA/epigenetic regulatory circuitry to cell fate determination in the neural lineage.

Polycomb group protein Ezh2 is a histone H3 Lys-27 histone methyltransferase orchestrating an extensive epigenetic regulatory program. Several nervous system-specific genes are known to be repressed by Ezh2 in stem cells and derepressed during neuronal differentiation. However, the molecular mechanisms underlying this regulation remain poorly understood. Here we show that Ezh2 levels are dampened during neuronal differentiation by brain-enriched microRNA miR-124. Expression of miR-124 in a neuroblastoma cell line was sufficient to up-regulate a significant fraction of nervous system-specific Ezh2 target genes. On the other hand, naturally elevated expression of miR-124 in embryonic carcinoma cells undergoing neuronal differentiation correlated with down-regulation of Ezh2 levels. Importantly, overexpression of Ezh2 mRNA with a 3′-untranslated region (3′-UTR) lacking a functional miR-124 binding site, but not with the wild-type Ezh2 3′-UTR, hampered neuronal and promoted astrocyte-specific differentiation in P19 and embryonic mouse neural stem cells. Overall, our results uncover a molecular mechanism that allows miR-124 to balance the choice between alternative differentiation possibilities through fine-tuning the expression of a critical epigenetic regulator.

Since their discovery in 1993, microRNAs (miRNAs), 19–25-nucleotide-long non-coding RNA molecules, have emerged as versatile regulators of developmental and physiological processes in a large fraction of eukaryotic organisms (1, 2). There are estimated to be over 1000 miRNAs in the human genome, and more than 50% of human genes are predicted to be miRNA targets (3, 4). Mature miRNAs regulate their cognate mRNAs as a part of an miRNA-induced silencing complex containing an Argonaute protein subunit (5). In plants, miRNAs often bind to fully complementary target sites typically located in the mRNA 3′-UTRs, which leads to gene repression through Argonaute-dependent mRNA “slicing” (6). On the other hand, animal miRNAs tend to be partially complementary to their target sequences, which affords regulation of target mRNAs through translational inhibition and slicer-independent destabilization (7, 8).

miRNAs are known to be crucial for neuronal differentiation, because conditional ablation of the endoribonuclease Dicer, an essential component of the microRNA maturation pathway, in neural stem cells or progenitors leads to dramatic defects in survival and differentiation of newborn neurons (9–11). One of the most abundant and perhaps best studied miRNAs in the brain is miR-124 (12–16). miR-124 is derived from three independent genes (miR-124-1, miR-124-2, and miR-124-3) contributing to the increased mature miR-124 levels during neuronal differentiation (4, 17, 18). Interestingly, however, knock-out of just the miR-124-1 gene in the mouse resulted in visible reduction of mature miR-124 levels, defective neuronal survival, and axonal outgrowth as well as smaller brain size (19).

miR-124 may regulate hundreds and possibly thousands of distinct target genes (18, 20–23). Important examples include genes encoding the SCP1 subunit of the global repressor of NS-specific genes REST, transcription factors Sox9 and cAMP-response element-binding protein, Notch ligand Jagged1, and the BAF53a subunit of a chromatin remodeling complex (24–27). We have previously shown that miR-124 also targets mRNA of Ptbp1 (polyypyrimidine tract-binding protein), a global regulator of pre-mRNA splicing (11). Ptbp1 is expressed...
at high levels in non-neuronal cells and neuronal precursors, where it suppresses the utilization of neuron-specific alternative exons. During neuronal differentiation, Ptbp1 expression is reduced by miR-124, which triggers a switch in alternative splicing patterns among a wide range of transcripts. Ptbp1 additionally controls the abundance of several neuron-specific mRNAs through nuclear and cytoplasmic RNA quality control mechanisms (11, 23, 28). Collectively, these studies demonstrate that miR-124 regulates several molecular pathways critical for proper progression of neuronal differentiation.

Neuron-specific genes are frequently modified by Ezh2-mediated H3K27 trimethylation (3meH3K27) in stem cells, whereas both the Ezh2 levels and the density of 3meH3K27 marks are down-regulated upon neuronal differentiation (29–31). Interestingly, overexpression of miR-124 in hepatocellular carcinoma cells, where it is normally present at negligibly low levels, has been shown to reduce Ezh2 expression (32). However, whether miR-124 contributes to down-regulation of Ezh2 expression during neurogenesis has not been investigated.

To this end, we first expressed miR-124 in mouse neuroblastoma Neuro2a (N2a) cells and showed that this treatment was sufficient to up-regulate a significant fraction of neuron-specific Ezh2 target genes. We further found that in P19 cells undergoing neuronal differentiation, the Ezh2 protein level was significantly reduced in an inverse correlation with increasing expression of mature miR-124. Importantly, miR-124-specific antisense inhibitor restored Ezh2 expression in differentiating P19 cells, whereas disruption of the putative miR-124 target site in exogenously expressed Ezh2 3′-UTR abolished the miR-124-mediated down-regulation and led to reduced neuronal differentiation. A similar effect of miR-124-regulated Ezh2 expression on neurogenesis was also observed in differentiating embryonic mouse neural stem cells. Thus, our results implicate Ezh2 as an important miR-124 target in the context of neuronal differentiation.

miR-124 Controls Ezh2 Expression during Neurodifferentiation

**EXPERIMENTAL PROCEDURES**

**Plasmids**—To generate the EGFP reporter construct for miRNA screening, 3′-UTR of Ezh2 was PCR-amplified from RP24–191K13 BAC clone and subcloned into the NotI site of pEGFP-N1 vector (Clontech). miRNA expression vectors were modified from pEM157 vector (11). A ~500-bp DNA fragment flanking precursor miRNA sequence of interest was PCR-amplified from human genomic DNA and subcloned into the SpeI and NotI site of the intronic region of dsRed2 in pEM157 vector. Various Ezh2 donor plasmids were modified from pRDRIPE plasmid (33) by replacing EGFP with Ezh2 or Ezh2-3′-UTR at Agel and BglII sites. The QuikChange site-directed mutagenesis kit (Stratagene) was used to destroy the miR-124 target site in Ezh2 3′-UTR (32).

**Cells**—HEK293T cells were cultured in DMEM/high glucose (PAA Laboratories, GmbH) supplemented with 10% fetal bovine serum (FBS), 1 mm sodium pyruvate, 2 mm L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 55 μm 2-mercaptoethanol (all from Invitrogen). P19 cells were routinely propagated in α-minimal essential medium (HyClone) supplemented with 2.5% FBS, 7.5% bovine calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin.
miR-124 Controls Ezh2 Expression during Neurodifferentiation

**A**
- [Diagram showing gene expression regulation]

**B**
- Heat map with 487 probes (414 genes) and 1646 probes (1255 genes)

**C**
- Venn diagram showing Ezh2 target genes and CNS-specific genes:
  - Ezh2 target genes: 634, 1254, 7617
  - CNS-specific genes: 52, 142, 198

**D**
- Bar graph showing relative fold change for genes:
  - Ezr2
  - Ascl1
  - Atf3
  - Dusp8
  - En2
  - Sypl2
  - Tpm1

Legend:
- Control
- miR-124
- miR-124 + Ezh2

Significance levels:
- ***p < 0.0001
- **p < 0.01
- *p < 0.05
miR-124 Controls Ezh2 Expression during Neurodifferentiation

Site-directed Mutagenesis—The predicted miR-124 binding site was mutated by base pair changes using DpnI-mediated site-directed mutagenesis (Stratagene). The primer sequences containing the Ago1(shAgo2-3')-UTR transgene expressed pre-miR-124-Sub reverse, 5′-CGACGAGGAGGCAAAAT-AGCAGAGGGCTGCC-3′; mTpm1 forward, 5′-TGAGGATAGTTGGG-3′; mDusp8 reverse, 5′-TACGCAGAGGAATAGCAGA-3′.

miRNA Northern Blot Analysis—Five μg of total RNA samples were separated on a 15% denaturing polyacrylamide gel containing 8 M urea and 1× TBE at 2.5 mA/cm² for 30 min. RNA was cross-linked to the membrane by UV irradiation (0.15 J/cm²), and the membrane was blocked with 6× SSC, 7% SDS at 42 °C for overnight. Hybridization probes were prepared by labeling the appropriate oligodeoxynucleotides using T4 polynucleotide kinase (New England Biolabs) and [γ-32P]ATP (PerkinElmer Life Sciences). The 32P-labeled probes were purified using Sephadex G-25 microspin columns (Geneaid) and added to the blocking solution. The hybridization was carried out for overnight at 42 °C. The membranes were washed four times with 3× SSC, 0.1% SDS at 42 °C and exposed to phosphorimaging plates.

Immunoblotting—Whole cell lysates were prepared by resuspending cells in Nonidet P-40 buffer (50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 10% glycerol) and sonicated (Bioruptor). Protein concentration was determined using the Bio-Rad DC protein assay (Bio-Rad) and analyzed by a Tecan infinite F200 plate reader (Tecan). Protein samples were subjected to SDS-PAGE. Antibodies used in the study were anti-Ezh2 (Cell Signaling Technology), anti-Tuj1 (Covance), anti-Erk1/2 (Sigma), anti-Suz12 (Abcam), and anti-Eed (Millipore).

Mousearray Analyses—N2a cell pools expressing different Argonaute paralog blends were described previously (35). Of these, N2a-WT cells containing the EGFP(shLuc) transgene had a native Argonaute expression dominated by Ago2, N2a-A1 cells containing the Ago1(shAgo2-3′)-UTR transgene expressed predominantly Ago1, and N2a-A2 cells containing the Ago2(shAgo2-3′)-UTR transgene overexpressed Ago2 (35). All three cell pools were cultured in DMEM (HyClone) containing 10% FBS (HyClone, characterized grade), 1 mM sodium pyruvate (Invitrogen), 100 IU/ml penicillin, 100 mg/ml streptomycin (Invitrogen), 5 μg/ml puromycin, and 2 μg/ml doxycycline (antibiotic-containing complete DMEM) for 72 h prior to siRNA/miRNA duplex transfections. One million cells were seeded per well of a 6-well plate in 2 ml of antibiotic-free complete DMEM and allowed to adhere for 1 h. 100 pmol of corresponding siRNA/miRNA duplex mixed with Lipofectamine 2000 and OptiMEM I (Invitrogen) was then added per well as recommended. Medium was changed 5 h post-transfection to antibiotic-containing complete DMEM, and the incubation was continued for another 24 h. Total RNAs were extracted with TRIzol (Invitrogen) and cleaned up using an RNeasy kit (Qiagen). The RNAs were hybridized with Agilent Mouse SurePrint G3 8 × 60K Gene Expression Microarrays as recommended. The data sets were normalized using RobiRNA (36), and genes showing consistent miR-124-induced up- or down-regulation were further analyzed using Ingenuity Pathway Analysis Software (Ingenuity).
miR-124 Controls Ezh2 Expression during Neurodifferentiation

A

Relative MFI

Control |

| Down-regulated |

| No changes |

| Up-regulated |

-log p value

B

ANOVA, p = 1.41x10^{-4}

C

Ezh2

1.0 0.8 0.6 0.4 0.3 0.3

-100kD

Tubulin

-50kD

D

Relative fold change

E

miR-124

3' cccuagugccgUACGGAAU 5'

Ezh2 3'-UTR

Mmu auaacuuuaau UGCCUU c 167

Hsa auaacuuuaau UGCCUU c 169

Rno auaacuuuaau UGCCUU c 167

Ocu auaacuuuaau UGCCUU c 171

Ptr auaacuuuaau UGCCUU c 168

Cfa auaacuuuaau UGCCUU c 174

Oga auaacuuuaau UGCCUU c 167

Mml auaacuuuaau UGCCUU c 170

Eca auaacuuuaau UGCCUU c 172

Bta auaacuuuaau UGCCUU c 170

Sub 3'-UTR Mmu auaacuuuaau ACGGAAU c 167

F

Relative fold change

WT 3'-UTR Del 3'-UTR Sub 3'-UTR

EV

miR-124

**
miR-124 Controls Ezh2 Expression during Neurodifferentiation

RESULTS

Overexpression of miR-124 in Neuroblastoma Cells Up-regulates Neuro-specific Ezh2 Target Genes—To better understand miR-124 functions in the neural lineage, we transfected three N2a cell populations expressing distinct blends of Argo-nauta paralogs (N2a-WT, N2a-Ago1, and N2a-Ago2; see Ref. 35 for details) with either a synthetic siRNA-like duplex designed to deliver mature 22-mer miR-124 or a non-targeting siRNA control and analyzed the samples by Agilent gene expression microarrays. N2a cells were chosen because they express endogenous miR-124 at negligibly low levels (11, 35). Hierarchical clustering of the microarray data suggested that all three cell populations responded to miR-124 in a largely similar manner (Fig. 1A). This allowed us to pool individual population-specific data sets and focus on highly reproducible gene expression changes. In line with previous studies suggesting that this miRNA may directly regulate hundreds distinct mRNA targets, miR-124 consistently down-regulated 1255 genes (≥1.5-fold, p < 0.001; t test). Analysis of this subset by gene set enrichment analysis (37, 38) showed a dramatic over-representation of predicted miR-124 targets (p = 0; data not shown).

Interestingly, 414 genes were consistently up-regulated (≥1.5-fold, p < 0.001; t test) in miR-124-transfected N2a cells, presumably as a result of indirect effects (Fig. 1B). Strikingly, gene set enrichment analysis of this group uncovered a highly significant enrichment of genes previously identified as targets of 3meH3K27 histone modification or Suz12 either by ChIP-on-chip or ChIP-sequencing (data not shown), indicative of possible regulation of these genes by the PRC2 complex containing Ezh2 as a catalytic histone methyltransferase subunit. Notably, when we compared the list of the miR-124-up-regulated central nervous system (CNS)-specific genes (39) with genes known to be regulated by Ezh2 in stem cells (40), a highly significant overlap was detected (p = 7.03 × 10⁻⁵; Fisher’s exact test) (Fig. 1C). On the other hand, the overlap between the corresponding subsets of miR-124-down-regulated CNS-specific genes and Ezh2 target genes could be explained by random sampling (p = 0.56; Fisher’s exact test) (data not shown). Six of the miR-124-up-regulated Ezh2 target genes were further validated by RT-qPCR, and their expression levels were down-regulated by simultaneous expression of miR-124-resistant Ezh2 (Fig. 1D). These results suggested that miR-124 might regulate extensive subsets of genes by targeting Ezh2.

miR-124 is a Potent miRNA Regulator of Ezh2 Expression—To examine the extent of miRNA-dependent regulation of Ezh2 expression, we co-expressed an EGFP-Ezh2 3’-UTR reporter construct with each of the 30 miRNAs (including miR-124) predicted to interact with Ezh2 3’-UTR by five miRNA target prediction algorithms (Target Scan, PicTar, miRBase, miRNA.org, and MicroInspector) or nervous system-specific miRNA miR-9 (41) lacking the predicted binding sites in the Ezh2 3’-UTR. FACS analysis showed that the miR-124-induced down-regulation of the EGFP-Ezh2 3’-UTR expression exceeded that of most other miRNAs and was comparable with the effects induced by miR-26a and miR-101 (Fig. 2, A and B), two miRNAs previously reported to regulate Ezh2 expression and contribute to tumorigenesis (42, 43). Moreover, overexpression of miR-124, as well as miR-26a and miR-101, caused a noticeable down-regulation of endogenous Ezh2 protein level in HEK293T cells (Fig. 2C). The miR-9 control had no effect on the EGFP-Ezh2 3’-UTR expression and the expression of endogenous Ezh2 protein, as expected. These findings were further confirmed by a secondary screen with a luciferase Ezh2 3’-UTR reporter co-transfected with miR-124 or several other high scoring miRNA candidates (Fig. 2D).

Notably, disruption of the predicted evolutionarily conserved miR-124 target site in the Ezh2 3’-UTR (Fig. 2E) by substitution or deletion (32, 44) abolished the miR-124-mediated down-regulation effect (Fig. 2F). We concluded that miR-124 is among the most efficient miRNA regulators of Ezh2 expression.

miR-124 Down-regulates Expression of Ezh2 but Not Other PRC2 Components during Neuronal Differentiation—To examine whether physiological levels of miR-124 could regulate Ezh2 expression during neuronal differentiation, we took advantage of the retinoic acid (RA)-induced P19 embryonic carcinoma in vitro differentiation model (45, 46). We found that the Ezh2 protein level was noticeably down-regulated in P19 cells undergoing neuronal differentiation (Fig. 3A), whereas Ezh2 mRNA levels remained virtually unchanged (Fig. 3B), thus

FIGURE 2. miR-124 regulates Ezh2 expression by targeting Ezh2 3’-UTR. A, screening for microRNAs regulating Ezh2 expression. An EGFP driven by the CMV promoter was fused to Ezh2 3’-UTR and used as a reporter in our fluorescence-based screening system. Individual miRNAs were expressed by dsRed2 containing vector pEM157. Both reporter and miRNA expression vectors were transfected into HEK293T cells and analyzed by FACS. miR-26a and miR-101 expression constructs were used as positive controls, and miR-9 was used as a negative control. EGFP mean fluorescence intensity (MF) of the EGFP'-dsRed2' population was assayed 48 h post-transfection. Relative mean fluorescence intensity was calculated after normalization against that of the empty vector (EV) control. B, miR-124 targets Ezh2 3’-UTR. Indicated miRNA expression vectors were co-transfected with EGP reporter constructs in HEK293T cells. C, endogenous Ezh2 expression was analyzed by Western blot, and Tubulin was used as a loading control. The -fold change of Ezh2 was normalized to EV control and is shown below the Ezh2 blot. Data shown are representative of three independent experiments. D, the indicated miRNA expression vectors were co-transfected with luciferase reporter construct in HEK293T cells. Luciferase assay was performed 48 h post-transfection. Relative luciferase activity was normalized against Renilla luciferase activity. Relative -fold change was calculated relative to the relative luciferase activity of the EV control. E, the miR-124 target site in Ezh2 3’-UTR is highly conserved among vertebrates. Alignment of the predicted miR-124 binding site in Ezh2 3’-UTR of different species is shown (Mus musculus; Mmu, Homo sapiens; Rno, Rattus norvegicus; Ocu, Oryctolagus cuniculus; Psa, Pan troglodytes; Cfa, Canis familiaris; Oga, Otolemur garnetti; Mml, Macaca mulatta; Eca, Equus caballus; Bta, Bos taurus). F, miR-124 target sites in Ezh2 3’-UTR was analyzed by luciferase reporter. Mutations of the miR-124 target site in Ezh2 3’-UTR specifically abolish miR-124-mediated down-regulation of the luciferase reporter. The seed region of the miR-124 target site in Ezh2 3’-UTR was either mutated by deletion (Del 3’ UTR) or substitution (Sub 3’ UTR) or left intact (WT 3’ UTR). The -fold change was calculated relative to the luciferase activity of the reporter with wild-type Ezh2 3’-UTR in the absence of additional miRNA expression (EV). All data shown in the bar graph are mean ± S.D. (error bars) of at least three independent experiments. The differences between groups were first determined by analysis of variance (ANOVA and Dunnett’s) and the significance of miRNA-mediated down-regulation compared with the control was determined by a two-tailed Student’s t test with equal variance (**, p < 0.05; ***, p < 0.01; ****, p < 0.001).
indicating possible involvement of a post-transcriptional regulatory mechanism. Indeed, we found that miR-124 expression was inversely correlated with Ezh2 expression during P19 neuronal differentiation (Fig. 3C). Interestingly, mRNA and protein expression dynamics of two other PRC2 components, Suz12 and Eed, showed similar trends (Fig. 3, D and E).

Because, unlike Ezh2, the Suz12 and Eed 3’-UTRs lacked putative miR-124 target sites, we hypothesized that miR-124 may directly down-regulate Ezh2 during neuronal differentiation, whereas Suz12 and Eed are probably regulated by distinct post-transcriptional mechanisms. To test this prediction, we disrupted the activity of endogenous miR-124 with a miR-124-specific locked nucleic acid antisense oligonucleotide in differentiating P19 cells.}

**FIGURE 3.** miR-124 down-regulates Ezh2 expression during P19 neuronal differentiation. A–E, inverse expression patterns of PRC2 members and miR-124 during P19 neuronal differentiation. Neuronal differentiation of P19 cells was induced by 1 μM all-trans-RA treatment for 4 days. Cell aggregates were dissociated into single cell suspensions at day 4 and recultured in neurobasal medium with N2 supplement. Cells were further cultured and collected at the indicated time points. A, Ezh2 and Tuj1 protein levels were analyzed by Western blot, and Erk1/2 served as a loading control. B, expression of Ezh2 mRNA level was determined by semiquantitative RT-PCR. Hprt served as a loading control. C, increased miR-124 expression level during P19 neuronal differentiation. miR-124 expression levels were determined by Northern blot, and U6 RNA served as a loading control. A representative figure of two independent experiments is shown. This observation is shown in an earlier report (41). D, Suz12, Eed, and Tuj1 protein levels were analyzed as described above, E, expression of Suz12 and Eed mRNA levels was analyzed by semiquantitative RT-PCR. HPRT served as a loading control. D–H, miR-124 inhibitor up-regulates endogenous Ezh2. miR-124 inhibitor was transfected into differentiating P19 cells at day 6 after the start of RA treatment. The levels of miR-124 and PRC2 members were analyzed at day 10. F, down-regulation of miR-124 expression level by inhibitor was confirmed by Northern blot analysis. A representative figure of two independent experiments is shown. The miR-124-specific inhibitor has been tested and published previously (11). G, Ezh2 protein expression was up-regulated upon miR-124 inhibitor treatment. H, Suz12 protein level was not altered upon miR-124 inhibitor treatment, whereas Eed protein level was below the detection limit of the Western blot assay. Ezh2, Suz12, Eed, and Tuj1 protein expression levels were normalized to Erk1/2. Their -fold changes were calculated relative to the protein levels at day 0 (Ezh2 and Suz12), day 10 (Tuj1), or in non-transfected controls (G and H) and indicated below their respective blots. N.D., not detectable. All data shown, unless otherwise stated, are representative of at least three independent experiments.
miR-124 Controls Ezh2 Expression during Neurodifferentiation

VOLUME 289 • NUMBER 30

miR-124 Controls Ezh2 Expression during Neurodifferentiation

miR-124 regulates Ezh2 expression in a critical manner to balance neurogenesis versus astrogensis. In P19 neuroblastoma cells, Ezh2 is the only PRC2 member directly regulated by miR-124.

FIGURE 4. Generation of doxycycline-inducible P19 cell lines expressing Ezh2. A, targeting strategy for the generation of doxycycline (Dox)-inducible P19 cell lines expressing Ezh2. The diagram shows the targeting construct and the acceptor locus before and after Cre-recombinase-mediated recombination. The empty and filled arrowheads indicate the LoxP2272 and LoxP sequence, respectively. B, schematic presentation of various targeting constructs that were used to generate Dox-inducible P19 cell lines expressing Ezh2 with various 3’-UTRs. EGFP was used as a control for the inducible system. Filled black boxes denote artificial β-globin 3’-UTR (EGFP control and Ezh2). Filled gray boxes designate Ezh2 3’-UTR (Ezh2 WT 3’ UTR). Black star, mutation in the miR-124 target site of wild-type 3’-UTR (Ezh2 Sub 3’ UTR). Puromycin was used as a positive selection marker for the screening of Cre-recombinase-mediated recombination events in P19 HILO-RMCE acceptor cell line (33). EF1α, elongation factor-1α promoter; TRE, tetracycline response element; rtTA3, reverse tetracycline transactivator.

The diagram shows the targeting construct and the acceptor locus before and after Cre-recombinase-mediated recombination. The empty and filled arrowheads indicate the LoxP2272 and LoxP sequence, respectively. B, schematic presentation of various targeting constructs that were used to generate Dox-inducible P19 cell lines expressing Ezh2 with various 3’-UTRs. EGFP was used as a control for the inducible system. Filled black boxes denote artificial β-globin 3’-UTR (EGFP control and Ezh2). Filled gray boxes designate Ezh2 3’-UTR (Ezh2 WT 3’ UTR). Black star, mutation in the miR-124 target site of wild-type 3’-UTR (Ezh2 Sub 3’ UTR). Puromycin was used as a positive selection marker for the screening of Cre-recombinase-mediated recombination events in P19 HILO-RMCE acceptor cell line (33). EF1α, elongation factor-1α promoter; TRE, tetracycline response element; rtTA3, reverse tetracycline transactivator.

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fluorescence staining and further verified by RT-qPCR for the expression of astrocyte-specific gene S100b (Fig. 6C). A similar astrogenesis-promoting effect was observed in cultured embryonic mouse neural stem cells expressing miR-124-resistant Ezh2 as well (Fig. 6D). Although Ezh2 is reported to prevent premature astrocyte differentiation in neurogenic phase by repressing astrocyte-specific genes in a Chd4-dependent manner (51), the Chd4 expression level was down-regulated at this late stage of P19 culture (Fig. 6E). The elevated recombinant Ezh2 expression at this stage therefore did not suppress but rather promoted astrocyte generation. Our results suggest that miR-124-dependent regulation of Ezh2 expression might be critical for a balanced production of astrocytes and neurons.

DISCUSSION

Neuron-enriched miRNA miR-124 provides a compelling example of a non-coding RNA modulating cellular gene expression at multiple levels (23). Importantly, this miRNA targets
several master regulators of elaborated transcriptional and post-transcriptional programs, including transcription factor Sox9 (24), transcriptional co-repressor SCP1 (26), chromatin remodeling component BAF53A (27), and RNA-binding protein Ptbp1 (11). Here we expand this list by showing that in cells undergoing neural differentiation (P19 as well as embryonic mouse neural stem cells), miR-124 represses the expression of a critical epigenetic factor, lysine methyltransferase Ezh2. We provide evidence that Ezh2 down-regulation by miR-124 in this context promotes neuronal and counters astrocyte-specific differentiation route.

What could be a molecular mechanism underlying this effect? Ezh2 is known to limit neurogenic competence of neural progenitor cells and repress expression of several neurogenesis-promoting genes (30, 31, 49, 52), including master regulator for neuronal lineage commitment and differentiation Ascl1/Mash1 (53, 54). Up-regulation of this gene is sufficient to promote neuronal differentiation (55, 56). Notably, Ascl1 is one of the genes consistently derepressed by miR-124 in N2a neuroblastoma cells (Table 1), and our future studies will determine the functional significance of this effect.

FIGURE 6. Ezh2 overexpression promotes P19 astrocyte differentiation. Neuronal differentiation of various stable P19 cell lines was induced as described in the legend to Fig. 5. Cells were analyzed at day 12. A, expression of miR-124 uncontrollable Ezh2 (Ezh2 or Ezh2 Sub 3’ UTR) promotes astrocyte differentiation. Astrocytes were defined by Gfap staining (red), and total cell number was determined by DAPI staining (blue). Representative images are shown. Scale bar, 50 μm. B, statistical analysis of astrocyte differentiation. The efficiency of astrocyte differentiation was calculated relative to that of Ezh2 WT 3’-UTR-expressing cells. The differences between groups were first determined by analysis of variance, and the significant difference between indicated pairs was determined by a two-tailed Student’s t test with equal variance. Data shown are mean ± S.D. (error bars) of three independent experiments. C, RT-qPCR analysis for the expression of Ezh2, Gfap, and S100b in the indicated P19 stable cell lines. Data are normalized against Hprt expression and corresponding cell lines without Dox treatment. Relative mRNA expression levels of the indicated genes were calculated as -fold change compared with the gene expression in cells expressing Ezh2 WT 3’-UTR. A representative result from three independent experiments with experimental triplicates is shown. Data shown are mean ± S.E. (error bars) of three independent experiments. E, down-regulation of Chd4 in differentiating P19 cells at the indicated time points was analyzed by RT-qPCR and normalized against Hprt. A representative result with experimental triplicates of three independent experiments is shown. Data shown are mean ± S.D. of triplicates. Significant differences between indicated pairs in all panels were determined by two-tailed Student’s t test with equal variance (*, p < 0.05; **, p < 0.01; ***, p < 0.001).
### TABLE 1

miR-124-up-regulated Ezh2 target genes

Among miR-124-up-regulated genes (fold change ≥ 1.5 and \( p < 0.001 \)), 74 genes are Ezh2 target genes, and 52 of them are CNS-specific genes as defined in Ref. 39. FC, -fold change.

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miR-124 Controls Ezh2 Expression during Neurodifferentiation

Our analysis also revealed that more than 1800 Ezh2 target genes are not up-regulated in miR-124-overexpressing cells. Non-CNS-specific genes (634 genes) are probably associated with silent chromatin in neuronal progenitors and therefore could not be up-regulated simply by miR-124-mediated Ezh2 down-regulation. A small fraction of Ezh2 target genes with predicted miR-124 target sites could potentially be down-regulated by miR-124 (81 genes; 7 non-CNS and 74 CNS-specific), but only 12 CNS-specific genes were down-regulated in miR-124-overexpressing cells, which is not statistically enriched. The remaining 1242 CNS-specific genes may require additional CNS-specific activators that are not expressed just 24 h after miR-124 transfection. Although our current analysis already revealed a significant overlap between Ezh2 target genes and the miR-124 up-regulated gene list, more Ezh2 target genes could be up-regulated by persistent miR-124 overexpression in differentiating neurons.

Interestingly, examination of published genomic maps of the Ezh2-specific 3meH3K27 modifications suggests that promoter regions of all three mouse miR-124 genes are associated with this repressive mark as well as Suz12, a component of the PRC2 complex, in ES cells (Table 2) (52). It is therefore possible that Ezh2 controls miR-124 levels in stem cells, synergizing with the repressive effect of REST (57). During the neurogenic phase, the H3K27-specific demethylase JmjD3 is up-regulated, leading to derepression of neuron-specific genes, possibly including miR-124 (58), that can now dampen Ezh2 expression. This hypothetical double-negative feedback between miR-124 and Ezh2/PRC2 would be similar to the previously reported relationship between miR-124 and SCP1/REST (26).

Although further work will be needed to address the miR-124-Ezh2/PRC2 cross-regulation model, the results of our preliminary studies are consistent with this possibility. Indeed, induction of Ezh2 expression following RA treatment led to dramatic down-regulation of miR-124 expression in differentiating P19 cultures (data not shown). Thus, it is possible that the stimulatory effect of miR-124-resistant Ezh2 on astrocyte generation observed in our study might be caused by Ezh2-mediated down-regulation of miR-124 expression. Underscoring the biological relevance of these regulatory events, miR-124 is naturally expressed in neurons but not in astrocytes (15), and it is predicted to directly down-regulate a number of astrocyte-enriched genes (22, 39).

In addition to its role in balancing neurogenesis versus astrogensis, the miR-124/Ezh2 circuitry may function in other biological scenarios. For example, it has been recently proposed to control aggressiveness of hepatocellular carcinoma (32). Another recent study showed that miR-124 may prevent the activation of microglia, immune cells residing in the central nervous system (16). Interestingly, Ezh2 is known to be up-regulated in activated lymphocytes and play an essential role in this process (59, 60), and it would be interesting to examine the role of Ezh2 in the context of microglia activation, which contributes to pathogen clearance in health or the progression of neurodegenerative and neoplastic diseases (61).

Although we show here that miR-124 represents one of the most potent miRNA regulators of Ezh2 expression, our data are also consistent with the possibility of combinatorial regulation by miRNA. Other than miR-124, miR-26a, and miR-101, six additional miRNAs consistently down-regulated the expression of Ezh2 3’-UTR reporter genes (Fig. 2D). Of these, only miR-20a, miR-26a, and miR-124 are known to be up-regulated in differentiating P19 cells (62), which predicts possible synergistic effects of these three miRNAs on Ezh2 abundance. However, miR-124 is likely to be the major regulator of Ezh2 expression in differentiating neurons, because it is the most abundant miRNA in the brain (12) and is also highly up-regulated in differentiating P19 cells (20 times for miR-124 versus 2 times for miR-20 and miR-26a) (62). The binding sites of these miRNAs are not overlapping. Other miRNAs identified in our study do not appear to be relevant for differentiating neurons.

In conclusion, our study suggests that miRNA control of an important epigenetic regulator can be used as a regulatory paradigm for modulating the choice between alternative differentiation scenarios.

Acknowledgment—We thank Weijun Dai for helpful discussions.

REFERENCES

miR-124 Controls Ezh2 Expression during Neurodifferentiation

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