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 areamped during neuronal differentiation by brain-enriched microRNA miR-124. Expression of miR-124 in a neuroblastoma cells 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.

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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 BAFl53a subunit of a chromatin remodeling complex (24–27). We have previously shown that miR-124 also targets mRNA of Ptbp1 (polyyyrimidine tract-binding protein), a global regulator of pre-mRNA splicing (11). Ptbp1 is expressed
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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 reducing differentiating embryonic mouse neural stem cells. Thus, our results implicate Ezh2 as an important miR-124 target in the context of neuronal differentiation.

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.

P19 Stable Cell Line—P19 stable cell lines were generated as described (33). For stable cell line selection, 2 µg/ml puromycin was added to the medium for 5 days. To turn on the Tet-inducible expression, doxycycline (Clontech) was added to a final concentration of 2 µg/ml.

Neuronal and Astrocyte Differentiation of P19 Cells—To differentiate P19 cells into neuron and astrocyte, we adapted a protocol as described before (34). Briefly, 1 × 10^5 cells/ml P19 cells were allowed to aggregate in a bacterial grade Petri dish (Fisher) and treated with 1 µM all-trans-retinoic acid (Sigma). The medium was changed at day 2. Cell aggregates were dissociated into a single cell suspension with 0.25% trypsin-EDTA (Invitrogen) at day 4. Cells were seeded onto a tissue culture grade Petri dish (Corning Inc.) at 1 × 10^5 cells/ml in neurobasal medium with N2 supplement (Invitrogen) and 0.4 mM L-glutamine (Invitrogen). To obtain neuron and astrocyte, cells were further cultured until day 6.5 and day 12, respectively, for immunofluorescence staining. For protein and RNA assays, cells were cultured until day 10 or 12, and samples were collected at the indicated time points.

Cell Culture, Nucleofection, and Differentiation of Neural Stem Cells (NSCs)—NSCs were obtained from cortices of mouse embryonic day 14 and maintained in the form of neurospheres in complete NeuroCult medium supplemented with 20 ng/ml recombinant human epidermal growth factor (rhEGF, StemCell Technologies). To overexpress EGFP, Ezh2 with artificial 3′-UTR (Ezh2), or Ezh2 with wild-type Ezh2 3′-UTR (Ezh2 WT 3′-UTR) in NSCs, 1.6 × 10^6 cells were co-nucleofected with 0.5 µg of pmaxGFP (Amaxa) together with 2 µg of the respective expression construct and plated onto poly-d-lysine-coated wells. 24 h postplating, the medium was changed to neurobasal medium containing N2 supplement for differentiation. Upon 72 and 120 h of differentiation, cells were harvested for RNA isolation and analyzed using quantitative RT-PCR.

Semi-quantitative and Quantitative RT-PCR Analysis—Total RNA was purified using TRIzol (Invitrogen) as recommended. Reverse transcription (RT) was performed using SuperScript III (Invitrogen) with random hexamer. cDNA was amplified with specific primers for Ezh2, Suz12, Eed, and Hprt. The abundance of transcripts of the housekeeping gene Hprt was used as a loading control. Quantification of PCR product was done using image processing software, ImageJ (National Institutes of Health). The primer sequences were as follows: mEzh2 forward, 5′-AACACCCAAACAGTGTCCATGCAC-3′; mEzh2 reverse, 5′-CTAAAAGGCAGCTGTTCAGAGAA-3′; mEed forward, 5′-CAACCACGACCACCCCTCAT-3′; mEed reverse, 5′-GGAGAGGTTTGGGCTGTT-3′; mSuz12 forward, 5′-AACCGAATATCGTGAGGATT-3′; mSuz12 reverse, 5′-CCATTTTCTTGTATCTGCATCTACT-3′; mHprt forward, 5′-GCTGTGTTGAAAAGAACGACCTC-3′; mHprt reverse, 5′-CACAGGACTAGAACACACTG-3′. For quantitative RT-PCR (RT-qPCR), cDNA was amplified with specific primers using an ABI StepOnePlus real-time PCR system (Applied Biosystems) and KAPA SYBR Fast ABI Prism 2x qPCR master mix (KAPA Biosystems). Data were normalized to the expression levels of the Hprt mRNA. The primer sequences were as follows: mEzh2 forward, 5′-TCCATGGCAACACACCACTAC-3′; mEzh2 reverse, 5′-GGGCTTGGCTAC-
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A

B

C

Ezh2 target genes

CNS-specific genes

634
1254
7617
22
142
198

Up-regulated genes
(miR-124 overexpression)

487 probes
(414 genes)

1646 probes
(1255 genes)

D

Relative Fold Change

Ezh2 Ascl1 Atf3 Dusp8 En2 Sypl2 Tpm1

Control
miR-124
miR-124 + Ezh2
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A representative result with experimental triplicates from three independent experiments is shown. Data shown are mean (±) S.D. of triplicates.

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).

Immunofluorescence—Immunofluorescence staining was performed according to a standard protocol. Anti-Tuj1 antibody was purchased from Covance. Anti-Gfap (ab4674) antibody was purchased from Abcam. Slides were mounted with mounting medium (Ibidi, GmbH) containing DAPI (Invitrogen).

Microarray Analyses—N2a cell pools expressing different Argonaute paralogs 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 transfected with 1, 5, or 30 pmol/ml miR-124 locked nucleic acid antisense oligonucleotide (Exiqon) using Lipofectamine 2000 (Invitrogen). Total protein and RNA were isolated 96 h post-transfection.

miRNA Northern Blot Analysis—Five µg of total RNA samples were separated on a 15% denaturing polyacrylamide gel containing 8% urea and 1× TBE. They were electrotransferred to Hybond N+ membrane (Amersham Biosciences) in 0.5× 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 oligodeoxyribonucleotides 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 overnight at 42 °C. The membranes were washed four times with 3× SSC, 0.1% SDS at 42 °C and exposed to phosphorimaging plates.

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A

Control Down-regulated No changes Up-regulated

Relative MFI

-\log p value

B

ANOVA, p = 1.41x10^4

MFI

EV  miR-9  miR-124  miR-191  miR-206  miR-124

C

Ezh2

Tubulin

D

ANOVA, p = 1.59x10^4

Relative fold change

-\log p value

E

miR-124

3' cccuauggcgccACGGAA 5'

Ezh2 3'-UTR Mmu aucaacuuuaau UGCCUU c 167 Hsa aucaacuuuaau UGCCUU c 169 Rno aucaacuuuaau UGCCUU c 167 Ocu aucaacuuuaau UGCCUU c 171 Pfr aucaacuuuaau UGCCUU c 168 Cfa aucaacuuuaau UGCCUU c 174 Oga aucaacuuuaau UGCCUU c 167 Mml aucaacuuuaau UGCCUU c 170 Eco aucaacuuuaau UGCCUU c 172 Bta aucaacuuuaau UGCCUU c 170 Sub 3'-UTR Mmu aucaacuuuaau AC GGAA c 167

F

Relative fold change

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 Arongaute 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 Suiz12 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 (\( p = 7.03 \times 10^{-5}; \) 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...
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 differ-
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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 efficiency of P19 neuronal differentiation was significantly reduced by the Ezh2 Sub 3'–UTR transgene lacking the miR-124 target site in its Ezh2-derived 3'–UTR. Our RT-qPCR and immunoblot analyses confirmed that these biological effects were accompanied by corresponding changes in the Ezh2 expression levels (Fig. 5C). Similarly, transient expression of recombinant Ezh2 in mouse embryonic neural stem cells undergoing neuronal differentiation down-regulated neuronal markers L1cam and Syp (Fig. 5D). On the other hand, expression of Ezh2 with WT 3'–UTR lowered expression of these genes to a lesser extent, an effect that was especially obvious for L1cam (Fig. 5D). Thus, down-regulation of Ezh2 protein expression by miR-124 is critical for efficient neuronal differentiation.

This treatment successfully restored Ezh2 but not Suz12 and Eed expression in a dose-dependent manner (Fig. 3, G and H). Although the amount of miR-124 was significantly reduced by treatment with a high dose inhibitor, the remaining miR-124 was still sufficient to partially suppress Ezh2 expression. It is therefore difficult to achieve more profound changes in Ezh2 protein levels through treatment with miR-124 inhibitor. These results, however, suggested that Ezh2 is the only PRC2 member directly regulated by miR-124.

miR-124-regulated Ezh2 Expression Is Critical for Efficient Neuronal Differentiation—To address functional significance of miR-124-induced Ezh2 down-regulation during neuronal differentiation, we generated several transgenic P19 lines stably expressing doxycycline-inducible Ezh2 mRNAs with various 3'–UTRs (Fig. 4, A and B). To avoid position effects caused by random integration, we utilized a site-specific transgene integration procedure developed earlier (33). This well-characterized P19 HILO-RMCE acceptor cell line, where all constructs are targeting to the same locus, provides a superior experimental system for comparing the effects of various Ezh2 constructs on neuronal differentiation. An additional cell line expressing EGFP with Globin 3'–UTR (EGFP) was generated as a control.

Using the expression of neuronal Tubulin βIII (TuJ1 immunofluorescence; Fig. 5, A and B) and expression of neuron-specific mRNAs, L1cam and Syp, as a readout (Fig. 5C), we found that expression of the Ezh2 transgene (Ezh2) lacking its natural 3'–UTR reduced the efficiency of P19 neuronal differentiation. Conversely, the Ezh2 transgene with the Ezh2 3'–UTR containing the miR-124 target site (Ezh2 WT 3'–UTR) failed to up-regulate Ezh2 protein level and cause this inhibitory effect (Fig. 5, B and C). Moreover, the efficiency of P19 neuronal differentiation was significantly reduced by the Ezh2 Sub 3'–UTR transgene lacking the miR-124 target site in its Ezh2-derived 3'–UTR. Our RT-qPCR and immunoblot analyses confirmed that these biological effects were accompanied by corresponding changes in the Ezh2 expression levels (Fig. 5C). Similarly, transient expression of recombinant Ezh2 in mouse embryonic neural stem cells undergoing neuronal differentiation down-regulated neuronal markers L1cam and Syp (Fig. 5D). On the other hand, expression of Ezh2 with WT 3'–UTR lowered expression of these genes to a lesser extent, an effect that was especially obvious for L1cam (Fig. 5D). Thus, down-regulation of Ezh2 protein expression by miR-124 is critical for efficient neuronal differentiation.

Ezh2 Regulation by miR-124 Balances Neurogenesis Versus Astrogenesis—miR-124 has been shown previously to promote neurogenesis and hinder gliogenesis using an in vitro differentiation model (48). Similarly, Ezh2-deficient neural progenitor cells are known to possess higher neurogenic and lower astrogenic potentials than their wild-type counterparts (49). To determine whether the miR-124/Ezh2 circuitry could control the choice between the two differentiation scenarios, we followed an established P19 astrogenesis protocol (50) and allowed transgenic P19 cells to differentiate for 12 days. We found that the efficiency of astrocyte differentiation was significantly enhanced in P19 cells expressing the miR-124-resistant Ezh2 Sub 3'–UTR transgene but not the miR-124-repressible Ezh2 WT 3'–UTR transgene (Fig. 6, A and B). The efficiency of astrocyte differentiation was determined by Gfap immuno-
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.
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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.

| Gene symbol | Gene name | FC  | CNS-specific |
|-------------|-----------|-----|--------------|
| Morc4       | Microrchidia 4 | 2.932 | No           |
| Tpm1        | Tropomyosin 1, α | 2.705 | Yes          |
| Col8a2      | Collagen, type VIII, α2 | 2.494 | No           |
| Dup8p       | Dual specificity phosphatase 8 | 2.371 | Yes          |
| Atf3        | Activating transcription factor 3 | 2.244 | Yes          |
| Pitr        | Prolactin receptor | 2.128 | Yes          |
| Mt1         | Metallothionein 1 | 2.102 | Yes          |
| Smox        | Spermine oxidase | 2.096 | Yes          |
| Klh122      | Kelch-like 22 (Drosophila) | 2.070 | Yes          |
| Pkna2       | Plexin A2 | 2.054 | Yes          |
| Fbn2        | Fibrillin 2 | 1.990 | Yes          |
| Sema7a      | Sema domain, immunoglobulin domain (lg), and GPI membrane anchor, (semaphorin) 7A | 1.989 | Yes          |
| Igfbp6      | Insulin-like growth factor-binding protein 6 | 1.946 | Yes          |
| Igfbp5      | Insulin-like growth factor binding protein 5 | 1.925 | Yes          |
| Atg1a3      | ATPase, Na⁺/K⁺ transporting, α3 polypeptide | 1.892 | Yes          |
| Chrm3       | Cholinergic receptor, muscarinic 3, cardiac | 1.862 | No           |
| Tgfb3       | Transforming growth factor, β3 | 1.852 | Yes          |
| Adora2b     | Adenosine A2b receptor | 1.841 | No           |
| Ap3m2       | Adaptor-related protein complex 3, μ2 subunit | 1.839 | Yes          |
| Shroom3     | Shroom family member 3 | 1.830 | Yes          |
| Otx         | Oxytocin | 1.816 | Yes          |
| Gnao1       | Guanine nucleotide-binding protein, αO | 1.780 | No           |
| Kcb9        | Potassium channel, subfamily K, member 9 | 1.777 | No           |
| Crhbp       | Corticotropin-releasing hormone-binding protein | 1.768 | Yes          |
| Slc35f1     | Solute carrier family 35, member F1 | 1.749 | Yes          |
| Faah        | Fatty acid amidase hydrolase | 1.746 | Yes          |
| Nphp2       | Nephrosis 2 homolog, podocin (human) | 1.743 | No           |
| Pxx7        | Paired box gene 7 | 1.729 | No           |
| Sypl2       | Synaptophysin-like 2 | 1.718 | Yes          |
| Gpc5        | Glycican 5 | 1.714 | Yes          |
| Kcnj3       | Potassium voltage-gated channel, kck-related subfamily, gene 3 | 1.707 | No           |
| Sp7         | Sp7 transcription factor 7 | 1.705 | Yes          |
| Epha5       | Eph receptor A5 | 1.703 | Yes          |
| Gpr4        | Glycican 4 | 1.701 | Yes          |
| Lpita       | Inositol 1,4,5-trisphosphate 3-kinase A | 1.691 | Yes          |
| Gpryc5c     | G protein-coupled receptor, family C, group 5, member C | 1.682 | No           |
| Iret5       | Iroquois-related homeobox 6 (Drosophila) | 1.680 | No           |
| Hhat        | Hedgehog acyltransferase | 1.678 | No           |
| Gpr45       | G protein-coupled receptor 45 | 1.678 | Yes          |
| Celsr2      | Cadherin, EGF LAG seven-pass G-type receptor 2 (flamingo homolog, Drosophila) | 1.664 | Yes          |
| Kirrel3     | Kinas of IRRE-like 3 (Drosophila) | 1.662 | Yes          |
| Cyp6a1      | Cytochrome P450, family 46, subfamily a, polypeptide 1 | 1.662 | Yes          |
| Hoxc12      | Homeobox C12 | 1.656 | No           |
| Tcfap2b     | Transcription factor AP-2B | 1.640 | No           |
| Nkx1-2      | NK1 transcription factor-related, locus 2 (Drosophila) | 1.638 | Yes          |
| Nol3        | Nucleolar protein 3 (apoptosis repressor with CARD domain) | 1.632 | Yes          |
| Rab15       | RAB15, member RAS oncogene family | 1.630 | Yes          |
| Nuak2       | NUAK family, SNF1-like kinase, 2 | 1.629 | No           |
| Tmem28      | Transmembrane protein 28 | 1.628 | Yes          |
| Hmx1        | H6 homeobox 1 | 1.625 | No           |
| Spry3       | SPRY domain-containing 3 | 1.624 | Yes          |
| Bbdh11      | BTB (POZ) domain containing 11 | 1.613 | Yes          |
| Adamtsa5    | ADAMTS-like 5 | 1.610 | No           |
| Cldn4       | Clq and tumor necrosis factor related protein 4 | 1.607 | Yes          |
| Caen2d2     | Calcium channel, voltage-dependent, α2/δ subunit 2 | 1.603 | Yes          |
| Dpp10       | Dipeptidylpeptidase 10 | 1.594 | Yes          |
| Zmip1       | Zinc finger, MIZ-type-containing 1 | 1.591 | Yes          |
| Hoxa9       | Homeobox A9 | 1.589 | No           |
| Calb1       | Calbindin 1 | 1.585 | Yes          |
| Pstpip2     | Proline-serine-threonine phosphatase-interacting protein 2 | 1.583 | Yes          |
| Plagl1      | Pleiomorphic adenoma gene-like 1 | 1.581 | Yes          |
| Nkx2-6      | NK2 transcription factor-related, locus 6 (Drosophila) | 1.579 | No           |
| Trim54      | Tripartite motif-containing 54 | 1.562 | No           |
| Gpr6        | G protein-coupled receptor 6 | 1.552 | Yes          |
| Apelin      | Apelin | 1.547 | Yes          |
| Srx22       | Sorting nexin 22 | 1.534 | Yes          |
| Alx4        | Aristless-like homeobox 4 | 1.534 | No           |
| Nspk4       | Neurexophilin 4 | 1.526 | No           |
| En2         | Engrailed 2 | 1.522 | Yes          |
| Tmem25      | Transmembrane protein 25 | 1.520 | Yes          |
| Tubb2b      | Tubulin, β2B | 1.513 | Yes          |
| Ybx2        | Y box protein 2 | 1.506 | No           |
| Il12rb1     | Interleukin 12 receptor, β1 | 1.505 | Yes          |
| Axl1        | Achaete-scute complex homolog 1 (Drosophila) | 1.502 | Yes          |
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 SCPI/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.

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\[^3\text{W. H. Neo and I.-H. Su, unpublished data.}\]
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