Reciprocal actions of REST and a microRNA promote neuronal identity

Cecilia Conaco[†], Stefanie Otto[†], Jong-Jin Han, and Gail Mandel[‡]

Department of Neurobiology and Behavior, Howard Hughes Medical Institute, State University of New York, Stony Brook, NY 11794

MicroRNAs (miRNAs) are implicated in both tissue differentiation and maintenance of tissue identity. In most cases, however, the mechanisms underlying their regulation are not known. One brain-specific miRNA, miR-124a, decreases the levels of hundreds of nonneuronal transcripts, such that its introduction into HeLa cells promotes a neuronal-like mRNA profile. The transcriptional repressor, RE1 silencing transcription factor (REST), has a reciprocal activity, inhibiting the expression of neuronal genes in nonneuronal cells. Here, we show that REST regulates the expression of a family of miRNAs, including brain-specific miR-124a. In nonneuronal cells and neural progenitors, REST inhibits miR-124a expression, allowing the persistence of nonneuronal transcripts. As progenitors differentiate into mature neurons, REST leaves miR-124a gene loci, and nonneuronal transcripts are degraded selectively. Thus, the combined transcriptional and posttranscriptional consequences of REST action maximize the contrast between neuronal and nonneuronal cell phenotypes.

noncoding RNA | repression | neuronal phenotype

Generation of cellular diversity during development requires coordination of gene expression mediated by both positive and negative transcriptional regulators. In neuronal development, inhibitory pathways have been shown to play a particularly important role (1, 2). Many neuronal genes are actively repressed in nonneuronal cells through a conserved 23-bp repressor element (RE1) that interacts with a zinc-finger protein known as the RE1 silencing transcription factor (REST/NRSF) (3, 4). REST mediates silencing of its target genes through the recruitment of histone deacetylases by corepressors associated with its repressor domains (5–8). During the transition from pluripotent cell to postmitotic neuron, REST is down-regulated, allowing genes encoding fundamental neuronal traits to be expressed (9). The extent to which REST contributes to mechanisms underlying other types of cellular specificity is not well established, although it has been shown recently to be a tumor suppressor in epithelial cells (10).

MicroRNAs (miRNAs) are also excellent candidates for regulating cellular phenotype. These small, highly conserved regulatory molecules modulate gene expression posttranscriptionally by targeting mRNAs for translational repression or degradation (11–13). miRNAs are produced from larger primary transcripts originating from distinct genes (14). The primary transcript is subjected to two processing steps, generating first a stem-loop precursor transcript that is exported to the cytoplasm and subsequently the ≈21- to 23-nt mature miRNA. One strand of the mature miRNA is preferentially incorporated into the RNA-induced silencing complex. This effector complex then is guided by the miRNA to target mRNAs bearing miRNA recognition elements in their 3′UTR (11–14). Since their discovery, miRNAs have been shown to play important roles in diverse biological processes, including developmental timing and patterning in *Caenorhabditis elegans* (15–21), apoptosis in *Drosophila melanogaster* (22–24), and insulin secretion of pancreatic islet cells (25). Evidence also supports the idea that miRNAs may be important during late vertebrate development (26, 27), particularly for lineage differentiation of various tissue types (28–30).

Although recent studies are starting to unravel how miRNAs fit into signaling pathways that govern cell fate decisions, the factors that control miRNA expression and their precise roles during the acquisition of a specific cell phenotype are just beginning to become elucidated. During cardiogenesis, for example, serum response factor induces the expression of muscle-specific miRNA, miR-1, which in turn controls cardiomyocyte proliferation by down-regulating the Hand2 transcription factor (30). Establishment of the left–right asymmetry of chemosensory neurons in *C. elegans* also involves a signaling network of transcription factors and miRNAs (19–20). In the mammalian nervous system, where cellular diversity is extreme, the factors responsible for miRNA gene regulation are only just emerging (31).

Here, we show that REST regulates a family of mouse miRNA genes by transcriptional repression. One of the target miRNAs, miR-124a, is known to be expressed to high levels throughout the mature nervous system. We show that similar to canonical REST-regulated genes, miR-124a is expressed as a result of the dismissal of REST from its binding sites on the chromatin during neuronal differentiation. Blocking miR-124a activity in mature neurons leads selectively to increased levels of nonneuronal transcripts. Our findings reveal that the establishment and maintenance of neuronal identity requires both derepression of REST-regulated genes as well as posttranscriptional down-regulation of nonneuronal transcripts by a miRNA that is also under REST control.

Results

A Family of Mouse miRNAs Contains Functional REST-Binding Sites. Serial analysis of chromatin occupancy (SACO) is an unbiased method for identifying functional transcription factor-binding sites genome-wide (32). By using this method for identifying REST-binding sites in a nonneuronal murine kidney cell line (TCMK1), we discovered a family of mouse miRNA genes that reside in close proximity to binding sites for the transcriptional repressor, REST (Fig. 1A; see also Table 1, which is published as supporting information on the PNAS web site). This family includes miR-9-1, -9-2, -9-3, -124a-1, -124a-2, -124a-3, and -132, all of which are expressed in brain (33–39). Each of the miR-9 gene loci gives rise to two mature miRNAs, miR-9 and -9*, whereas the three miR-124a loci give rise to only one mature miRNA, miR-124a. Both miR-9 and -9*, as well as miR-124a, are highly enriched throughout the brain and are up-regulated during rat brain corticogenesis (34) and during neuronal differentiation of P19 embryonal carcinoma cells (36). In contrast, the

Fig. 1. REST occupies RE1 sites associated with a family of miRNAs. (*A*) miRNA genes identified as potential REST targets in a REST SACO screen bind REST *in vivo*. (*Left*) Schematic diagram showing chromosomal locations of RE1 sites (vertical lines) with respect to the predicted stem-loop precursors of the miRNA transcripts (gray boxes). (*Right*) ChIP analysis showing REST occupancy at the miRNA loci in TCMK1 kidney cells. REST-N and REST-C refer to Abs directed against the N and C termini of REST. Rabbit IgG served as control. (*B* and *C*) Quantitative PCR analysis showing that endogenous miRNA genes are regulated by REST. Levels of miRNAs were measured 24 h after transduction of MEFs with dnREST (*B*) or of E15.5 postmitotic cortical neurons with REST (*C*). miRNA levels were normalized to endogenous GAPDH mRNA. Fold changes in miRNA expression are shown as the ratio of geometric means of transcript levels in cells expressing dnREST or full-length REST relative to cells transduced with an adenovirus expressing only GFP. The limits of the 95% confidence intervals indicate variability of the changes (*, $P \leq 0.05$; **, $P \leq 0.005$, Student's *t* test).

product of the miR-132 gene is expressed at a lower level and is restricted to particular areas in the brain (39).

Chromatin immunoprecipitation (ChIP) analysis in kidney cells verified that REST occupied the RE1 sites associated with each of the SACO-identified miRNA genes (Fig. 1*A*). Evidence that REST regulates the expression of these miRNAs was provided initially by the introduction of a dominant negative form of REST (dnREST), which contains the DNA-binding domain but lacks repressor domains, into mouse embryonic fibroblasts (MEFs) by adenoviral transduction. Quantitative PCR was used to measure the expression level of each miRNA. With the exception of miR-124a, for which it was possible to design a single primer set that could detect the transcripts from all three loci, primers for the other miRNA genes were designed to detect each unique precursor transcript. Expression of dnREST resulted in an increase in endogenous transcript levels for miR-9, -124a, and -132 (Fig. 1*B*), an induction comparable with that seen with other REST-regulated genes (9, 40–42). Consistent with their regulation in MEFs, the ectopic expression of REST in cortical neurons that do not express this transcriptional repressor resulted in a decrease in the steady RNA levels of miR-9 and -124a after 24 h (Fig. 1*C*) and miR-132 after 48 h (data not shown).

REST Confers Neuronal-Specific Expression of miR-124a. We initially chose to study REST regulation of miR-124a in more depth because of its evolutionary conservation, abundance, and re-

striction to the nervous system. Vertebrate genomes possess three highly conserved miR-124a genes, whereas invertebrates only have one. The single miR-124 gene in the invertebrates *C. elegans* and *D. melanogaster* gives rise to a mature miRNA of the same sequence as vertebrate miR-124a, although their precursor transcripts differ at several nucleotides (Fig. 2*A*). Interestingly, although invertebrates possess a miR-124 gene, they appear to lack REST. However, Dallman *et al.* (43) have shown previously in flies that REST may be replaced functionally by the transcriptional repressor, Tramtrack 88 (ttk88). Consistent with this finding, a consensus ttk88 binding site is present in the promoter region of the single locus encoding miR-124 in *D. melanogaster*. In the mouse, the miR-124a genes (miR-124a-1, -124a-2, and -124a-3) are located on chromosomes 14, 3, and 2, respectively (Fig. 2*B*). Each miR-124a locus is associated with either ESTs or annotated mRNAs. However, these mRNAs do not code for any known proteins, suggesting that they may be part of the primary miRNA transcript. Active transcription at all three miR-124a loci could explain the abundance of this miRNA in neuronal tissues.

We examined the expression of REST protein and mature miR-124a in mouse embryonal carcinoma (P19) cells undergoing neuronal differentiation in response to retinoic acid (Fig. 3*A*). Differentiation was monitored by the appearance of the neuronal marker, neuronal βIII-tubulin (TuJ1). The expression of mature miR-124a at 4 days after retinoic acid treatment coincided with the disappearance of REST protein and terminal differentiation, as evidenced by the expression of TuJ1. In contrast, expression levels of the REST corepressor, CoREST, which is expressed in both neural progenitors and mature neurons (9), were independent of changes in REST levels. The reciprocity of REST and miR-124 gene expression during P19 differentiation was accounted for by changes in the occupancy of REST on the chromatin. ChIP analysis (Fig. 3*B Left*) showed that REST occupied the RE1 sites of miR-124a-1, -124a-2, and -124a-3, as well as the RE1 of the REST-regulated glutamic acid decarboxylase (GAD1) gene, in primary cortical progenitors, but was dismissed from the chromatin in mature cortical neurons. The dismissal of REST from the RE1 sites coincided with the appearance of mature miR-124a, measured by ribonuclease protection analysis, in cortical neurons; miR-124a was barely detectable in the dividing cortical progenitors (Fig. 3*B Right*).

To determine that the repression of miR-124a was because of REST bound specifically to the RE1 sites, a luciferase reporter assay was performed in the murine kidney cell line TCMK1. Luciferase reporter genes were constructed with flanking sequences containing the RE1 sites of miR-124a-3 or GAD1 cloned upstream of the thymidine kinase (TK) promoter that is active in both neuronal and nonneuronal cell types (TK-miR-124a-3 RE1, TK-GAD1 RE1). As predicted, luciferase activity was lower for the constructs bearing the RE1 of miR-124a-3 or GAD1 compared with constructs in which the RE1 site had been deleted (TK-miR-124a-3 ΔRE1, TK-GAD1 ΔRE1) because of the presence of endogenous REST protein in these cells (Fig. 3*C*). To confirm that binding of REST to the RE1s was responsible for the lower activity, the cells were cotransfected with the reporter and either dnREST or vector alone. Expression of dnREST resulted in a doubling of luciferase activity for the construct containing the miR-124a-3 RE1 and a 7-fold increase in activity for the construct containing the RE1 of the canonical REST-regulated gene, GAD1. Constructs lacking RE1 sites were not affected by the presence of dnREST (Fig. 3*D*).

REST Regulation of miR-124a Promotes a Neuronal Phenotype. miRNAs can regulate their target transcripts either by blocking translation or by causing mRNA degradation (11–13). In a recent study, Lim *et al.* (44) demonstrated that overexpression of miR-124a in HeLa cells resulted in selective down-regulation of nonneuronal
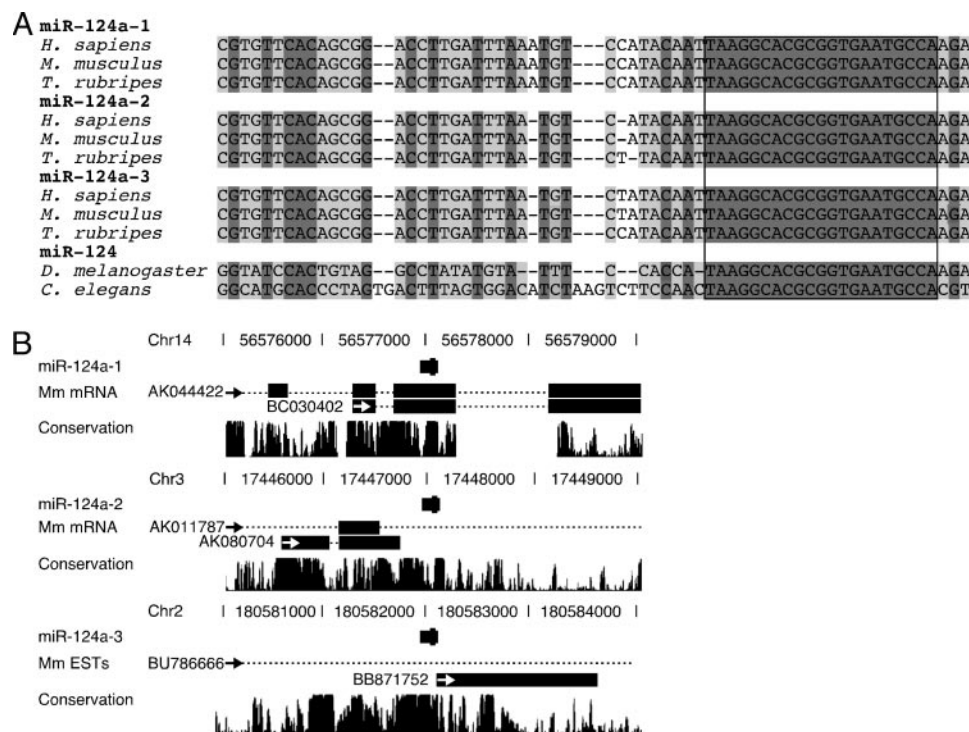
Fig. 2. Conservation of miR-124a. (*A*) Comparison of miR-124a stem-loop precursor sequences across species. Nucleotides conserved across all species are shown in dark gray, and regions conserved in vertebrates, but not in invertebrates, are in light gray. The box indicates the sequence of mature miR-124a. (*B*) Predicted stem-loop precursors for each miRNA are depicted as short filled rectangles with a vertical bar representing the mature miRNA. miR-124a-1 and -124a-2 are located within GenBank transcripts, and miR-124a-3 is located within a mouse EST (arrows indicate direction of transcription). Alignment scores, indicated by black bars in the conservation track, show that the three miR-124a loci are located in conserved regions of the mouse genome (UCSC, Mm5 assembly).

transcripts. Sequence analysis of the 3′UTR of these transcripts revealed a preponderance of matches to the seed region (nucleotides 2–7) of miR-124a, suggesting that the miRNA was directly targeting these mRNAs for degradation. One caveat of this study, however, was that it was based exclusively on an overexpression paradigm in cells in which miR-124a is not normally expressed. To address this problem, we depleted miR-124a from primary cultures of terminally differentiated cortical neurons with antisense 2′-*O*-methyl (2′-OMe) oligoribonucleotides. Because of their resistance to ribonuclease cleavage, the irreversible binding of 2′-OMe oligoribonucleotides to cognate miRNAs can efficiently inhibit interaction of the miRNA with its mRNA targets (45). The mRNA levels for 17 nonneuronal transcripts identified in Lim *et al.* (44) were measured by quantitative PCR 48 h after 2′-OMe transfection into neurons. A significant increase in transcript levels was observed for 10 of these transcripts in miR-124a 2′-OMe-treated neurons relative to neurons treated with an antisense 2′-OMe to a muscle-specific miRNA, miR-1 (Fig. 4*A*). Transcript levels for CoREST, a gene expressed abundantly in neurons, did not change. Conversely, overexpression of miR-124a in MEFs resulted in a decrease in all 17 nonneuronal transcripts relative to cells treated with miR-124a mut5–6, an inactive mutated version of miR-124a (Fig. 4*B*).

Discussion

To date, regulation by REST has been ascribed largely to neuronal genes that encode proteins expressed in most terminally differentiated neurons. Among these proteins are axon guidance molecules, ion channels, synaptic vesicle proteins, and neurotransmitter receptors (3, 4, 9, 40, 41, 46–57). The unbiased SACO screen in a kidney cell line has now revealed that noncoding miRNAs represent yet another set of target genes for REST. Moreover, REST regulation of miR-124a is similar to that of canonical REST-regulated neuronal genes. During neurogenesis, for example, REST repression is lost as REST is dismissed from the RE1 sites on the miRNA chromatin.

In nonneuronal cells, the neuronal phenotype is suppressed by REST repression of neuronal genes. This finding raises the question of whether there is a reciprocal mechanism in neurons for the suppression of nonneuronal genes. miR-124a is a good candidate to mediate such a mechanism because its overexpression in HeLa cells and MEFs results in the selective downregulation of many nonneuronal transcripts (ref. 44 and Fig. 4*B*). Moreover, as shown in this work, inhibition of miR-124a function in neurons, where it is normally expressed, results in the selective up-regulation of nonneuronal transcripts. This result provides further support for the role of this miRNA as a constitutive repressor of mRNAs encoding a large diversity of nonneuronal proteins. Interestingly, however, not all of the transcripts downregulated by overexpression of miR-124a in nonneuronal cells were affected by depletion of miR-124a in neurons (Fig. 4*A*). Possible explanations for this outcome are as follows: (*i*) Overexpression causes off-target down-regulation of transcripts; (*ii*) down-regulation of some transcripts in nonneuronal cells is an indirect effect of direct targets of miR-124a, such as transcriptional activators or RNA binding proteins that may function to stabilize nonneuronal transcripts; and (*iii*) in neurons, a single nonneuronal mRNA may be regulated by multiple miRNAs such that blocking miR-124a alone is not sufficient to cause up-regulation. Further studies will be required to discriminate among these possibilities.

On the basis of our results, and the work of Lim *et al.* (44), we propose a simple model for how contrast, exemplified by distinct mRNA profiles, is achieved between neuronal and nonneuronal cell phenotypes (Fig. 4*C*). In nonneuronal cells, REST binds to
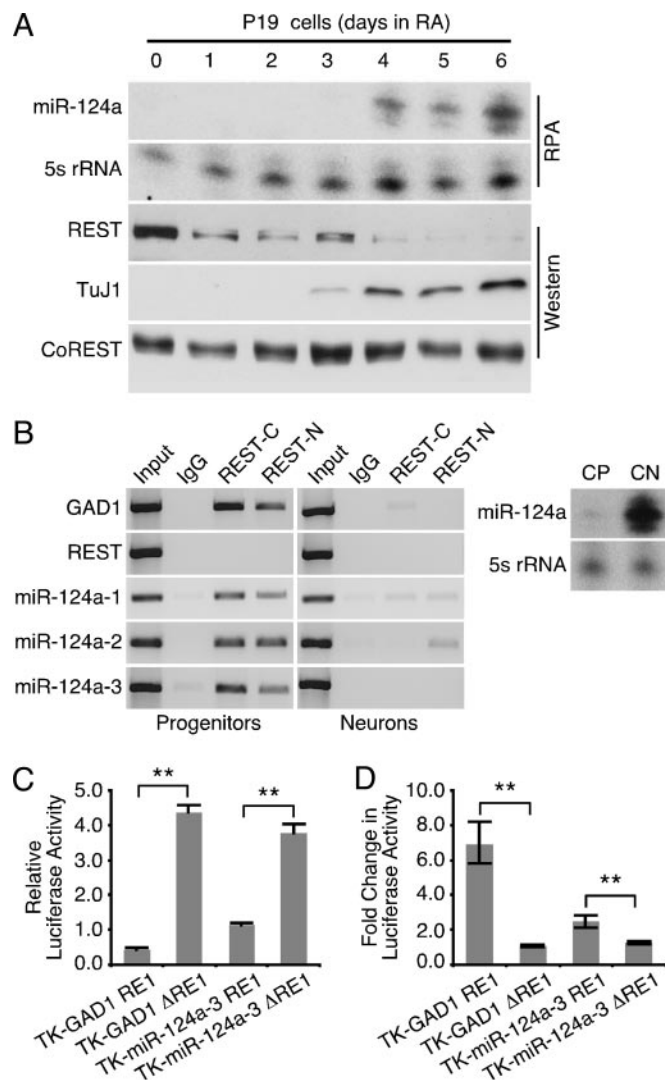
Fig. 3. REST confers neuronal specificity of miR-124a. (*A*) Western blot and ribonuclease protection assay (RPA) showing the disappearance of REST protein and appearance of mature miR-124a during neuronal differentiation of P19 cells with retinoic acid (RA). Neuronal βIII-tubulin (TuJ1) indicates presence of mature neurons. Levels of 5s rRNA and CoREST protein serve as controls. (*B Left*) ChIP analysis showing REST leaving the chromatin of the three miR-124a loci as cortical progenitors differentiate into cortical neurons. The neuronal-specific GAD1 gene and REST coding sequence serve as positive and negative controls, respectively. (*Right*) RPA analysis showing miR-124a expression in cortical neurons (CN) but not in cortical progenitors (CP). (*C*) Luciferase reporter analysis showing that RE1-containing constructs (TK-miR-124a-3 RE1, TK-GAD1 RE1) transfected into TCMK1 kidney cells have lower luciferase activity compared with constructs lacking the RE1 (TK-miR-124a-3 ΔRE1, TK-GAD1 ΔRE1). Relative luciferase activity was measured after 48 h. Error bars represent SD from three independent experiments (**, $P \leq 0.005$, Student's *t* test). (*D*) Luciferase reporter assay in TCMK1 kidney cells showing derepression of reporters bearing the RE1s of miR-124a-3 and GAD1 upon introduction of dnREST. Fold changes in luciferase activity are shown as the ratios of the geometric means of reporter activity with and without dnREST. Variability is indicated by the limits of the 95% confidence intervals (**, $P \leq 0.005$, Student's *t* test).

consensus RE1 sites in neuronal genes and miR-124a and blocks their transcription. The repression of miR-124a, in turn, results in persistence of hundreds of nonneuronal transcripts, thus greatly favoring the nonneuronal phenotype. In neurons, where REST is absent, global derepression of neuronal genes and miR-124a occurs in conjunction with *en masse* down-regulation

of nonneuronal transcripts mediated by miR-124a. Together, these two events now alter the balance in favor of the neuronal phenotype. The requirement for persistent down-regulation of nonneuronal transcripts might seem uneconomical. In actuality, however, the use of neuronal miRNAs to regulate mRNA concentrations posttranscriptionally allows for basal transcription of these genes in neurons by the same transcription factors and cognate genetic elements used in nonneuronal cells, an economy of genomic function.

A large number of miRNAs are expressed during the progression of cortical development (33–38). It seems likely that their distinct spatiotemporal expression patterns reflect specific roles in coordinating the gene expression profiles that characterize neuronal subtypes. Indeed, a recent study in *C. elegans* has revealed a dual miRNA circuit that regulates a terminal cell fate decision between two distinct neuronal types (19). It remains to be seen how the REST-regulated miRNAs, other than miR-124a, function during mammalian nervous system development or in the fine-tuning of neuronal functions.

Materials and Methods

Cell Culture. A murine kidney cell line (TCMK1) and P19 embryonal carcinoma cells were obtained from the American Type Culture Collection. TCMK1 cells were maintained in MEM with 10% FBS, 1% sodium pyruvate, 1% nonessential amino acids, and 1.5 mg/ml sodium bicarbonate. P19 cells were cultured in αMEM with 7.5% bovine calf serum and 2.5% FBS and maintained subconfluent before neuronal induction. Neuronal induction was performed essentially as described in ref. 58. Briefly, 1×10^6 P19 cells were allowed to aggregate in polystyrene Petri dishes (Fisher) in the presence of 500 nM retinoic acid (Sigma). After 4 days, the cells were dissociated and plated at 6×10^6 cells on 10-cm tissue culture dishes (Falcon). Primary MEFs were derived from embryonic day (E) 14.5 embryos and grown in DMEM with 10% FBS, 2 mM L-glutamine, and 1% nonessential amino acids. Cortical progenitors were isolated from E12.5 mouse embryos and grown in Neurobasal medium supplemented with 40 ng/ml bFGF (Chemicon), 2% B27, and 500 μM L-glutamine (59). Cortical neurons were isolated from E15.5 mouse embryos as described in ref. 9 and maintained in Neurobasal medium with 2% B27 and 500 μM L-glutamine. After 3 days *in vitro*, the cortical neurons were treated with 5 μM cytosine arabinoside (Sigma) to inhibit the growth of proliferating cells.

SACO. A REST SACO library was constructed from TCMK1 cells according to Impey *et al.* (32). Sequence analysis was performed by the Cold Spring Harbor Laboratory Genome Research Center.

ChIP Analyses. ChIP was performed as described in ref. 8. Crosslinked chromatin was immunoprecipitated by using the following polyclonal antibodies (Abs): anti-REST-N (3) and anti-REST-C (9). After the reversal of crosslinks, the DNA was purified by using the QIAquick PCR purification kit (Qiagen, Valencia, CA). All DNA samples were subjected to 50 cycles of PCR.

Western Blotting and Ribonuclease Protection Assay. P19 cells undergoing neuronal differentiation were collected for protein and RNA extraction every day for 6 days. Whole cell protein extracts were prepared as described in ref. 6. Protein extracts were quantitated by using the Coomassie Plus Protein Assay Reagent (Pierce), and 50 μg of protein was resolved on 7% polyacrylamide gels. The following Abs were used for Western blotting: anti-REST-N (3), anti-CoREST (5), and anti-βIII-tubulin (TuJ1; Covance, Richmond, CA). For ribonuclease protection assay of miRNAs, total RNA was extracted by using TRIzol
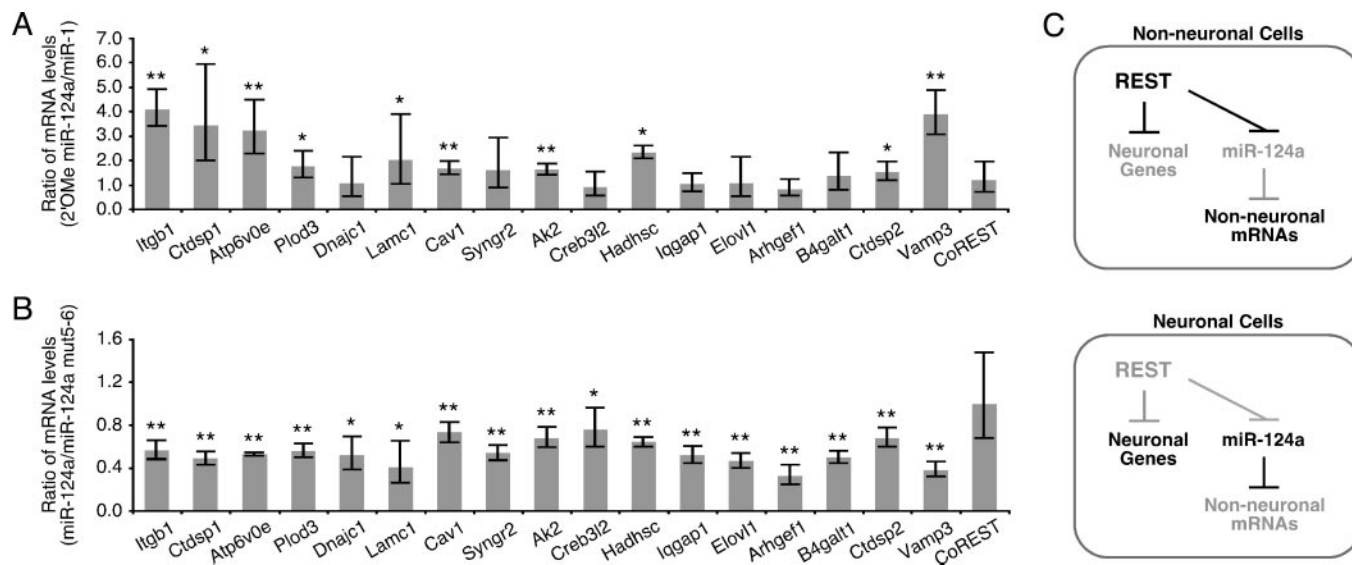
Fig. 4. Inhibition of miR-124a in neurons unmasks regulation of nonneuronal transcripts. (*A*) Quantitative PCR showing that depletion of miR-124a using an antisense 2′-OMe oligoribonucleotide results in increased levels of nonneuronal transcripts in cortical neurons. The level of the nontarget transcript for CoREST did not change. Expression of target mRNAs was measured after 48 h and normalized to GAPDH. Fold changes in mRNA expression are shown as the ratio of the geometric means of transcript levels in cells transfected with an antisense 2′-OMe to miR-124a relative to cells transfected with an antisense 2′-OMe to muscle-specific miR-1. Variability is indicated by the limits of the 95% confidence intervals (∗, $P \le 0.05$, ∗∗, $P \le 0.005$, Student's t test). (*B*) Quantitative PCR showing that overexpression of miR-124a down-regulates nonneuronal transcripts in MEFs. Expression of target mRNAs was measured after 24 h and normalized to GAPDH. Fold changes in mRNA expression are shown as the ratio of the geometric means of transcript levels in cells overexpressing miR-124a relative to cells treated with an inactive mutated version of miR-124a (miR-124a mut5–6). Variability is indicated by the limits of the 95% confidence intervals. (*C*) A hierarchy of two global negative regulators, REST and miR-124a, promotes a neuronal phenotype. (*Upper*) REST transcriptionally represses neuronal genes and miR-124a in nonneuronal cells and neural progenitors. (*Lower*) The dismissal of REST from chromatin during neurogenesis results in *en masse* expression of neuronal genes and down-regulation of competing nonneuronal transcripts through miR-124a function.

(Invitrogen). Two micrograms of RNA was used with the mirVana miRNA detection kit (Ambion, Austin, TX) following the manufacturer's protocol. Probes for miRNA detection were synthesized by Integrated DNA Technologies (Coralville, IA) and end-labeled with ^{32}P by using the mirVana probe and marker kit (Ambion).

Adenoviral Vectors and Transduction. Adenoviral vectors expressing full-length REST and dnREST that lacks both N- and C-terminal repressor domains are described in ref. 3. Primary cortical neurons (E15.5) were infected after 5 days *in vitro* with adenovirus at a multiplicity of infection of 50–100. Calcium phosphate precipitation was used to introduce adenovirus into MEFs (60). Briefly, virus was resuspended in MEM and precipitates were allowed to form with the addition of 25 mM $CaCl_2$. The precipitate was applied to the MEFs for 30 min and then replaced with fresh medium.

RNA Isolation and Quantitative Real-Time PCR Analysis of miRNAs. Total RNA from adenovirus transduced primary cortical neurons and MEFs was extracted by using the RNeasy kit (Qiagen) and treated with DNase (DNA-free kit, Ambion). Reverse transcription was performed by using Superscript III (Invitrogen). Quantitative real-time PCR was performed in an Applied Biosystems PRISM 7900HT Fast Real-Time PCR System with SYBR green PCR master mix (Applied Biosystems). Primers (see Data Set 1, which is published as supporting information on the PNAS web site) for miRNA detection were designed within the precursor stem-loop for each individual miRNA gene locus, with the exception of miR-124a, for which a single set of primers was able to recognize precursor transcripts from all its gene loci. The relative abundance of each miRNA was determined by using a standard curve generated from 10-fold serial dilutions of cortical neuron cDNA and normalized to GAPDH mRNA. To

analyze changes in miRNA expression, ratios of the geometric means between control and experimental samples were calculated. The limits of a 95% confidence interval were determined to indicate variability of the mean ratios for each miRNA. Significance was determined by using Student's t test.

Luciferase Assay. A vector encoding the firefly luciferase gene, pGL3-TK, was constructed by ligating the TK promoter from pRL-TK *Renilla* luciferase expression vector (Promega) into the BglII/HindIII sites of pGL3-basic (Promega). Flanking sequences containing the RE1 sites of the miR-124a-3 or GAD1 genes were cloned into pCR4-TOPO by using the TA TOPO cloning kit (Invitrogen). The inserts were excised with SpeI/BglII and inserted into the NheI/BglII sites of pGL3-TK to create the TK-miR-124a-3 RE1 and TK-GAD1 RE1 reporter vectors. The RE1 sites on these vectors were deleted by using the Quikchange II site-directed mutagenesis kit (Stratagene) to create TK-miR-124a-3 ΔRE1 and TK-GAD1 ΔRE1. Deletion of RE1 sites was verified by sequencing. TCMK1 cells (1×10^5 cells per well on a 12-well dish) were transfected with 250 ng of each of the reporter vectors along with 20 ng of pRL-TK and 250 ng of an expression vector for dnREST (3) or an empty vector (pcDNA, Invitrogen). The ratio of firefly vs. *Renilla* luciferase activity was measured after 48 h using the dual luciferase reporter assay system (Promega) following the manufacturer's protocol. Changes in luciferase activity were determined by taking the ratios of the geometric means for reporters cotransfected with dnREST and pcDNA. Variability of mean ratios for each reporter was determined by calculating the limits of a 95% confidence interval. Significance was determined by using Student's t test.

Inhibition and Overexpression of miR-124a. For inhibition of miR-124a, E15.5 primary cortical neurons at 4 days *in vitro* were

transfected with 500 nM 2′OMe oligoribonucleotides (Integrated DNA Technologies) by using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. For overexpression of miR-124a, MEFs were plated at 1×10^5 cells per 6-cm dish and transfected by calcium phosphate precipitation as described in ref. 61. Briefly, 500 μl of 2× Hepes buffered saline (HBS) was added dropwise to 100 nM miR-124a or miR-124a mut5–6 RNA duplex (Integrated DNA Technologies) (44) in 500 μl of 125 mM CaCl$_2$ to allow precipitates to form. Precipitates were added to cells and medium was renewed after 5 h. After the indicated time, RNA was extracted by using the RNeasy kit (Qiagen) and DNase treated (DNA-free, Ambion).

Relative abundance of selected mRNAs was determined by quantitative real-time PCR as described above by using standard curves generated from 10-fold serial dilutions of MEF cDNA (see Data Set 1 for primer sequences). Statistical analyses were performed as described above for quantitative PCR of miRNAs.

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