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MicroRNA miR-124 Regulates Neurite Outgrowth during Neuronal Differentiation

Jenn-Yah Yu^{1,2,†}, Kwan-Ho Chung^{1,2,†}, Monika Deo^{1,†}, Robert C. Thompson^{1,2,3}, and David L. Turner^{1,2,4,*}

¹Molecular and Behavioral Neuroscience Institute, University of Michigan, Ann Arbor, MI 48109-2200

²Program in Neuroscience, University of Michigan, Ann Arbor, MI 48109-2200

³Department of Psychiatry, University of Michigan, Ann Arbor, MI 48109-2200

⁴Department of Biological Chemistry, University of Michigan, Ann Arbor, MI 48109-2200

Abstract

MicroRNAs (miRNAs) are small RNAs with diverse regulatory roles. The miR-124 miRNA is expressed in neurons in the developing and adult nervous system. Here we show that overexpression of miR-124 in differentiating mouse P19 cells promotes neurite outgrowth, while blocking miR-124 function delays neurite outgrowth and decreases acetylated α -tubulin. Altered neurite outgrowth also was observed in mouse primary cortical neurons when miR-124 expression was increased, or when miR-124 function was blocked. In uncommitted P19 cells, miR-124 expression led to disruption of actin filaments and stabilization of microtubules. Expression of miR-124 also decreased Cdc42 protein and affected the subcellular localization of Rac1, suggesting that miR-124 may act in part via alterations to members of the Rho GTPase family. Furthermore, constitutively active Cdc42 or Rac1 attenuated neurite outgrowth promoted by miR-124. To obtain a broader perspective, we identified mRNAs downregulated by miR-124 in P19 cells using microarrays. mRNAs for proteins involved in cytoskeletal regulation were enriched among mRNAs downregulated by miR-124. A miR-124 variant with an additional 5' base failed to promote neurite outgrowth and downregulated substantially different mRNAs. These results indicate that miR-124 contributes to the control of neurite outgrowth during neuronal differentiation, possibly by regulation of the cytoskeleton.

Introduction

MicroRNAs (miRNAs) are 20–25 nucleotide (nt) endogenous non-coding RNAs that are involved in diverse biological processes [1–3]. MiRNA containing ribonucleoprotein complexes regulate target gene expression through translational repression and/or target mRNA degradation in a sequence-dependent manner [4]. Recent studies have revealed that miRNAs are involved in multiple biological pathways in a variety of animals. In *C. elegans* and *Drosophila*, miRNAs have been shown to affect a number of biological processes including

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*Author for correspondence: David L. Turner, University of Michigan, MBNI, 5062 BSRB, 109 Zina Pitcher Place, Ann Arbor, MI 48109-2200, 734-647-6891.

†Current addresses: Jenn-Yah Yu: Department of Biochemistry, University of Washington, Seattle, WA; Kwan-Ho Chung: National Eye Institute, Bethesda, MD; Monika Deo: Fred Hutchinson Cancer Research Center, Seattle, WA

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developmental timing [5], left/right asymmetric neuronal cell fate [6,7], programmed cell death [8], muscle development [9], and fat metabolism [10]. In mammals, miRNAs have been implicated in a broad range of processes including hematopoietic lineage differentiation, developmental patterning, heart and skeletal muscle differentiation and function, insulin secretion, and immune function [11–21]. miRNAs also appear to have important roles in the mammalian central nervous system (CNS). Numerous miRNAs are expressed in the CNS [22–24], and many neural miRNAs are expressed in spatial and/or temporal patterns that suggest roles in the regulation of CNS development [25–30]. MiRNA miR-132 has been shown to regulate neuronal morphogenesis through decreasing levels of GTPase-activating protein, p250GAP [31] and (along with miR-219) has been implicated in the regulation of circadian rhythms [32]. MiR-133b is expressed in dopaminergic neurons in the midbrain, and regulates the differentiation through repressing Pitx3, a paired-like homeodomain transcription factor [33]. MiR-134 has been shown to regulate dendritic spine development by inhibiting expression of the Limk1 protein kinase [34].

miR-124 is one of the most abundantly expressed miRNAs in the nervous system, being widely expressed in neurons in the brain, retina, and spinal cord [22,25,26,28,35–37]. There are three miR-124 genes, miR-124-1, miR-124-2, and miR-124-3, located on three different chromosomes in mouse and human genomes [38]. All three miR-124 genes are widely expressed in overlapping patterns in the mouse nervous system [28]. The expression of mammalian miR-124 can be first detected in differentiating neurons and persists in mature neurons, suggesting that miR-124 plays important roles during neural development [25,28,37,39,40]. The expression of miR-124 appears in part to mediate the repression of non-neuronal genes in neurons [41–43], as well as the downregulation of genes expressed in neural progenitors [44]. Overexpression of miR-124 (and other brain-enriched miRNAs) in mouse embryonic stem cells promotes neuronal fates [45], and a recent study indicates that miR-124 can promote neuronal differentiation via inhibition of the Ctdsp1/SCP1 phosphatase, a component of the REST/NRSF transcriptional repression complex [46]. miR-124 can also target PTBP1, a global repressor of alternative splicing, thereby regulating alternative splicing during neuronal differentiation [47]. Since miR-124 is expressed abundantly in differentiating and differentiated neurons and may target hundreds of mRNAs [41], it is possible that miR-124 may affect many aspects of the neuronal differentiation process.

Mouse P19 embryonal carcinoma cells [48] can be differentiated into neurons by transient expression of neural basic helix-loop-helix (bHLH) transcription factors [49,50]. Here, we show that expression of miR-124 together with the bHLH protein MASH1 in P19 cells enhances neurite initiation and outgrowth, while blockade of miR-124 action using antisense 2'-O-Methyl (2'-O-Me) RNA oligonucleotides delays or reduces neurite outgrowth. Further, expression of miR-124 in uncommitted P19 cells led to substantial changes in the cytoskeleton, and altered the level or localization of two members of the RhoGTPase family, Cdc42 and Rac1. Expression of activated Cdc42 or Rac1 attenuated neurite outgrowth promoted by miR-124, suggesting that Cdc42 and Rac1 may function downstream of miR-124 in promoting neurite outgrowth. We also demonstrate that miRNA overexpression or functional blockade in primary cultures of differentiating neurons from embryonic mouse cerebral cortex results in alterations in neurite outgrowth. In an effort to discern the mechanisms by which miR-124 modulates neurite outgrowth, we evaluated global alterations in gene expression concurrent with expression of miR-124 in P19 cells. We observe that mRNAs encoding proteins involved in cytoskeletal regulation are enriched among mRNAs downregulated by miR-124 expression. A variant of miR-124 with an additional base at the 5' end failed to promote neurite outgrowth, showed altered target specificity, and downregulated a substantially different set of mRNAs in P19 cells. These results indicate that miR-124 contributes to the regulation of neurite outgrowth during neuronal differentiation, and that this effect is likely to be mediated at least in part via alterations in cellular cytoskeleton.

Material and Methods

Expression plasmids

Plasmids were constructed using standard techniques. All expression vectors are based on the US2 plasmid expression vector, a variant of CS2 [51,52] in which the simian CMV promoter has been replaced with the human ubiquitin C promoter and first intron [53]. US2-MASH1, US2-Luc, US2-c β gal, and US2-eGFP are US2 variants of previously described CS2 vectors [49,50,52,54]. US2-Ngn2, US2-Rac1-CA, and US2-Cdc42-CA contain the coding regions for mouse Neurogenin-2, human Rac1 G12V, and human Cdc42 G12V respectively. Additional vector information is available at <http://sitemaker.umich.edu/dlturner.vectors>

Partial primary transcript sequences for the three mouse miR-124 genes were amplified by PCR from embryonic telencephalon cDNA and cloned into US2 using the listed primers with the indicated restriction sites: miR-124-1 (386nt fragment);

Forward-EcoR1: GAGAATTCGCACGCGTCGCCAGCTTTTTC;

Reverse-Xba1: TCTCTAGATGCAGCTGCAGCGCTGAGATC. miR-124-2 (445nt fragment);

Forward-EcoR1: GAGAATTCACCCGCTACTCTTCAACCTTG;

Reverse-Xho1: GACTCGAGGGATTTCAGATTCTCGCTG. miR-124-3 (795nt fragment);

Forward-EcoR1: GAGAATTCTGCACCCGTCAGAAGACTG;

Reverse-Xba1: GATCTAGAAAGCTGCACACTTCCCTCC.

The miR-124-1 m mutant expression vector was constructed by PCR with the 124-1m1R primer:

ACCCAAGGTGCTCAGACAGCCCCATTCTTGGCATTACCGCG**getageAA**
TTGTATGG AC. The complement of the mature miR-124 miRNA sequence is in bold, with the mutated sequence in lowercase. An endogenous Sty1 site used for cloning is underlined. Since the base pairing of miR-124 hairpin loop structure in 124-1m is disrupted, biogenesis of the mutant miR-124 miRNA may be interrupted.

The partial 3'UTR sequence for mouse integrin beta1 (Itgb1) was amplified by RT-PCR from P19 cell RNA and inserted into US2-Luc after the luciferase coding region with the primers: Itgb1 3'UTR (1064nt fragment);

Forward-Xho1: CTCTCGAGTGAATCCTACTCAGGCGGA;

Reverse-Xba1: TCTCTAGAGCACTGTCAAAATGAAAAGGC.

Mutations in each predicted miR-124 site were introduced by PCR with specific primers.

2'-O-Me RNA oligos

Antisense 2'-O-methyl oligonucleotide complementary to miR-124: 2'-O-Me-124-as, UUACUUGGCAUUCACCGCGUGCCUUAUUAUU. Scrambled sequence of 2'-O-Me-124-as used as a control: 2'-O-Me-124-sc, UUGGCAUUGUGCACUACACCCUGCUUUAUUU.

Quantification of F-actin

F-actin content of P19 cells was measured using the method of Machesky et al [55,56]. In brief, cells in plates were washed twice with D-PBS (Invitrogen), and then scraped in 100 μ l of fixative extraction buffer (10mM PIPES, 190mM K₂HPO₄, 10mM KH₂PO₄, 5mM EGTA,

2mM MgCl₂, 0.1% Triton, 3.7% formaldehyde) with 0.5μM Alexa 546 phalloidin. Cell extracts were transferred to 1.5ml tubes and rocked for 1 hr at room temperature, then pelleted for 2 min in a microcentrifuge, and washed once with 200μl of washing buffer (0.1% saponin, 190mM K₂HPO₄, 10mM KH₂PO₄, 10mM PIPES, 5mM EGTA, and 2mM MgCl₂). The pellets were resuspended in 200μl of methanol and rocked for 1 hr at room temperature to extract the bound phalloidin. The cell extracts were pelleted again and the supernatant containing bound phalloidin was measured for fluorescence emission at 572 nm following excitation at 340 nm (Wallac Victor 1420 multilabel counter).

P19 Cell culture and transfections

Mouse P19 cells were maintained as described [49]. For transfection, cells were plated on dishes at 70–90% confluence without antibiotics. Transfections with DNA plasmids were performed with FuGENE 6 (Roche), while transfections with synthetic miRNA duplexes or 2'-O-Me RNA oligos were performed with Lipofectamine 2000 (Invitrogen), as directed by the manufacturers. Transfected cells were split onto dishes or coverglasses coated with poly-L-lysine and mouse natural laminin (Invitrogen). For experiments with uncommitted P19 cells (Fig. 3–5), 1 μg US2-mt, US2-124-1, or US2-124-1m was transfected with 0.5 μg US2-puro and 0.6 μg US2-eGFP into P19 cells in 12-well plates. Cells were split 10–14 hr after transfection in 1:20 dilution and 15 μg/ml puromycin was added. Media was replaced with OPTI-MEM1 (Invitrogen, CA) supplemented with 1% fetal bovine serum 8–12 hr after split with 15 μg/ml puromycin. Cells were fixed with 4% paraformaldehyde at room temperature for 15 min. Quantity of plasmids transfected in each experiment is listed as follows: Fig. 2: 0.6 μg US2-eGFP, 0.6 μg US2-MASH1 were cotransfected with 0.8 μg US2-mt, US2-124-1, US2-124-1m. For neuronal differentiation assays in Fig. 1: 0.6 μg US2-eGFP, 0.7 μg US2-MASH1 were cotransfected with 35 pmol of RNA duplexes. Fig. 1: 0.6 μg US2-eGFP, 0.7 μg US2-MASH1 were cotransfected with 1 μg of 2'-O-Me oligos. Fig. 6: 0.5 μg US2-eGFP, 0.5 μg US2-MASH1, and 0.5 μg US2-124-1 were cotransfected with 0.6 μg US2-mt or US2-CA-Rac1/Cdc42. Cells were split 1:20 at 10–14 hr after transfection and the media was replaced with OPTI-MEM1 (Invitrogen, CA) with 1% fetal bovine serum 1 hr later. Cells were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature.

Luciferase assays

In 12-well plates, 50 ng US2-luc, US2-luc-124a_target or US2-luc-Itgb1 (WT, s1m, s2m, s12m) plasmids were cotransfected with 30 pmol of RNA duplexes, 50–100 ng US2-cβgal, and 500 ng US2-mt per well. Reporter activity was assayed 22 hr after transfection using the Dual-Light system (Applied Biosystems/Tropix, Foster City, CA). Luciferase activity was normalized to β-galactosidase activity to control for transfection efficiency variation.

Antibodies and indirect immunofluorescence

Primary antibodies and dilutions for immunohistochemistry: anti-acetylated tubulin, clone 6-11B-1, mouse monoclonal, Sigma, 1:2000; anti-α-tubulin, clone DM1A, mouse monoclonal, Sigma, 1:2000; anti-GFP, rabbit polyclonal, Molecular Probes, 1:2000; anti-actin, rabbit polyclonal, Sigma, 1:2000; anti-Cdc42, mouse monoclonal, BD Biosciences/Transduction Laboratories, 1:250; anti-Cdc42, rabbit polyclonal, clone sc-87, Santa Cruz, 1:250; anti-Rac1, clone 23A8, mouse monoclonal, Upstate, 1:1000; anti-RhoA, clone 26C4, mouse monoclonal, Santa Cruz, 1:500; anti-neuronal class III beta-tubulin, clone TuJ1, mouse monoclonal, Covance, 1:2000. Secondary antibodies and dilutions: Alexa Fluor488 goat anti rabbit antibody, Molecular Probes, 1:2000; Alexa Fluor546 goat anti-mouse antibody, Molecular Probes, 1:2000. Alexa Fluor546 phalloidin from Molecular Probes was used at a dilution of 1:60. Cells were photographed with a DAGE 330 video camera/digital capture system on an inverted microscope.

Western Blots

P19 cells cultured in 6-well plates were transfected with 2.4 μ g of US2-mt, US2-124a1, or US2-124a1m. In addition to the expression constructs, 1.2 μ g of US2-puromycin and 0.8 μ g US2-GFP were used for each transfection in Fig. 4A–C, 5. Cells were transfected by using FuGENE6 (Roche) according to the manufacturer's instructions. For the experiments in Fig. 4D, 1 μ g US2-MASH1 and 1.2 μ g US2-puro were cotransfected with 3 μ g of 2'-O-Me oligos into P19 cells cultured in 6-well plates by using Lipofectamine 2000 (Invitrogen). Approximately 12–16 hours after transfection, cells were split onto two 10 cm plates and 15 μ g/ml puromycin was added. Media was replaced with OPTI-MEM1 (Invitrogen, CA) supplemented with 1% fetal bovine serum 8–12 hr after split with 15 μ g/ml puromycin. Cell extracts were prepared at 44–48 hours using modified RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM EDTA, 1% aprotinin, 1 mM PMSF, 1 mM sodium orthovanadate) and subjected to sonication. After sonication, Triton X-100 was added to a final concentration of 1%; insoluble material was removed by centrifugation at 14,000 rpm at 4 °C. The extracts were subjected to SDS-PAGE followed by western blot analysis. The same antibodies were used as listed for indirect immunofluorescence.

Northern blot analysis

P19 cells were transfected with 2 μ g of expression vectors per well in 6-well plates and lysed at 48 hours after transfection for total RNA isolation using TRIzol (Invitrogen). Northern blot analysis was performed as described previously [53] using 15 μ g of RNA per sample. Images were obtained with IPLab Gel H 1.5g software (Signal Analytics) from the PhosphorImager 445 SI (Molecular Dynamics) and were assembled with ImageJ software [57]. Blots were stripped and reprobed for U6 snRNA as loading control.

Culture and transfection of primary cortical neurons

Culture of primary cortical neurons has been described previously [58]. In brief, mouse embryos were dissected from a timed-pregnant female at E14.5. Dorsal part of the telencephalons were dissected and dissociated by trituration with fire-polished Pasteur pipettes into single cell suspension. 10^7 cells were electroporated with 5 μ g US2-mt, US2-124-1, or US2-124-1m with 3 μ g US2-eGFP (Fig. 7A–C), or 14 μ g 2'-O-Me oligos with 4 μ g US2-eGFP (Fig. 7D, E) using a Nucleofector and Mouse NSC Nucleofector Kit (Amaxa). $3\text{--}5 \times 10^5$ primary cortical cells (non-electroporated) were plated onto 12-well coated with poly-L-lysine and mouse natural laminin (Invitrogen) prior to the plating of electroporated cells to maintain a high density of cortical cells. 2.5×10^5 electroporated cortical cells were plated and cultured in L15 medium with N2 supplement, B27 supplement, Glucose 30 mM, NaHCO₃ 26 mM, 100 units/ml penicillin, 100 μ g/ml streptomycin, and 20 ng/ml FGF2. Half the medium was replaced with fresh medium without FGF2 every other day. Cells were fixed by 4% paraformaldehyde at 43 hr after electroporation and stained with an antibody against GFP.

Microarray analysis and miRNA target site predictions

P19 cells in 6-well dishes were cotransfected with 1 μ g US2-puro and 50nM (final concentration) of synthetic RNA duplexes for miR-124, miR-124UU21 (see Fig. 2 for sequences), or a control short interfering RNA (siRNA) against the *Xenopus* XASH3 mRNA, using Lipofectamine 2000 as described above. Transfected cells were selected using puromycin as described above and RNA was isolated from two independent transfections for each synthetic RNA duplex at 25 hours after transfection using TRIzol. 200ng of total RNA was amplified and labeled using the Illumina Total Prep RNA Amplification Kit (Ambion). 1.5 μ g of labeled cRNA was hybridized at 55° C for 22 hrs to Sentrix-6 Mouse V1.0 BeadChip microarrays (Illumina). Microarrays were washed and scanned for data collection as directed by the manufacturer. Microarray data was analyzed using BeadStudio software (Illumina).

Differential gene expression was determined using quantile normalization and the Illumina Custom error model. mRNAs for analysis were selected based on mRNAs detected in at least one condition with $P < 0.01$. For differential expression analyses, a cutoff of $P < 0.01$ was used. All analyses used a subset of Illumina probes that matched sequences in the Refseq database and that mapped to the mouse genome at a single location (Pinglang Wang and Fan Meng, University of Michigan, personal communication).

The mouse 3' UTR database (UTRdb release 16) [59] was searched for exact matches to the six seed sequences complementary to miR-124 and miR-124-UU21 using custom software (DLT, unpublished). Evolutionary conservation of the seed sequences was not required, as previous analyses have shown that both conserved and non-conserved seed sequences can mediate miRNA repression [42]. Using Microsoft Access, 3' UTRs that contained seed sequences were matched to the Refseq annotated Illumina probes using accession numbers. If more than one 3' UTR sequence was linked to an individual Illumina probe, the UTR sequence with the largest total number of seed sequences was used. The R software package was used for statistical analysis of seed sequence frequencies. Mapping of regulated genes to Gene Ontology categories and to the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways [60] was performed using WebGestalt [61].

Results

miR-124 is upregulated in P19 cells expressing neural bHLH proteins and contributes to neurite outgrowth

Transient expression of neural bHLH proteins such as MASH1 can direct neuronal differentiation of uncommitted mouse P19 embryonal carcinoma cells [49,50]. Endogenous miR-124 was upregulated in P19 cells following transfection of MASH1 or Ngn2 expression vectors, but not after transfection of a control vector (Fig. 1A). We also observed upregulation of the miR-124-1 primary transcripts by semi-quantitative RT-PCR in P19 cells expressing MASH1 (data not shown). To determine if miR-124 is required for neuronal differentiation of P19 cells transfected with bHLH expression vectors, antisense 2'-O-methyl (2'-O-Me) RNA oligonucleotides (oligos) were used to inhibit miR-124 [62,63]. An antisense 2'-O-Me oligo complementary to miR-124 (O-Me-124-as) or a 2'-O-Me oligo with a scrambled version of the same sequence (O-Me-124-sc) was cotransfected into P19 cells with MASH1 and Green Fluorescent Protein (GFP) expression vectors. Inhibition of miR-124 by 2'-O-Me-124-as did not prevent expression of a neuronal specific β -tubulin protein detected by the TuJ1 antibody, but the percentage of TuJ1 labeled cells with neurites in differentiating P19 cells decreased two-fold (Fig. 1B, C). The ability of the 2'-O-Me-124-as oligo to block miR-124-mediated repression by miR-124 was confirmed using a luciferase reporter with a 3' UTR regulated by miR-124 (Supplemental Fig. S1).

To investigate if increased expression of miR-124 could cooperate with bHLH proteins to promote neuronal differentiation of P19 cells, we constructed expression vectors for miR-124 in which partial pri-miR-124 sequences containing the miR-124 stem-loop precursors from all three miR-124 genes (miR-124-1/2/3) were expressed under control of the human ubiquitin C promoter. The miR-124-1, 124-2, and 124-3 vectors all produced mature miR-124 in transfected P19 cells, as did a vector expressing a longer ~3.8 Kb cDNA for pri-miR-124-1 (Fig. 1A) (also see [28]). As a control, nucleotides 2–7 of the mature miR-124 sequence in the miR-124-1 vector were mutated to make the miR-124-1m expression vector. These nucleotides have been shown to be essential for target recognition by miRNAs [64–68]. The miR-124 expression constructs were cotransfected with plasmids expressing MASH1 and GFP into P19 cells. MiR-124-1 promoted neurite initiation in transfected P19 cells in comparison with a control vector, while P19 cells transfected with the mutant miR-124-1m expression vector did not differ from P19 cells transfected with the control plasmid (Fig. 1D, E). 46 hr after

transfection, ~24% of the miR-124-1 transfected cells had neurites in comparison with ~2% of the transfected cells in the control group (Fig. 1D). The expression constructs for miR-124-2 and miR-124-3 as well as the longer miR-124-1 cDNA also showed a similar ability to promote neurite initiation (Fig. 1D and data not shown). Enhanced neurite outgrowth in miR-124 expressing cells was still present 64 hr after transfection (Fig. 1F).

Since chemically synthesized RNAs can function as miRNAs [69,70], we cotransfected P19 cells with a MASH1 expression vector and various synthetic miR-124 RNA duplexes (Fig. 2A, B). Synthetic miR-124 miRNA paired with either a perfectly complementary strand (miR-124-as) or with a synthetic version of the endogenous miR-124* strand promoted neurite outgrowth in the transfected cells relative to a control RNA duplex (Fig. 2B). In contrast, a mutant version of the synthetic miR-124 (miR-124-mutant) failed to promote neurite outgrowth in a duplex with either miR-124-as or miR-124*. A synthetic miR-124 target sequence cloned into the 3' UTR of a luciferase expression vector was used as a sensor to confirm that the transfected miR-124 miRNAs were incorporated into RISC and could inhibit reporter gene expression efficiently and similarly (Fig. 2A, B) [6,8,30,37]. These observations indicate that the miR-124 miRNA itself is sufficient to promote neurite outgrowth, and that additional pri/pre-miRNA sequences present in the miR-124-1/2/3 expression vectors are not required.

An alternate 21nt sequence variant of mature miR-124 was isolated from adult brain [22], shifted 5' by one nucleotide at both ends, most likely reflecting variation in the site of Drosha cleavage. We refer to the second sequence variant as miR-124-UU21 (based on the UU sequence present at the 5' end) (Fig. 3A, B). A 23nt miR-124 clone with UU at the 5' end was also isolated from rat neurons [71]. Cotransfection of a miR-124-UU21 synthetic duplex with MASH1 did not promote neurite outgrowth, despite effectively inhibiting the luciferase sensor (Fig. 2B). To determine whether the functional difference arose from the additional U at the 5' end or the missing A at the 3' end of miR-124-UU21, a 22nt variant of miR-124 (miR-124-UU22) with both the additional 5' U and the 3' A was tested. The miR-124-UU22 duplex was unable to promote neurite outgrowth with MASH1, although it effectively inhibited the luciferase sensor (Fig. 2B), indicating that the precise 5' end of the miR-124 miRNA is essential for its ability to promote neurite outgrowth.

Expression of miR-124 alters actin filaments and microtubules

The miR-124-1 expression vector, the miR-124-1m mutant expression vector, or a control vector was cotransfected into uncommitted P19 cells with a vector expressing GFP and a vector expressing the puromycin resistance gene, permitting selection of transfected cells. Expression of miR-124 was not sufficient to activate expression of neuronal markers in uncommitted P19 cells, nor was it able to trigger neurite outgrowth in P19 cells when expressed without a neural bHLH protein (Fig. 3 and data not shown). However, expression of miR-124 did alter the cellular morphology of the uncommitted P19 cells: cell bodies of cells transfected with miR-124 expression vectors became less spread out and their lamellipodia were disrupted (Fig. 3). Alexa-546-conjugated phalloidin was used to detect filamentous actin (F-actin) in transfected P19 cells. miR-124 expression not only disrupted lamellipodia, but also led to the formation of multiple fine F-actin containing projections at the edges of most cells, while reducing overall F-actin levels (Fig. 3). α -tubulin acetylation is a post-translational modification widespread in neurons and other cell types. Long-lived and stabilized microtubules are enriched in acetylated α -tubulin [72]. Expression of miR-124 in uncommitted P19 cells increased acetylated α -tubulin relative to the control vector or the miR-124-1m expression vector, as detected by immunofluorescence at 44 hr after transfection (data not shown). Changes in F-actin, total actin, α -tubulin, and acetylated α -tubulin were quantitated in cells transfected with miR-124-1 or control vectors (Fig. 4). The amount of F-actin in the miR-124-1 transfected cells decreased by ~20%, while the total actin protein level decreased

by ~30% in P19 cells expressing miR-124 (Fig. 4A–C). In contrast, the level of α -tubulin protein was not reduced, although expression of miR-124 led to a three-fold increase in acetylated α -tubulin (Fig. 4A, B). F-actin, actin, α -tubulin, and acetylated α -tubulin were not significantly different in P19 cells transfected with the miR-124-1m mutant expression vector when compared to P19 cells transfected with the myc tag control expression vector (Fig. 4A, B). We also examined the levels of α -tubulin, and acetylated α -tubulin after blocking miR-124 function in MASH1 transfected P19 cells. 2'-O-Me-124-as decreased levels of acetylated α -tubulin, but not total α -tubulin (Fig. 4D), consistent with the possibility that miR-124 promotes the accumulation of acetylated α -tubulin.

Rac1 and Cdc42 act downstream of miR-124 in regulating neurite outgrowth

Small GTPases in the Rho family, including Rac, Rho, and Cdc42, have been shown to regulate both microtubules and actin filaments in a variety of cells [73,74]. In fibroblasts, activation of Rac and Cdc42 promotes formation of lamellipodia and filopodia respectively, while RhoA activation leads to the formation of stress fibers and focal adhesions [74]. Since miR-124 expression affected both microtubules and actin filaments in P19 cells, we tested whether miR-124 alters the expression of Rho GTPase family members. The miR-124-1 expression vector, the miR-124-1m expression vector, or a control vector was cotransfected with vectors expressing GFP and the puromycin resistance gene, as described above. Western blot analysis showed that the protein level of Cdc42, but not Rac1 and RhoA, was reduced to ~50% of the control by miR-124 (Fig. 5A, B). Compartmentalization of Rho GTPases is important for the regulation of their enzyme activity, signaling cascades, and stability [75–77]. Expression of miR-124 resulted in strong nuclear accumulation of Rac1 and reduced cytoplasmic Rac1 in comparison with P19 cells transfected with the control vector or the miR-124-1m mutant expression vector (Fig. 5C). The altered subcellular localization of the Rac1 protein suggests that miR-124 may negatively regulate Rac1 function in the cytoplasm.

Both Rac1 and Cdc42 are important regulators involved in various aspects of neurite outgrowth during neuronal differentiation [78,79]. Since miR-124 negatively regulates Cdc42 levels and alters Rac1 localization, we hypothesized that Cdc42 and Rac1 are downstream of miR-124 in regulating neurite outgrowth. For both Cdc42 and Rac1, a valine substitution at amino acid 12 creates a constitutively active (CA) form. If inhibition of these proteins is required for miR-124 function, expression of CA-Cdc42 or CA-Rac1 might be expected to block the enhanced neurite outgrowth resulted from miR-124 expression. Expression constructs for miR-124, MASH1, and GFP were cotransfected into P19 cells with either a control plasmid or expression vectors for CA-Cdc42 or CA-Rac1 (Fig. 6). Expression of either CA-Cdc42 or CA-Rac1 attenuated neurite outgrowth in MASH1 transfected P19 cells 48 hrs after transfection. At 65 hrs after transfection, CA-Cdc42 continued to prevent neurite outgrowth, while substantial neurite outgrowth was observed in the presence of CA-Rac1 at 65 hrs after transfection.

miR-124 alters neurite outgrowth in primary cortical neurons

miR-124 is present in the developing cerebral cortex [25,26,28]. To further investigate the role of miR-124 during neuronal differentiation, we cotransfected a GFP expression vector with miR124 expression vectors or miR-124 2'-O-Me oligos into cortical progenitors isolated from E14.5 mouse embryos and allowed the cortical cells to differentiate *in vitro*. The miR-124-1 expression vector did not alter the timing of neurite outgrowth, and blocking miR-124 function with 2'-O-Me oligos did not prevent neurite outgrowth from the transfected cortical neurons. However, we observed altered numbers of primary neurites arising from the cell bodies of transfected cells (Fig. 7). The miR-124-1 expression vector increased the number of primary neurites in cortical neurons 43 hr after electroporation, while electroporation of the miR-124-1m mutant expression vector did not alter the number of primary neurites relative to a control vector (Fig. 7A–C). In contrast, blocking miR-124 function during neuronal

differentiation with the miR-124 2'-O-Me antisense oligo decreased the number of primary neurites relative to cells electroporated with the 2-O-Me oligo scrambled control (Fig. 7D, E).

miR-124 and miR-124-UU21 regulate distinct sets of genes in P19 cells

Since miR-124 but not miR-124-UU21 promotes neurite outgrowth, it is likely that at least some target mRNAs are not regulated by both forms of miR-124. Basepairing between nucleotides 2–8 of a miRNA (numbered from the 5' end), often referred to as the “seed sequence” [68], and a target mRNA are thought to be essential for target mRNA recognition and inhibition [64,66–68]. However, the terminal 5' nt of the miRNA (position 1) is not required to basepair with the target mRNA sequence for inhibition. Target sequences that are complementary to nucleotides 2–8 of miR-124 should be inhibited by miR-124 efficiently. In contrast, the same target sequences would be expected to have nucleotides at positions one and two unpaired in a duplex with miR-124-UU21, likely reducing or preventing inhibition by miR-124-UU21. The *Itgb1* mRNA is downregulated by miR-124 [41,43,44]. The 3'UTR of mouse *Itgb1* contains two putative miR-124 binding sites in which the 3' nt of each putative target site is mismatched with the terminal 5' nt of miR-124, while miR-124-UU21 is mismatched with the two terminal 5' nt (Fig. 8A). To test if the 3'UTR of mouse *Itgb1* responds differently to miR-124 and miR-124-UU21 expression, the 3'UTR of *Itgb1* (*Itgb1*-WT) was inserted after a luciferase reporter gene. miR-124 repressed reporter activity approximately three-fold (Fig. 8B). The ability of miR-124 to inhibit the luciferase activity was attenuated by mutations in either or both predicted miR-124 binding sites on *Itgb1* 3'UTR (Fig. 8B), indicating that these sites mediate miR-124 inhibition. However, synthetic miR-124-UU21 RNA duplexes cotransfected with the luciferase reporter plasmid did not inhibit luciferase activity (Fig. 8C). This result indicates that miR-124 and miR-124-UU21 can regulate different target mRNAs.

Bartel and coworkers found that expression of synthetic miR-124 or other miRNAs led to decreased levels of numerous mRNAs in HeLa cells [41]. To identify mRNAs regulated by miR-124, but not by miR-124-UU21, we performed microarray analysis on RNA isolated from uncommitted P19 cells 25 hours after transfection with a control siRNA, a synthetic miR-124 duplex, or a synthetic miR-124-UU21 duplex. Transfection of either miR-124 or miR-124-UU21 led to decreased expression of a subset of mRNAs (Fig. 9A and Supplemental Table 1). Most downregulated genes were affected by one of the two miR-124 forms, but not both (Fig. 9A). Conaco et al. [43] used both gain and loss of function assays for miR-124 in combination with qRT-PCR to verify miR-124 regulation of 17 mRNAs first identified as downregulated by miR-124 by Lim et al.[41]. Our microarray analysis detected 13 of these mRNAs in control RNA samples, and probes for 10 of these 13 mRNAs were downregulated by miR-124 (with $P < 0.01$; two additional mRNAs among the 13 were downregulated with $P < 0.05$; see Supplementary Table 2). These observations indicate that our microarray analysis was highly specific and many of the regulated genes are consistent with the prior reports [41,43].

To determine if specific functional pathways were overrepresented among the genes regulated specifically by miR-124 or miR-124-UU21, we mapped the functions of the downregulated genes to the Gene Ontology (GO) cellular components. The 228 genes downregulated in P19 cells by miR-124, but not the 186 genes downregulated by miR-124-UU21, were significantly enriched for genes that encode components of the cytoskeleton, including the actin cytoskeleton, as well as vesicle transport and cell junctions (Fig. 9B and Supplemental Table 3). miR-124-UU21 downregulated genes were enriched for components of the cell-matrix junctions (Fig. 9B). We also mapped the sets of downregulated genes to KEGG pathways and among genes downregulated by miR-124, but not by miR-124-UU21, we observed significant enrichment ($P < 0.001$) for genes involved in regulation of the actin cytoskeleton (not shown).

Since target sites in the *Itgb1* 3' UTR could be regulated by miR-124, but not miR-124-UU21, we expected that different seed sequences would be present in the 3' UTRs in the three sets of genes downregulated at the mRNA level by miR-124, miR-124-UU21, or both miRNAs in P19 cells [41,42,80]. We searched the 3' UTRs of mouse genes in the UTRdb database [59] for six different seed sequences partially or completely complementary to the 5' end of miR-124, miR-124-UU21, or both miRNAs (Supplemental Fig. S2, Supplementary Table 1). We observed significant enrichment for distinct subsets of seed sequences complementary to the transfected miRNA in each of the three sets of regulated genes, relative to the frequency of various seed sequences in the 3' UTRs of mRNAs detected in the control. Strikingly, among the 19 genes downregulated by miR-124 and classified as components of the cytoskeleton, nine contained seeds complementary to miR-124 (seeds 1 or 5, Supplementary Table 3). Taken together with previous results, these results suggest that many of the mRNAs downregulated by miR-124 or miR-124-UU21 are likely to be direct miRNA targets, and at least part of the differences in which mRNAs are regulated by each miRNA arises from the presence of distinct seed sequences.

DISCUSSION

MiR-124 is an evolutionarily conserved miRNA expressed in differentiating and mature neurons. MiR-124 has been implicated in the regulation of neuronal gene expression but its cellular role is not completely understood. Here we demonstrate that miR-124 modulates neurite outgrowth and affects the cytoskeleton. In P19 cells expressing MASH1, blocking endogenous miR-124 function by transfection of 2'-O-Me oligos complementary to miR-124 reduced neurite outgrowth, while overexpression of miR-124 promoted neurite initiation and extension. In dissociated primary mouse cortical neurons differentiating *in vitro*, blocking miR-124 function with 2'-O-Me oligos decreased the number of primary neurites, while overexpression of miR-124 increased the number of primary neurites extended from neuronal cell bodies. The reciprocal phenotypes observed in each cell type strongly suggest that the observed changes reflect specific functional effects of miR-124. The onset of miR-124 expression in the CNS coincides with the start of neuronal differentiation, indicating that miR-124 is expressed during process outgrowth. The phenotypes we observed with blocking or overexpression of miR-124 in differentiating P19 cells and cortical neurons are similar but not the same, which suggests that miR-124 may affect different aspects of neurite outgrowth in different types of neurons and/or under different conditions.

Our observations on neurite outgrowth are consistent with those of Makeyev et al. [47], who observed that miR-124 promoted neurite outgrowth in neuroblastoma cells as well as stimulated neuronal differentiation in retinoic acid (RA) treated and aggregated P19 cells. They found that the mRNA for the splicing factor PTBP1 is a direct target of miR-124 repression, and that part of the effects of miR-124 could be attributed to alternate mRNA splicing arising from the downregulation of PTBP1. Visvanathan et al. [46] also reported that miR-124 function in the embryonic chicken neural tube promoted neuronal differentiation and cell cycle exit, while Krichevsky et al. [45] found that coexpression of miR-124 and miR-9 in ES cells differentiated *in vitro* increased the fraction of neuronal cells. Previous studies demonstrated that miR-124 is likely to downregulate the level of numerous direct target mRNAs [41,42], as well as indirectly upregulate neuronal transcription by inhibition of the REST/NRSF transcriptional repressor complex [43,46]. In addition to REST/NRSF and PTBP1, other targets are likely to contribute to miR-124 functions. Cao et al. [44] observed defects in the basal lamina in response to ectopic miR-124 expression in chicken neural tube, and they linked these defects to reduced expression of integrin $\beta 1$ and laminin proteins. In addition, since miRNAs can inhibit translation without reducing mRNA levels, it is likely that there are additional miR-124 target genes yet to be identified [81].

While coexpression of miR-124 with MASH1 in P19 cells promoted neurite outgrowth from the transfected cells, we found that expression of miR-124 without MASH1 in undifferentiated P19 cells led to remodeling of the cytoskeleton in transfected cells, including reduced lamellipodia formation as well as changes in both microtubules and actin filaments. These changes reflect changes in both structural and regulatory components of the cytoskeleton. MiR-124 expression disrupted actin filaments, and reduced total actin levels, while acetylation of α -tubulin was increased, suggesting that miR-124 may increase stability of microtubules. The level of the Cdc42 protein was reduced, while Rac1 showed altered subcellular localization. The Cdc42 3' UTR does not contain seed sequences complementary to miR-124, suggesting that it is regulated indirectly by miR-124. Makeyev et al. [47] reported that miR-124 expression regulates alternate splicing of Cdc42, leading to a switch to a neuron-specific isoform with a different C-terminus. The antibody we used to detect Cdc42 recognizes an N-terminal epitope, indicating that miR-124 expression leads to a decrease in total Cdc42 levels.

In fibroblasts, activation of Cdc42 leads to filopodia formation, while activation of Rac1 leads to the lamellipodia and membrane ruffles [74]. Localization of Rac1 to the membrane and cytoplasm is important for Rac1 activity and function in promoting lamellipodia formation [75–77]. Nuclear accumulation of Rac1 has been shown to affect stability of Rac1 and eventually lead to Rac1 protein degradation [77,82,83]. Both the disruption of lamellipodia and localization of Rac1 to the nucleus in P19 cells expressing miR-124 suggest that miR-124 may indirectly downregulate the activity of Rac1 in the cytoplasm. Changes in the cytoskeleton controlled by the Rho family of small GTPases, including Cdc42 and Rac1, are known to regulate neurite outgrowth, raising the possibility that the effects of miR-124 on neurite outgrowth may arise from the cytoskeletal changes. We found that expression of CA-Cdc42 or CA-Rac1 inhibited or delayed neurite outgrowth in differentiating P19 cells expressing MASH1 and miR-124, consistent with the possibility that reduced levels/activity of these small GTPases is required for the miR-124 to promote neurite outgrowth. Although Cdc42 and Rac1 promote neurite formation in some cell types [reviewed in 79], inhibition of neurite outgrowth has been observed in rat primary cortical neurons [84] and chicken dorsal root ganglion neurons [85]. Localized Rac1 and Cdc42 activity may also be necessary for neurite outgrowth [76].

We found that a miR-124 sequence variant (miR-124-UU21) with an additional 5' end U failed to promote neurite outgrowth, and it did not inhibit a reporter based on the 3' UTR of mouse *Itgb1*. While the 5' end of miR-124 has a single mismatched base at both target sites in the *Itgb1* UTR, the 5' end of miR-124-UU21 has two mismatched bases with each site, preventing repression. These observations suggest that mRNAs involved in neurite outgrowth regulated by miR-124 may contain miR-124 target sites with a single base mismatch at the 5' end of miR-124. Lim et al. pioneered the use of microarrays to identify mRNAs downregulated by miRNA expression, including human mRNAs regulated by miR-124 in HeLa cells [41]. We used a similar microarray-based analysis to identify mouse mRNAs downregulated by mature miR-124 or the miR-124-UU21 variant in undifferentiated P19 cells. The mRNAs downregulated by miR-124 were consistent with other reports, and included known targets of miR-124 [41] [47] [43] [81]. Comparison of mRNAs downregulated by miR-124 and miR-124-UU21 showed surprisingly limited overlap between mRNAs regulated by each miRNA. Seed sequences complementary to the transfected miRNA were enriched in each set of downregulated mRNAs, consistent with previous studies on miR-124 [41,42,80]. In addition, we found that seed sequences in which the 5' terminal base of miR-124 is not complementary to the seed sequence were enriched in mRNAs regulated specifically by miR-124, while seeds with different patterns of complementarity were enriched in mRNAs regulated specifically by miR-124-UU21, or by both miRNAs. We found statistically significant enrichment for genes encoding components of the cytoskeleton among the mRNAs downregulated by miR-124, but not among mRNAs downregulated by miR-124-UU21. Makeyev et al. [47] observed enrichment for cytoskeletal components among the products of alternately spliced mRNAs

regulated by miR-124 via repression of PTBP1, but our observations indicate that enrichment for cytoskeletal components extends to other mRNAs regulated by miR-124. Many of the mRNAs for cytoskeletal components contain seed sequences for miR-124 in their 3' UTR, suggesting that they may be direct targets. In P19 cells, both miR-124 and miR-124-UU21 downregulated PTBP1 and some alternately spliced mRNAs regulated by PTBP1 (e.g. Pdlim7, Sept6 [47]) were upregulated after transfection of either miRNA (Supplemental Table 3), suggesting that the functional difference between the two miRNA sequences may reflect targets other than PTBP1.

Our observations suggest that one function of miR-124 is to regulate genes encoding proteins involved in cytoskeletal reorganization. The regulation of cytoskeletal components by miR-124 suggests a likely mechanism for its effects on neurite outgrowth and possibly other aspects of neuronal differentiation. The control of cellular morphology during neuronal differentiation is complex and involves the coordinated function of many genes. It has been suggested that a single miRNA may repress multiple targets in the same pathway to modulate a complex biological process [86]. Recently, Kosik and coworkers showed that in cultured hippocampal neurons, miR-124 is preferentially localized in the neuronal somas rather than dendrites [87]. This raises the intriguing possibility that miR-124 could preferentially inhibit expression of cytoskeletal and other proteins from mRNAs present in the cell soma, but not inhibit expression of the same proteins if they are locally translated from mRNAs in processes or growth cones.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(A) Northern blot analysis detects mature miR-124 in mouse P19 cells transiently transfected with MASH1 or Ngn2 expression vectors. miR-124 is also detected in P19 cells transfected with expression vectors for three different mouse miR-124 genes (miR-124-1, miR-124-2, and miR-124-3), or a vector expressing a 3.8 Kb cDNA for miR-124-1, but not from a mutant form of miR-124-1 (miR-124-1m). Blots were stripped and reprobed for the U6 snRNA as loading control. (B) P19 cells were transfected with 2'-O-Me RNA oligos complementary to miR-124 (O-Me-124-as) or a control scrambled miR-124 sequence (O-Me-124-sc), in combination with plasmids expressing GFP and MASH1. Cells were fixed at 57 hr after transfection and stained with anti-GFP and TuJ1 antibodies. (C) Quantification of neurite initiation for 2'-O-Me-124-as or 2'-O-Me-124-sc transfected cells at 57 hr. GFP positive cells with processes longer than 2 cell-body diameters were considered as neurons with neurites. Average percentage of GFP positive neurons with neurites is shown, with standard error indicated. ** $p < 0.01$, Student's t-test. (D-F) miR-124 expression vectors or a control expression vector expressing a myc-epitope tag (mt) were cotransfected with plasmids expressing GFP and MASH1. Transfected P19 cells were fixed 46 hr after transfection, stained with anti-GFP and TuJ1 antibodies, and neurite initiation was quantitated (D). Experiments were done in three repeats. Average percentage of GFP positive neurons with neurites with standard error indicated. ** $p < 0.01$, Student's t-test. (E) Enhanced neurite outgrowth in cells cotransfected with miR-124-1 vector, but not with the miR-124-1m vector. (D) Transfected cells fixed at 64 hr after transfection and stained with anti-GFP and TuJ1 antibodies.

B

Synthetic RNA duplex	Enhanced neurite outgrowth	Relative luciferase activity
Control RNA duplex (XASH3 siRNA)	no	100.0%
5' - UAAGGCACGCGUGAAUGCCA 124 uaAUUCCGUGCGCCACUUACG-5' 124-as	yes	10.7±1.0% **
5' - UAAGGCACGCGUGAAUGCCA 124 : : : : uaGUUCCAGGCGACACUUGUG-5' 124*	yes	15.6±0.8% **
5' - CAGGGAUCGUCGUGAACACCA 124-mutant : : uaAUUCCGUGCGCCACUUACG-5' 124-as	no	93.2±8.8%
5' - CAGGGAUCGUCGUGAACACCA 124-mutant : : : uaGUUCCAGGCGACACUUGUG-5' 124*	no	99.2±6.6%
5' - UUAAGGCACGCGUGAAUGCC 124-UU21 uuAAUCCGUGCGCCACUUAC-5' 124-UU21-as	no	9.9±0.7% **
5' - UUAAGGCACGCGUGAAUGCCA 124-UU22 uAAUCCGUGCGCCACUUACG-5' 124-as	no	13.2±0.3% **

Figure 2. A synthetic miR-124 RNA duplex is sufficient to promote neurite outgrowth

(A) Schematic graph shows a luciferase sensor construct used to assess miRNA transfection and function. One copy of a sequence completely complementary to miR-124 and miR-124-UU21/22 is present after the luciferase coding region. (B) Sequences of synthetic miRNA duplexes are shown in the first column. Presence or absence of the enhanced neurite outgrowth phenotype as described in Fig. 1 is indicated. Average relative luciferase activities for each miRNA duplex cotransfected with the sensor construct in A were measured at 22 hr after transfection is shown ± standard error. ** $p < 0.01$, Student's t-test.

Figure 3. miR-124 expression alters the morphology and actin cytoskeleton of P19 cells. P19 cells were cotransfected with the miR-124-1 expression vector, the miR-124-1m expression vector or the mt control vector in combination with plasmids expressing GFP (green) and puromycin resistance genes. Transfected cells were selected in puromycin, fixed at 44 hr after transfection and stained with Alexa-546-conjugated phalloidin (red). Inset shows an example of fine F-actin containing processes frequent at the edges of miR-124 expressing cells (position of inset indicated by arrowhead). Arrows indicate lamellipodia in the control and miR-124-1m transfections. Scale bar = 100 μ m.

Figure 4.

Actin, F-actin, and acetylated α -tubulin levels in miR-124 expressing P19 cells. Transfected cells were cultured and selected as described for Fig. 3. For A–C, proteins were extracted from transfected P19 cells 44 hour after transfection. (A) Western blot analysis of total actin, acetylated α -tubulin and α -tubulin. (B) Quantification of western blot analysis in A. Density of the bands on X-ray films was scanned, quantified by Image J. (C) Quantification of methanol extracted F-actin bound to Alexa-546 conjugated phalloidin. (D) Quantification of western blot analyses for acetylated α -tubulin and α -tubulin in MASH1 transfected P19 cells in which miR-124 function was blocked with 2-O-Me-124-as oligonucleotides. α -tubulin levels are reduced relative to the 2-O-Me-124-sc control. Proteins were extracted from the transfected cells at 48 hr after transfection. Average densities are shown with standard error indicated. * $p < 0.05$, ** $p < 0.01$, Student's t-test.

Figure 5. miR-124 alters Cdc42 levels and Rac1 localization

(A) Western blot analysis of Cdc42, Rac1, and RhoA. (B) Quantification of Cdc42 levels in (A). Bar graph is shown as average with standard error indicated as error bar. ** $p < 0.01$, Student's t-test. (C) miR-124 increases nuclear accumulation of Rac1. Transfected P19 cells were fixed at 44 hr after transfection and processed with antibodies against GFP and Rac1.

Figure 6. Constitutively active forms of Cdc42 or Rac1 inhibit neurite outgrowth promoted by miR-124 in MASH1 transfected P19 cells. Cdc42V12 (CA-Cdc42), Rac1V12 (CA-Rac1), or mt control expression constructs was co-transfected with plasmids expressing miR-124 (124-1), GFP, and MASH1. Transfected cells were allowed to differentiate as described for Fig. 1. P19 cells were transfected with mt or CA-Cdc42 in (A), mt or CA-Rac1 in (B). Cells were fixed at 48hr or 64 hr as indicated after transfection and processed with the TuJ1 antibody.

Figure 7.

Expression of miR-124 increases number of primary neurites extended from cell bodies of cortical neurons and blocking of miR-124 decreases number of primary neurites. Cortical progenitors freshly dissociated from E14.5 cortex were transfected with GFP plasmid and different expression constructs or 2'-O-Me oligos as indicated. Cells were cultured for 43 hr *in vitro* before fixation, cells were processed for indirect immunofluorescence with an antibody to GFP, and neurite outgrowth was analyzed. (A) Cortical neurons transfected with mt, 124-1, and 124-1m. (B) Quantification of primary neurites extended from the transfected neurons. GFP positive neurons were categorized according to number of primary neurites (processes longer than one cell-body diameter) extended directly from their cell bodies. Histograms of 124-1 group and control (mt) group were plotted side by side. miR-124-1 shifted the distribution toward fewer neuritis per cell. Difference of the two distributions is significant according to Wilcoxon's rank-sum test ($p < 0.01$). (C) Histograms of 124-1m group and control

(mt) group. Difference of the two distributions is not significant ($p>0.05$). (D) Primary neurons transfected with 2'-O-Me oligos with either antisense sequences of miR-124 (O-Me-124-as) or control scrambled miR-124 sequence (O-Me-124-sc) (E) Histograms of 2-O-Me-124-as and group and control group. Difference of the two distributions is significant ($p<0.01$).

Figure 8. Two forms of miR-124 differ their ability to regulate the Itgb1 3' UTR

(A) Sequences of two predicted miR-124 binding sites in the Itgb1 3' UTR and complementarity between the predicted sites and miR-124/miR-124-UU21 (see Fig. 3). The additional 5' U for miR-124-UU21 is indicated in lowercase. (B) The 3' UTR of Itgb1 was expressed as the 3' UTR for a luciferase reporter (Itgb1-WT), and the predicted miR-124 target sites in the reporter were mutated individually (Itgb1-s1m and s2m) or together (Itgb1-s12m). The altered sequences in the Itgb1 s1m and s2m mutations are indicated above the sites in A. Normalized luciferase reporter activity shows that the 3' UTR of Itgb1 was inhibited by cotransfected miR-124 RNA duplex, but not a control siRNA duplex (XASH3). Mutation of either predicted binding site partially reduced miR-124 inhibition, while inhibition of both sites reduced inhibition further. (C) While cotransfected miR-124 inhibited the luciferase activity of Itgb1-WT, but miR-124-UU21 did not. Luciferase activities were assayed 22 hr after

transfection. Average of three assays is shown, with standard error indicated. ** $P < 0.01$, Student's t-test.

A **B**

<u>Enriched GO cellular components</u>	<u># of genes</u>	<u>P-value</u>
<i>downregulated by miR-124a:</i>		
cytoskeleton	19	3.52e-3
actin cytoskeleton	10	4.80e-4
actin filament	3	4.60e-3
myosin complex	4	3.64e-3
clathrin-coated vesicle	6	5.01e-3
trans-Golgi network transport vesicle	3	7.03e-3
cell junction	9	6.37e-4
intercellular junction	7	1.62e-3
tight junction	4	9.30e-3
basolateral plasma membrane	5	4.72e-3
<i>downregulated by miR-124a-UU21:</i>		
Cell-matrix junction	3	9.93e-3
Cell-substrate adherens junction	3	8.58e-3
Focal adhesion	3	7.35e-3

Figure 9.

Microarray analysis was used to identify mRNAs downregulated in P19 cells transfected with miR-124 or miR-124-UU21 synthetic RNA duplexes (see Fig. 2). (A) Venn diagram with number of mRNAs downregulated by miR-124, miR124a-UU21, or both miRNAs, compared to control siRNA transfections. A detection probability with $P < 0.01$ in the control RNA samples and decreased expression with $P < 0.01$ was used as the threshold to identify downregulated mRNAs. Most regulated mRNAs are downregulated by only one of the two miRNAs. (B) Categories of GO cellular components enriched ($P < 0.01$) among genes downregulated by miR-124 or miR-124-UU21 include components of the cytoskeleton.