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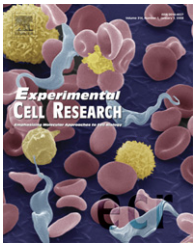

Research Article

MicroRNA miR-124 regulates neurite outgrowth during neuronal differentiation

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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

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miRNAs are involved in multiple biological pathways in a variety of animals. In *Caenorhabditis elegans* and *Drosophila melanogaster*, miRNAs have been shown to affect a number of biological processes including 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-cβgal, 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: GACTCGAGGGATTTCCCAGATTCTCGCTG. miR-124-3 (795nt fragment); forward-EcoR1: GAGAATTCTGCACCCGTCAGAAGACTG; reverse-Xba1: GATCTAGAAAGCTGCACACTTCCCTCC.

The miR-124-1m mutant expression vector was constructed by PCR with the 124-1m1R primer: ACCCAAGGTGCTCAGACAGCCCCATTCT**TGGCATTCACCGCGgctagcAA**TTGTATGGAC. 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.