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Mouse microRNA-124 regulates the expression of *Hes1* in P19 cells

Chong Wang[1,2], Nan Yao[1,2], Cai-Ling Lu[2], Dan Li[1,2], Xu Ma[1,2]

[1]Graduate School of Peking Union Medical College, Number 5 Dong Dan San Tiao, Beijing 100005, China, [2]Department of Genetics, National Research Institute for Family Planning, 12 Da Hui Si, Beijing 100081, China

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1. ABSTRACT

MicroRNAs (miRNAs) belong to a conserved class of small non-coding RNAs that are typically 18–25 nucleotides long. They are found in both animals and plants. These small RNAs can regulate gene expression at translational level by interacting with their target messenger RNAs, and they play an essential role in the development of plants and animals. To date, more than 200 miRNAs have been identified in mammals; however, their mRNA targets have not yet been identified. In this study, we demonstrate that the expression of *Hes-1*, which is a basic helix-loop-helix transcriptional repressor, is regulated by miRNA-124 in P19 cells. Reduction in the levels of miR-124 mediated by locked nucleic acids resulted in the accumulation of Hes-1 and hindered the retinoic acid-induced neuronal differentiation of P19 cells. Thus, our results indicate that miR-124 regulates the expression of *Hes-1* at the post-transcriptional level and is involved in the retinoic acid-induced neuronal differentiation of P19 cells.

2. INTRODUCTION

Non-coding RNAs, including ribosomal RNA, small nuclear RNA, small nucleolar RNA, and transfer RNA play essential roles in a wide variety of processes such as chromosome maintenance, gene imprinting, transcriptional regulation, pre-mRNA splicing, and the regulation of messenger RNA (mRNA) translation. Micro RNAs (miRNAs) are an extensive class of non-coding RNAs that are 18–25 nucleotides long; they are involved in the post-transcriptional regulation of mRNA (1–6). Thus far, a large number of miRNAs have been identified in animals and plants. The enzymatic processes involved in the biogenesis of miRNAs are highly conserved in plants and animals. The biogenesis of miRNAs includes the following steps: (1) initially, a long RNA strand is transcribed; (2) this strand is processed into an miRNA hairpin precursor (about 70–75 nucleotides long); (3) this precursor is then transported to the cytoplasm; and (4) finally, the RNase III enzyme dicer cleaves the precursor to

yield an ~22 nucleotides long stable miRNA, which is derived from 1 arm of the hairpin precursor (7).

The biological functions of many miRNAs have been identified in plants and animals (8). The most apparent difference between the miRNAs derived from plants and those derived from animals is the mechanism by which they interfere with target genes. It is generally considered that animal miRNAs recognize target mRNAs via incomplete base-pairing, thereby resulting in the translational inhibition of the target. Alternatively, mature miRNAs are incorporated into ribonucleoprotein complexes (miRNPs), which function in RNA interference (RNAi)-mediated gene silencing (9, 10). This miRNA–miRNP complex represses target mRNAs by binding to their partially complementary sequences in the 3′ untranslated regions (UTRs) (11, 12). Such miRNA complexes, like small interfering RNA (siRNA), can induce the cleavage and degradation of mRNAs. Thus, miRNAs have 2 functions—to repress mRNA translation and induce cleavage of mRNAs.

In mammals, certain miRNAs have been found to have specialized functions such as maintenance of cells in the pluripotent state during early embryogenesis (13); moreover, another study suggests that miRNAs might also be involved in tissue-specific or organ-specific development (14). Thus far, hundreds of miRNAs have been cloned from mouse and human organs and cell lines (15, 16) and the presence of numerous other miRNAs have been predicted on the basis of computational algorithms (17). Researchers have begun to explore the possible roles of these numerous miRNAs in mammalian development; for example, they have identified a small subset of brain-expressed miRNAs that was induced in all-trans retinoic acid (RA)-treated mouse and human embryonal carcinoma (EC) cells. The induction of this subset of miRNAs closely coincided with the time of terminal neuronal differentiation. The fact that the expression of these miRNAs is evolutionarily conserved suggests that they may play a role in the specification and/or progression of the neuronal lineage.

P19 cells derived from mouse embryos have been extensively used as a model system for investigating *in vitro* differentiation. It has been observed that P19 cells cultured in a medium containing RA differentiate into neurons and glia cells (18). It was observed that numerous genes in these cells are activated in response to RA treatment, including genes encoding several neural basic helix-loop-helix (bHLH) proteins such as Mash1 and NeuroD (19–22). In addition, P19 cells at key differentiation stages have been collected and their total RNA analyzed by northern blotting. Remarkably, 19 miRNAs were induced in the RA-treated P19 cell line. Of these 19 miRNAs, 4 were brain-specific (miR-9, –124a, –124b, and –135), 4 were brain-enriched (miR-9*, –125a, –125b, and –128), and 11 were brain-nonenriched (let-7a, let-7b, miR-30a, –30b, –30c, –30e, –98, –100, 103-1, –156, and –218) (23). At day 4 after RA treatment, the expression of all these 19 miRNAs was induced in P19 cells, and thereafter, progressive accumulation of these miRNAs till they reached higher levels was observed. It was concluded that these miRNAs could collaborate with or fine-tune the regulation mediated by neurogenic proteins that specify neuronal identity. These data strongly suggest that the role of these miRNAs in the specification and/or progression of neuronal fate is conserved in mammals.

In our study, we investigated which gene is regulated by miR-124 at post-transcriptional level during the RA-induced neuronal differentiation of P19 cells and attempted to explain how miR-124 contributes in the regulation of neuronal differentiation. We performed loss-of-function experiments for this miRNA in the P19 cells in order to understand their regulatory contribution during neuronal differentiation.

3. MATERIALS AND METHODS

3.1. Reagents and drugs

Retinoic acid was purchased from Sigma-Aldrich and dissolved in dimethyl sulfoxide (DMSO, Sigma). Alpha medium, Dulbecco's minimal essential medium (DMEM), fetal bovine serum (FBS), horse serum, and penicillin-streptomycin were purchased from Hyclone.

3.2. Retinoic acid preparation

RA stock was prepared in DMSO at a concentration of 5×10^{-4} M. The stock solution was directly diluted with the culture medium in order to obtain the desired concentration, which was 2.5×10^{-6}M in our experiments.

3.3. Cell culture and cell differentiation

P19 cells were purchased from Peking Union Medical College and cultured in alpha medium supplemented with 10% FBS, 2 mM L-glutamine and 100 U/ml of penicillin-streptomycin; this culture medium was maintained at 37°C in a humidified atmosphere containing 5% CO_2. In order to detach the P19 cells from the tissue culture surface, they were treated with phosphate-buffered saline (PBS) containing 0.25% trypsin and 1 mM EDTA. The cells were plated at a density of 10^6 cells/ml into 25-cm^2 cell culture dishes containing RA-treated medium in order to promote cell aggregation. The medium was replaced with fresh medium after 4 days. The aggregates were plated on tissue culture dishes at day 5 and cultured in DMEM containing 5% horse serum and N2 supplement.

3.4. Transfections

For miRNA knock-down studies, P19 cells treated with RA for 4 days were grown in DMEM containing 5% horse serum and N2 supplement. After 12 hours, cells were transfected with locked nucleic acids (LNAs) by using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instructions. All the transfections were independently repeated at least 3 times. The final amount of LNA used for the transfections was 100 nM. Each reaction required 20 µl of Lipofectamine™ 2000. Cells were harvested 3 days after transfection for subsequent analyses. The sequences of the oligonucleotides used in this study are as follows: LNA–miR-124, 5′-gGcaTtcACCgCgtGccTta-3′; LNA–miR-9, 5′-tcAtaCagCtAgAtAacCaaAga-3′ (the capital

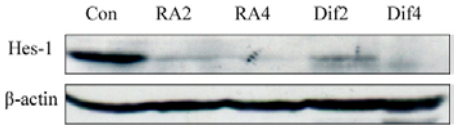

Figure 1. Expression of Hes-1 at different time-points during the differentiation of P19 cells, as revealed by immunoblotting analysis. Con: normal P19 cells treated without retinoic acid (RA); RA2: 2 days after RA treatment; RA4: 4 days after RA treatment; Dif2: 2 days after induction of cell differentiation; Dif4: 4 days after induction of cell differentiation.

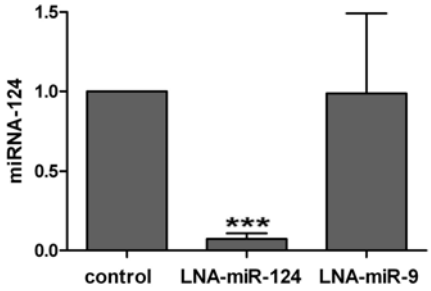

Figure 2. Quantitative RT-PCR performed with miRNA-specific Taqman® probes, for measuring the expression levels of miR-124 in P19 cells. Con: 4 days after induction of cell differentiation; LNA–miR-124: 3 days after transfection with LNA–miR-124; LNA–miR-9: 3 days after transfection with LNA–miR-9. *t*-tests were performed to analyze the difference between the different groups. Each measurement was obtained in triplicate. ***, $p < 0.001$.

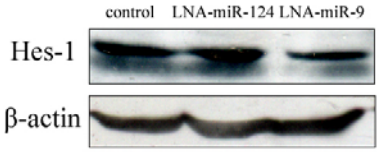

Figure 3. P19 cells were transiently transfected with LNA–miR-124 or LNA–miR-9. Whole cell extracts were harvested 3 days after transfection and assayed for Hes-1 protein expression by western blotting. Beta-actin (Santa Cruz) was used as the loading control.

letters indicate LNAs). LNA–miR-9 was used as a scramble control.

3.5. Total RNA isolation and miRNA expression assays

Total RNA was isolated by using TriZol (Invitrogen), as per the manufacturer's instructions. The purity and concentration of the RNA was determined by measuring its absorbance at 260 and 280 nm. Reverse transcription of the RNA was performed by using specific miRNA stem-loop primers and Taqman® miRNA reverse transcription (RT) kit (Applied Biosystems, Australia). Expression of mature miRNAs was measured by performing Taqman® microRNA assays (Applied Biosystems, Australia) according to the manufacturer's instructions. The expression level of the miRNAs was normalized against the expression level of U6 small nuclear RNA.

3.6. Semi-quantitative reverse transcription-polymerase chain reaction for the detection of mRNA expression

cDNA was synthesized from 1 μg of total RNA in a 20 μl reaction mixture with an RT kit (Takara) as follows: 30°C for 10 min, 42°C for 1 h, 99°C for 5 min, and 4°C for 5 min. Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed on the basis of the amounts of templates available. Each PCR reaction involved a 5-min initial denaturation step at 94°C, which was followed by the following amplification cycles (30 cycles for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 30 cycles for enhancer of Split homologue-1 (Hes-1)) at 94°C for 30 s, annealing at 55°C for 30 s, and final extension at 72°C for 1 min. The primers used were as follows: GAPDH-forward, 5′-TGAAGGTCGGTGTGAACGGATTTGGC-3′; GAPDH-reverse, 5′-CATGTAGGCCATGAGGTCCACCAC-3′; Hes-1-forward, 5′-ACACCGGACAAACCAAAGAC-3′; Hes-1-reverse, 5′-ATGCCGGGAGCTATCTTTCT3′.

3.7. Protein extraction and western blotting

In order to detect the levels of Hes-1 in the cells, whole-cell lysates were prepared in a radioimmunoprecipitation assay (RIPA) lysis buffer, and the resultant extracts were assayed by western blotting. The proteins in the extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Immunoblotting was performed with the rat anti-Hes-1 antibody (MBL). The expression level of β-actin (Santa Cruz) was used as the loading control for the western blots. The blots were developed with a chemiluminescent substrate (enhanced chemiluminescence, GE). Primary antibodies were detected with anti-rat horseradish peroxidase (HRP) (ZSGB); this was followed by electrochemiluminescent (ECL) detection.

4. RESULTS

4.1 Down-regulation of Hes-1 during neurogenesis

To confirm whether Hes-1 mRNA might be a target of miR-124, we used mouse P19 cells that differentiate into neural cells after treatment with RA. We first examined the expression of Hes-1 during RA-induced neuronal differentiation of P19 cells by western blot analysis (Figure 1). P19 cells were treated with RA for 4 days. As shown in Figure 1, Hes-1 expression was readily detectable in undifferentiated P19 cells, whereas it was barely detectable in differentiated P19 cells. In contrast, it has been reported that miR-124 was barely detectable in undifferentiated P19 cells but was clearly detectable in differentiated P19 cells (23). These results suggest that the expression of miR-124 might be associated with the differentiation of P19 cells. In addition, it is possible that a relationship exists between miR-124 and Hes-1.

4.2 Regulation of *Hes-1* expression by miR-124

To study the function of miR-124, we attempted to decrease the levels of endogenous miR-124 by using synthetic LNA that targeted mature miR-124. When 100 nM synthetic LNA–miR-124 was introduced into differentiated P19 cells, the intracellular level of mature miR-124 reduced significantly (Figure 2). In contrast, the level of Hes-1 protein increased in the presence of LNA–miR-124 (Figure 3); however, the level of Hes-1 mRNA

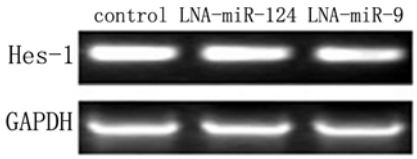

Figure 4. P19 cells were transiently transfected with LNA–miR-124 or LNA–miR-9. Total RNA was harvested 3 days after transfection and assayed for Hes-1 mRNA expression by semi-quantitative RT-PCR. GAPDH was used as the control.

was unchanged (Figure 4). During P19 cell differentiation, since the level of miRNA-9 was observed to be the same as that of miRNA-124, we selected LNA–miR-9 as a control. We observed that in P19 cells, LNA–miR-9 did not affect the expression levels of miR-124 or Hes-1. These results indicate that synthetic LNA–miR-124 interfered with the function of miR-124, resulting in the accumulation of Hes-1 protein. These results strengthen our hypothesis that miR-124 regulates the expression of *Hes-1*.

4.3 *Hes-1* as a target of miR-124 in P19 cells

To verify that *Hes-1* may be a target of miR-124 in P19 cells, we searched the NCBI database for exact homologs of miR-124. We observed that *Hes-1* exhibited 50% complementarity to miR-124. The region that was nearly complementary to miR-124 was located in the coding region of the Hes-1 mRNA.

5. DISCUSSION

The bHLH transcription factors play essential roles in mammalian neurogenesis (24–26). *Hes-1* is one of the bHLH transcriptional factors that are expressed in undifferentiated but not in differentiated cells. In contrast to bHLH genes such as *Mash1* and *NeuroD*, which are considered to positively regulate neuronal development during lineage commitment and post-mitotic differentiation, *Hes-1* (27) negatively regulates the transcription of the bHLH genes; therefore, this gene is required for the negative regulation of neuronal differentiation. It is involved in the Notch signaling pathway in mammals and inhibits neuronal differentiation. *Hes-1* was initially identified as a mammalian homolog of *hairy*, which is a downstream target of the Notch signaling pathway in Drosophila (28).

We already know that the brain-specific miR-124 is induced during P19 cell differentiation. Our aim was to verify whether miR124 could regulate the expression of *Hes-1*. To this end, we used LNA to reduce the levels of miR-124 in the P19 cells. LNA is a family of conformationally locked nucleotide analogues that, amongst other benefits, imparts highly unprecedented affinity and very strong nuclease resistance to DNA and RNA oligonucleotides (29–33). It has been reported that LNAs used in antisense constructs selectively inhibit target RNAs with increased potency *in vitro* and *in vivo* and cause minimal toxicity. Scientists have used LNAs to decrease miRNA levels in chick embryos. In this study, we aimed to use LNAs for complete complementation with miR-124 so that like siRNA, they could degrade mature miR-124. We observed that LNA actually decreased the levels of miR-124. MiRNAs can mediate post-transcriptional gene silencing by either mRNA degradation or translational inhibition. In the latter case, gene silencing can be confirmed by a decrease in the expression levels of only the target protein. In our study, we transfected P19 cells with LNA, and we observed that the Hes-1 protein levels increased; however, its mRNA level was not changed. However, there was a partial sequence similarity between the Hes-1 mRNA and mouse miRNA-124. Thus, we concluded that miR-124 mediated post-transcriptional gene silencing by translational inhibition.

Neurogenesis is a process that consists of complex transcriptional networks involving many transcription factors such as *neuroD*, *hes-1*, *mash-1*, and *math-1*. In addition, many miRNAs play essential roles during neurogenesis; for example, miR-124 regulates neurite outgrowth during neuronal differentiation (34). In the transcriptional network, these miRNAs function to suppress the genes that act as negative regulators during the process of neurogenesis. Before the onset of neurogenesis, *Hes-1* binds to the promoters of brain-specific genes, thereby silencing these genes. The levels of miR-124 increase during neurogenesis, leading to post-transcriptional suppression of *Hes-1* expression; thus, *Hes-1* cannot exert its suppressive effects on neurogenesis.

In our study, we used P19 cells that were committed to terminal differentiation. We concluded that the up-regulation of miR-124 results in the post-transcriptional silencing of *Hes-1*. However, it is unlikely that miR-124 alone is responsible for the complete suppression of *Hes-1* during neuronal differentiation; it may be involved in attenuating its expression during neurogenesis so that neurogenic transcription factors can bind to brain-specific promoters and promote neurogenesis. In conclusion, we demonstrate that the up-regulated expression of miR-124, which is one of the brain-specific miRNAs that are up-regulated during neurogenesis, is required during terminal differentiation of neural cells for the inhibition of the negative regulator of neurogenesis, i.e., Hes-1. These findings provide us with a new method for elucidating the mechanism of cell differentiation and nerve development.

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Abbreviations: UTR: untranslated regions ; RA: all-trans-retinoic acid; EC cells: embryonal carcinoma cells; bHLH, basic helix-loop-helix; LNA, locked nucleic acid; DMSO: dimethyl sulfoxide; DMEM: Dulbecco's minimal essential medium; FBS: fetal bovine serum

Key Words: P19, Hes-1, MiRNA-124, Neurogenesis, Cell Differentiation

Send correspondence to: Xu Ma, Department of Genetics, National Research Institute for Family Planning, 12 Da Hui Si, Beijing 100081, China, Tel:86-10-62176870, Fax: 86-10-62179059, E-mail: genetic@263.net.cn