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# Identification of target genes of microRNAs in retinoic acid-induced neuronal differentiation*

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*Abstract*: MicroRNAs (miRNAs) are phylogenetically widespread, small noncoding RNAs of 18–25 nucleotides in length, and are expressed in animals and plants. These small RNAs can regulate gene expression at the translational level through interactions with their target messenger RNAs, and have a role in the development of *Caenorhabditis elegans*, plants, and mammals. Although more than 200 miRNAs have been found in mammals, it is not easy to identify their targets. We investigated the target genes of miRNAs and analyzed the function of these miRNAs during retinoic acid (RA)-induced neuronal differentiation.

## INTRODUCTION

Noncoding RNAs, including ribosomal RNA, small nuclear RNA, small nucleolar RNA, and transfer RNA, have roles in a great variety of processes such as chromosome maintenance, gene imprinting, transcriptional regulation, pre-mRNA splicing, and the control of mRNA translation [1]. One class of noncoding RNAs, called microRNAs or miRNAs, are small RNAs of 18–25 nucleotides in length that regulate mRNA at the posttranscriptional level [2–5]. To date, a large number of miRNAs have been discovered in animals and plants [2–5]. These miRNAs are expressed as long RNAs, pre-miRNAs, from noncoding regions of the genome referred to as JUNK DNA, and processed to pre-miRNAs of approximately 70 nucleotides by RNase III *Drosha* [6,7]. These pre-miRNAs are transported to the cytoplasm via interaction with exportin 5 and processed by the RNase III enzyme Dicer to produce mature miRNAs [8–12]. Mature miRNAs are incorporated into ribonucleoprotein complexes (miRNPs) containing eIF2C2, which function in RNA interference (RNAi)-mediated gene silencing [13–15].

These miRNA-miRNP complexes repress mRNA translation by partially base-pairing to the 3'-UTR of target mRNAs [2–5]. However, *Arabidopsis thaliana* miR-171 and miR-165/166 are perfectly complementary to the coding regions of two scarecrow-like (SCL) putative transcription factor family member mRNAs, *PHAVOLUTA* (*PHV*) and *PHABULOSA* (*PHB*), respectively [16,17], except for a few mismatches at the 3' end of miR-165/166. These miRNAs can induce cleavage of the mRNAs similar to small interfering (si)RNA-mediated mRNA degradation. Thus, miRNAs have two functions, namely, repressing mRNA translation and mediating cleavage of mRNAs (Fig. 1).

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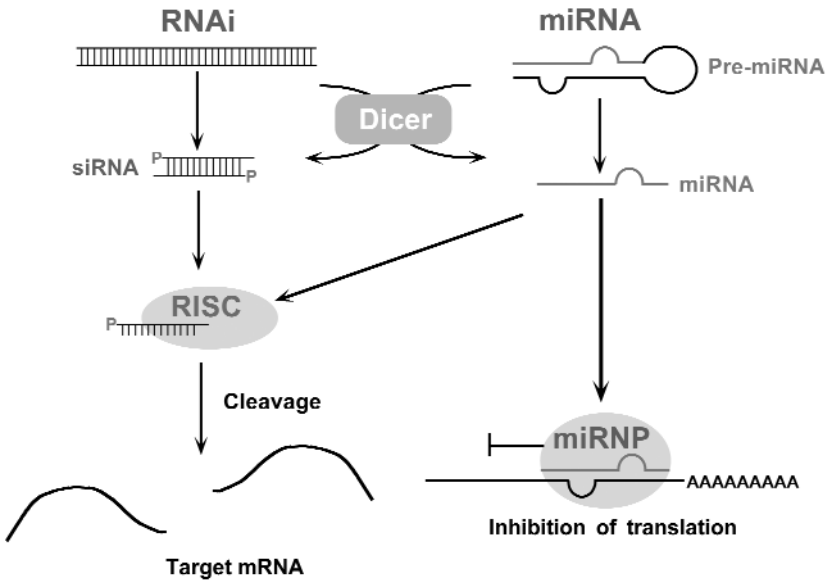


**Fig. 1** Diagram showing siRNA and miRNA action. siRNAs are produced from long dsRNAs by the ribonuclease Dicer. The siRNAs are then incorporated in RISC and the RISC cleaves the target RNAs. miRNAs are expressed as short hairpin RNAs (pre-miRNAs) and transported to the cytoplasm. Pre-miRNAs are processed to mature miRNAs by Dicer. The miRNAs are incorporated in miRNPs and then miRNPs inhibit the translation of target mRNAs by forming partial duplexes with the target mRNAs. In addition, miRNA can function as siRNAs in cases of complete matching with the target mRNAs.

## IDENTIFICATION OF TARGET GENES OF miRNAs IN MAMMALIAN CELLS

To date, more than 200 miRNAs have been identified in diverse mammalian organisms. However, the mechanism and function of these miRNAs are not well understood. In addition, despite a recent report on the prediction of target genes of miRNAs in mammals [18], the real target mRNAs of miRNAs remains unknown. The fact that miRNAs require only a partial homology (in some cases, about 70 %) makes such identification even more difficult. It has been reported that some of the *Drosophila* miRNAs that align to the K box motif (5'-UGUGAU-3') mediate a negative posttranscriptional regulation of the Hairy/enhancer of split (*HES*) gene family in *Drosophila* [19–21]. A human miR-23 containing the antisense sequence to the K box motif has also been identified, although its target gene is unknown.

We initiated a study to investigate whether the human Hairy *HES* gene was the target of human miR-23 [22]. Hairy HES1 [23; Accession No. NM_005524] is a basic helix-loop-helix (bHLH) transcriptional repressor that is expressed in undifferentiated cells, but not in differentiated cells [24]. It participates in the Notch signaling pathway in mammals and acts as an anti-differentiation factor. miR-23 aligned to a coding region of human *HES1* (NM_005524) mRNA near the termination codon and to mouse *Hes1* mRNA (at nearly the same position as in human *HES1* including the stop codon; Fig. 2a). A duplex of *HES1* (NM_005524) mRNA and miR-23 was also observed using a prediction program for mRNA secondary structure (Mulfold), suggesting that miR-23 may be conserved phylogenetically as a regulator of human and mouse Hes1. In addition, we also showed that miR-23 partially base-pairs with another mRNA also called *HES1* [25; human homolog of *Escherichia coli* and *Zebrafish*, Accession No. Y07572] at nearly the same position as in human *HES1* (NM_005524) and mouse *HES1* including the stop codon. Although the function of homolog *HES1* (Y07572) remains unknown, a related protein with the same ElbB domain is involved in an early stage of the biosynthesis of isoprenoid compounds. Although Hairy HES1 (NM_005524) has no similarity to homolog HES1 (Y07572) at the amino acid level, the target sequences for miR-23 in both genes have 70 % similarity at the mRNA level.

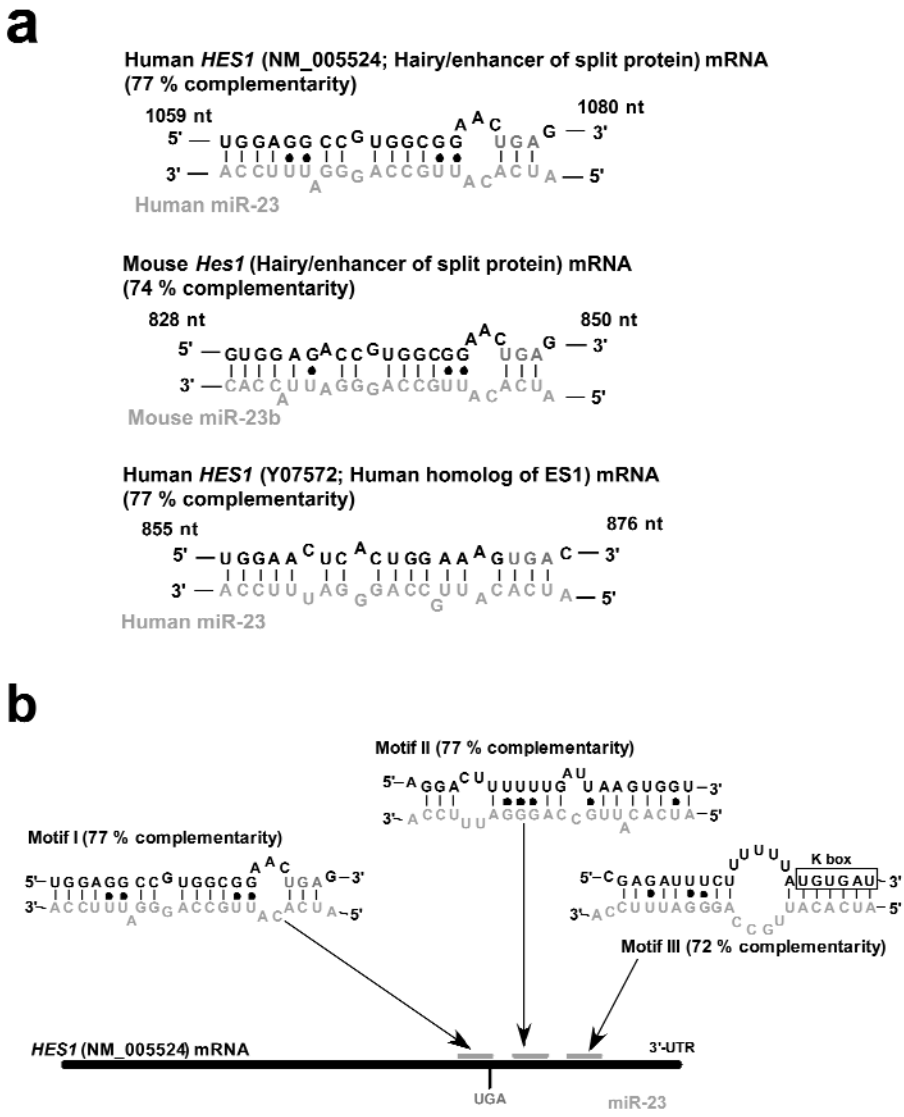


**Fig. 2** HES1 is a target of miR-23. (a) Prediction of secondary structures between miR-23 and its target RNAs. The region nearly complementary to human and mouse miR-23 (blue) is located in the coding region, near the termination codon (red), of human Hairy *HES1* (NM_005524), mouse *Hes1* and human homolog *HES1* (Y07572) mRNAs (top). (b) Human Hairy *HES1* (NM_005524) mRNA has three target regions (motifs I, II, and III) for miR-23 (bottom). Motif III has a K box sequence (black box) that is known, at least in the case of *Drosophila*, to be involved in posttranscriptional negative regulation.

Moreover, we investigated the effect of two additional independent miRNA target motifs (Fig. 2b) that were found in the 3'-untranslated region (UTR) of Hairy *HES1* (NM_005524) mRNA, but did not match the 3'-UTR of homolog *HES1* (Y07572; Fig. 3). Analysis of the activity of a luciferase reporter that was linked to the 5' of the miR-23 target site or the sequences of the other two unique potential target motifs in Hairy *HES1* (NM_005524) mRNA revealed that the three independent target motifs (Fig. 2b) act as targets of miR-23 in *HES1* (NM_005524) mRNA and that each of these sites has only about 72–77 % homology with miR-23. In addition, the activity of the luciferase attached to five copies of the putative target sequence of miR-23 in *HES1* (Y07572) mRNA was regulated by miR-23. Thus,

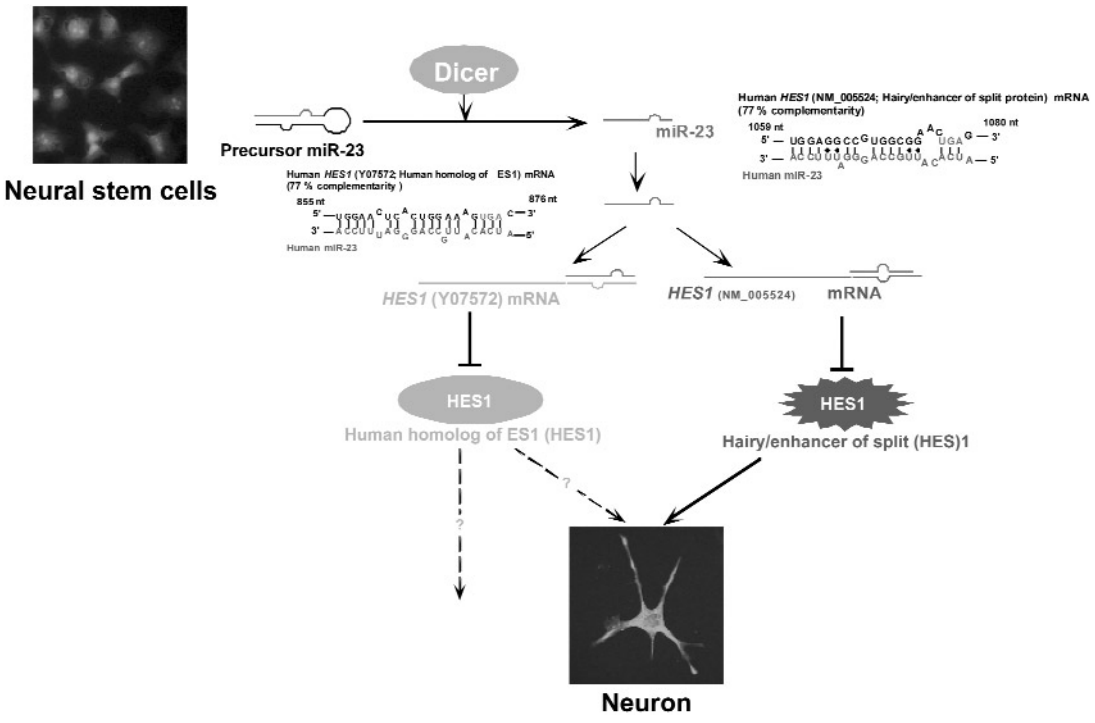


**Fig. 3** A model for the function of miR-23 in RA-induced differentiation. When cells are treated with RA, pre-miR-23 is expressed. The pre-miR-23 is then processed to mature miR-23 by the ribonuclease Dicer. The mature miR-23 negatively regulates the expression of the Hairy *HES1* gene strongly and the homolog *HES1* gene weakly. These events promote neuronal differentiation of NT2 cells.

these results suggested that miR-23 could regulate both *HES1* (NM_005524) and *HES1* (Y07572) gene expression by partially base-pairing to the mRNAs.

## ROLE OF miR-23 DURING RETINOIC ACID-INDUCED NEURONAL DIFFERENTIATION

To investigate the function of miR-23 during retinoic acid (RA)-induced neuronal differentiation, we used human embryonal carcinoma NT2 cells. When subjected to RA-induced differentiation, NT2 cells showed suppression of HES1 expression that was independent of the level of *HES1* (NM_005524) mRNA as detected by northern blotting analysis. On the other hand, miR-23 was easily detected in differentiated NT2 cells in contrast to the undifferentiated cells. Introduction of synthetic miR-23, but not mutant miR-23, into undifferentiated NT2 cells resulted in reduction of the Hairy HES1 protein. Thus, the level of *HES1* (NM_005524) mRNA did not change in response to synthetic miR-23, suggesting that synthetic miR-23 caused gene silencing at the translational level. As expected, targeting of endogenous miR-23 by synthetic siRNA (siRNA-miR-23) resulted in elevation of the Hairy HES1 protein and the mutant siRNA-miR-23 had no effect. These data strongly supported that Hairy HES1 is a target for miR-23 in NT2 cells. NT2 cells did not undergo RA-induced neuronal differentiation (as confirmed by cell morphology and differentiation markers such as MAP2) in the presence of siRNA-miR-23, suggesting that miR-23 plays a critical role during RA-induced neural differentiation.

A model for the function of miR-23 in the regulation of RA-induced neuronal differentiation of NT2 cells is proposed (Fig. 3). When cells are treated with RA, pre-miR-23 is expressed. The pre-miR-23 is then processed to mature miR-23 by the ribonuclease Dicer. The mature miR-23 negatively regulates the expression of the Hairy *HES1* (NM_005524) gene strongly and the homolog *HES1* (Y07572) gene weakly. These events promote neuronal differentiation of NT2 cells. However, the mechanisms for

the expression of pre-miR-23 and the inhibition of translation of both *HES1* mRNAs by miR-23 remain to be addressed in future studies.

## CONCLUSION

miRNAs are endogenous small RNAs that can regulate the expression of genes at the posttranscriptional level. The genes that encode these small RNAs are hidden in noncoding regions of the genome, and it is believed that miRNAs participate in cell differentiation and development in *C. elegans* and plants. We demonstrated that a human miRNA, miR-23, regulates the expression of the transcriptional repressor *HES1* (NM_005524) gene and is necessary for RA-induced neuronal differentiation. In addition, it has recently been reported that miRNAs modulate the hematopoietic lineage differentiation of mouse stem cells [26]. Kuwabara et al. used a randomized ribozyme library to isolate genes involved in neuronal development, and she identified small RNAs that determine the fate of cells [27]. These findings suggest that small RNAs also participate in cell differentiation and development in mammals. Moreover, siRNAs can also induce DNA methylation of a promoter in plants and mammals [28,29]. It is becoming clear that nature has not only structural and functional genes for proteins, but also noncoding functional genes for siRNA and miRNA. An era of investigating unexplored functional RNAs has begun and is predicted to go a long way toward providing information regarding the regulation of biological phenotypes.

## EXPERIMENTAL PROTOCOL

### Culture and transfection of cells

Human NT2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (FBS). Transfections were performed with the Effectin$^{TM}$ reagent (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. Luc-TS23-expressing and Luc-mTS23-expressing NT2 cells were selected by incubation with puromycin for a week. RA was used at 5 μM to be induced neuronal differentiation of NT2 cells for 3 weeks.

### Assay of luciferase activity

miR-23-siRNAs, synthetic miR-23, and mutant miR-23 were introduced into NT2 cells that expressed Luc-TS23 or Luc-mTS23 using Oligofectamin$^{TM}$ (Invitrogen) according to the manufacture protocol. After incubation for 72 h, cells were harvested and lysed. Total protein was assayed for luciferase activity using a luminometer (Lumat LB9501; Berthold, Bad Wildbad, Germany).

### Amplified sandwich enzyme-linked immunosorbent assay (ELISA)

Amplified ELISA has been described elsewhere [30]. NT2 cells, grown in the presence or absence of RA (5 μM, for 3 weeks), were harvested. Total protein was used in this assay. ELISA plates were coated with specific polyclonal antibodies against Hes1 (Santa Cruz, CA, USA). After the plates had been washed three times, biotinylated second antibodies, followed by horseradish peroxidase-conjugated (HRP-conjugated) streptavidin, were added at room temperature. Absorbance was monitored at 490 nm with a microplate reader after addition of phenylenediamine (Sigma-Aldrich Co., MO, USA).

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