

MICRO-RNAS-10a AND -10b CONTRIBUTE TO RETINOIC ACID-INDUCED DIFFERENTIATION OF NEUROBLASTOMA CELLS AND TARGET THE ALTERNATIVE SPLICING REGULATORY FACTOR SFRS1 (SF2/ASF)

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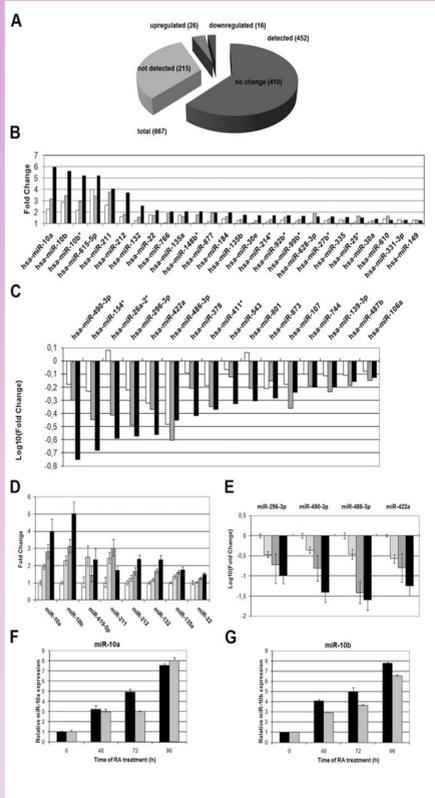
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MicroRNAs (miRNAs) are an emerging class of non-coding endogenous RNAs involved in multiple cellular processes, including cell differentiation. Treatment with Retinoic Acid (RA) results in neural differentiation of neuroblastoma cells. We wanted to elucidate whether miRNAs contribute to the gene expression changes induced by RA in neuroblastoma cells, and whether miRNA regulation is involved in the transduction of the RA signal. We show here that RA treatment of SH-SY5Y neuroblastoma cells results in profound changes in the expression pattern of miRNAs. Up to 42 different miRNA species significantly changed their expression (26 upregulated and 16 downregulated). Among them, the closely related miR-10a and -10b showed the most prominent expression changes. Induction of miR-10a and -10b by RA also could be detected in LA-N-1 neuroblastoma cells. Loss of function experiments demonstrated that miR-10a and -10b are essential mediators of RA-induced neuroblastoma differentiation and of the associated changes in migration, invasion, and *in vivo* metastasis. In addition, we found that the SR-family splicing factor SFRS1 (SF2/ASF) is a target for miR-10a and -10b in HeLa and SH-SY5Y neuroblastoma cells. We show here that changes in miR-10a and -10b expression levels may regulate SFRS1-dependent alternative splicing and translational functions. Taken together, our results give support to the idea that miRNA regulation plays a key role in RA-induced neuroblastoma cell differentiation. The discovery of SFRS1 as direct target of miR-10a and -10b support the emerging functional interaction between two post-transcriptional mechanisms, microRNAs and splicing, in the neuronal differentiation context.

1. miRNA expression profiling during RA-induced differentiation of neuroblastoma SH-SY5Y cells.



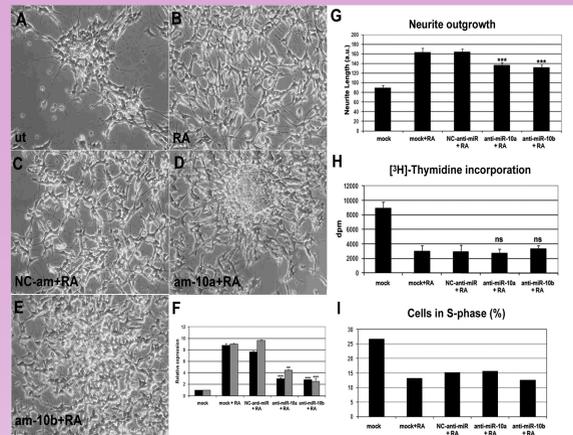
A. Summary of TaqMan microRNA LDA expression profiling results. From 667 miRNAs analyzed, 452 were detected. Among them 26 were upregulated and 16 downregulated by RA treatment.

B and C. Relative expression values detected in TaqMan microRNA LDA for microRNAs with FDR<0.05, for upregulated (B) and downregulated miRNAs (C). The values for 24 (empty bars), 48 (grey bars) and 96 (black bars) h of RA treatment are represented.

D and E. RT-qPCR validation experiments for selected upregulated (D) and downregulated (E) microRNAs. Data were normalized for the expression of U6 small nuclear RNA and the relative expression level to the value of 0 h of RA treatment represented (mean ± SD from the two independent determinations). The values for 0 (empty bars), 24 (light grey bars), 48 (dark grey bars) and 96 (black bars) h of RA treatment are shown.

F and G. Expression levels of miR-10a (F) and -10b (G) during RA treatment of SH-SY5Y and LA-N-1 neuroblastoma cells. The cells were treated with RA for the times indicated in the Figure and the levels of miR-10a and -10b analyzed by RT-qPCR as indicated for Fig. 1 D and E. The graph shows the mean ± SD from two independent determinations. Black bars: SH-SY5Y cells. Grey bars: LA-N-1 cells.

2. Suppression of miR-10a and -10b endogenous expression results in impairment of RA-induced differentiation.

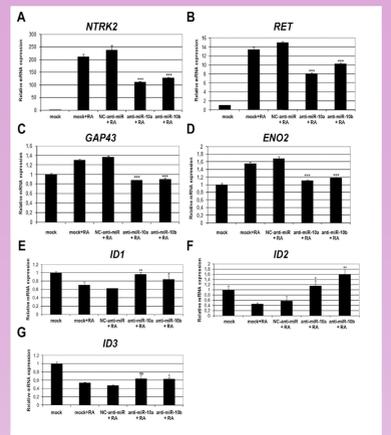


A-E. Morphological differentiation induced by RA was impaired after experimental reduction of miR-10a and -10b levels.

F. RT-qPCR analysis of miR-10a and -10b expression levels after transfection of SH-SY5Y cells with anti-miR-10a, -10b and negative control (NC) anti-miR and subsequent RA treatment for 96 h. For comparison, the values obtained with mock-transfected cells treated with vehicle (mock) or RA (mock+RA) were shown.

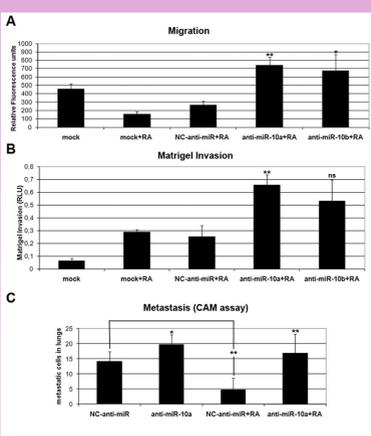
G. miR-10a or -10b knockdown results in reduced RA-induced neurite outgrowth. Quantification of neurite length was performed with Neuron J software on photomicrographs obtained after transfection of SH-SY5Y cells with anti-miR-10a, -10b and negative control (NC) anti-miR and subsequent RA treatment for 96 h. The graph represents the values (mean ± SEM) obtained from at least 100 cells for each condition.

H and I. Suppression of miR-10a and -10b expression did not affected the proliferation arrest induced by RA. G. [³H]-Thymidine incorporation in control mock-transfected SH-SY5Y neuroblastoma cells and after RA treatment for 96 h of mock-transfected cells, or cell transfected with NC-anti-miR, anti-miR-10a or anti-miR-10b. The graph represents the values obtained from triplicate experiments (mean ± SD). H. Effect of RA treatment and miR-10a or -10b knockdown in the fraction of cells at the S-Phase of the cell cycle. The percentage of cells in S-phase was obtained analyzing the DNA content by cytometry after staining with Propidium iodide. The effects of RA treatment in cells transfected with NC-anti-miR, anti-miR-10a or anti-miR-10b were compared with those observed in mock-transfected cells.



A-G. Experimental reduction of miR-10a and -10b levels impaired gene expression changes associated with RA-induced differentiation (A-G). RT-qPCR analysis of the mRNA levels of *NTRK2* (*trkB*, A), *RET* (B), *GAP43* (C), *ENO2* (neuron-specific enolase, D), *ID1* (E), *ID2* (F) and *ID3* (G). mRNA expression levels after RA treatment of SH-SY5Y cells transfected with anti-miR-10a, -10b or negative control (NC) anti-miR.

3. Knockdown of miR-10a and -10b impaired the RA-induced reduction of migratory and metastatic abilities of neuroblastoma SH-SY5Y cells.

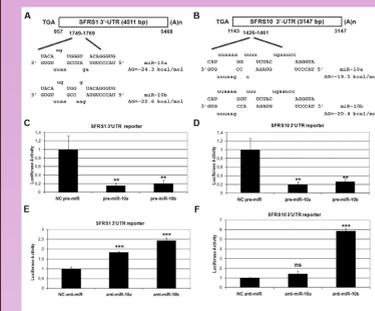


A. Migration. Mock-transfected cells and cells transfected with Negative Control (NC) anti-miR, anti-miR-10a or anti-miR-10b were treated with RA or vehicle for 96 h. Cells from the different treatment groups were labeled with Calcein AM and migration transwell assays were set with Fluoroblock light-opaque inserts. The graph shows a representative experiment performed in triplicate (mean ± SD).

B. Invasion. Mock-transfected cells and cells transfected with Negative Control (NC) anti-miR, anti-miR-10a or anti-miR-10b were treated with RA or vehicle for 96 h. Cells from the different treatment groups were used to set transwell Matrigel invasion assays. The graph shows the average of three experiments performed in triplicate (mean ± SD).

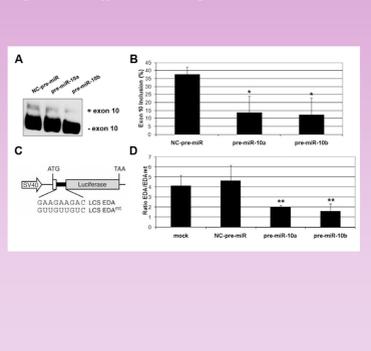
C. Metastasis. Cells transfected with Negative Control (NC) anti-miR or anti-miR-10a were treated with RA or vehicle for 96 h. Cells from the different treatment groups were transferred to the upper chorion-allantoic-membrane of 10-day-old chicken embryos and the number of metastasized cells into the lungs evaluated 7 d later. The graph represents the values obtained from six parallel assays (mean ± SD).

4. Identification and validation of splicing regulatory factor SFRS1 (SF2/ASF) as direct target of miR-10a and -10b in neuroblastoma cells.



A-F. Location of the putative miR-10a and -10b target sites in SFRS1 (A) and SFRS1 3'UTR (B). Unpaired bases are indicated in lowercase above and below the duplex. The predicted ΔG value for this structure is calculated by mFold analysis. C and D. Effects of miR-10a and -10b overexpression on the activity of SFRS1-3'UTR (C) and SFRS1-3'UTR (D) Luciferase reporter constructs in HeLa cells. HeLa cells were co-transfected with pre-miR-10a and -10b or negative control pre-miR. Values (mean ± SD) are represented as relative light units obtained from three independent experiments performed in triplicate. E and F. Effects of miR-10a and -10b silencing on the activity of SFRS1-3'UTR (E) and SFRS1-3'UTR (F) Luciferase reporter constructs in HeLa cells. HeLa cells were co-transfected with anti-miR-10a and -10b or negative control anti-miR. Values (mean ± SD) are represented as relative light units obtained from an experiment performed in triplicate.

5. Experimental alteration of miR-10a and -10b expression levels impaired the functions of SFRS1 in the regulation of alternative splicing and specific mRNA translation.

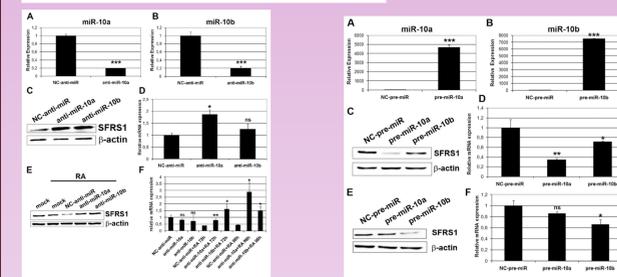


A. Alternative splicing of *tau* protein exon 10 is altered by transfection of anti-miR-10a and -10b. RT-PCR was performed on RNA extracted from anti-miR-10a, -10b or negative control (NC) anti-miR transfected SH-SY5Y cells. *tau* Exon 10 flanking primers were used in RT-PCR reaction.

B. Quantification of the percentage of exon 10 inclusion. The graph shows the value (mean ± SD) from 3 independent experiments.

C. Schematic representation of the reporter genes used for the analysis of mRNA translation regulation.

D. The effect of the alteration of miR-10a and -10b levels on the translation of the LCS-EDA luciferase reporters. Neuroblastoma cells were transfected in parallel reactions with either the LCS-EDA or the LCS-EDA^{mi} Luciferase reporters and the corresponding pre-miR-10a, -10b or NC-pre-miR, together with a promoterless Renilla Luciferase reporter as internal control. After transfection, luciferase activities were determined and the ratio between the normalized activities of the LCS-EDA and the LCS-EDA^{mi} reporters is represented in the graph for the different transfection groups (mean ± SD of two triplicate experiments).



A-F. miR-10a/-10b knockdown leads to increased SFRS1 protein and mRNA levels in HeLa and SH-SY5Y cells. A and B. RT-qPCR analysis of miR-10a (A) and -10b (B) endogenous expression levels after transfection of HeLa cells with anti-miR-10a, and -10b as compared to negative control (NC) anti-miR. C. Western blot of SFRS1 protein expression after anti-miR-10a, -10b and NC-anti-miR transfection of HeLa cells. D. RT-qPCR analysis of SFRS1 mRNA levels in the same conditions. The graph shows expression levels relative to that of cells transfected with NC-anti-miR (mean ± SD of a triplicate experiment). E. Western blot of SFRS1 protein expression after anti-miR-10a, -10b and negative control NC-anti-miR transfection of SH-SY5Y cells followed by RA treatment. F. RT-qPCR analysis of SFRS1 mRNA levels in same conditions. The graph shows expression levels relative to that of RA untreated, NC-anti-miR transfected cells (mean ± SD of a triplicate experiment).

A-F miR-10a and -10b overexpression leads to a reduction of SFRS1 protein expression in HeLa and SH-SY5Y cells through a decrease of its mRNA levels. A and B. RT-qPCR analysis of miR-10a (A) and -10b (B) endogenous levels after transfection of HeLa cells with pre-miR-10a, -10b as compared to negative control (NC) pre-miR. C. Western blot analysis of SFRS1 protein expression in HeLa cells after transfection of negative control (NC) pre-miR, pre-miR-10a, or pre-miR-10b. D. RT-qPCR analysis of SFRS1 mRNA expression in same conditions. The graph shows expression levels relative to that of cells transfected with NC-pre-miR (mean ± SD of a triplicate experiment). E. Western blot of SFRS1 protein expression after pre-miR-10a, -10b and NC-pre-miR transfection of SH-SY5Y cells. F. RT-qPCR analysis of SFRS1 mRNA levels in same conditions. The graph shows expression levels relative to that of RA untreated, NC-pre-miR transfected cells (mean ± SD of a triplicate experiment).