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Original

N-Myc-activated microRNAs Inhibit Protein Synthesis of RUNX1 and RUNX3 in Neuroblastoma Cell Lines

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SUMMARY

RUNX1 and RUNX3 are master transcription factors in sensory neuron lineage specifications. Protein levels of such developmental regulators are tightly controlled during carcinogenesis, in order to block differentiation and drive proliferation. Here we report that neuroblastoma specific microRNAs inhibit protein syntheses of RUNX1 and RUNX3 through 3'UTR sequences. Computational prediction identified two putative binding sequences for N-Myc-activated microRNAs both in *RUNX1* and *RUNX3* 3'UTRs. Streptavidin RNA aptamer-tagged 3'UTR sequences pulled down miR-17, miR-18a, miR-19a, miR-20a or miR-130a from neuroblastoma cell lysate. 3'UTR target protection from N-Myc-activated microRNAs increased protein synthesis of RUNX1 or RUNX3 and induced differentiation in neuroblastoma cell lines. Together, protein levels of RUNX1 and RUNX3 are post-transcriptionally regulated by N-Myc-activated microRNAs, highlighting the mutual negative feedback between N-Myc oncogene and RUNX3 tumor suppressor in neuroblastoma.

Key Words : Neuroblastoma, RUNX transcription factor, N-Myc, microRNA, Streptavidin RNA aptamer

INTRODUCTION

Runt related transcription factors, RUNX1 and RUNX3, are lineage specific developmental regulators in dorsal root ganglion neurogenesis[1~3)]. Although RUNX1 and RUNX3 share an evolutionary conserved DNA recognition domain, functional redundancy is surprisingly low, suggesting the spatio-temporal gene regulations by transcriptional and post-transcriptional mechanisms. Previously, we reported that protein levels of RUNX1 and RUNX3 were critical for neuroblastoma cell growth[4)]. Neuroblastoma is a heterogeneous pediatric malignancy, and lower expression of *RUNX3* results in poorer outcome[5)]. Interestingly, ectopic synthesis of RUNX3 induces protein degradation of N-Myc oncogene[5)], suggesting the function of RUNX3 as a tumor suppressor. On the other hand, clinical relevance of RUNX1 in neuroblastoma remains largely unknown. Although mRNA expression of *RUNX1* is elevated during neuroblastoma carcinogenesis[6)], protein level of RUNX1 is maintained very low in neuroblastoma cell lines[4)]. One plausible explanation of this discrepancy is the 3'UTR mediated post-transcriptional regulation through microRNAs[7~9)]. In this study, we investigated the post-transcriptional regulation of *RUNX* genes in neuroblastoma cell lines and discovered that N-Myc-activated microRNAs inhibited protein syntheses of both RUNX1 and RUNX3 through 3' UTR sequences.

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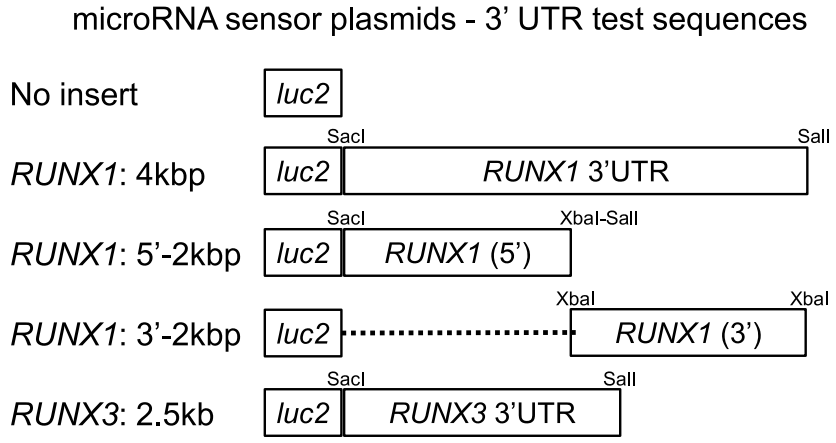

Fig. 1

Schematic diagram of microRNA sensor plasmids. Each 3'UTR test sequence was fused after Firefly luciferase coding sequence (*luc2*). Full length *RUNX1* 3'UTR (4 kbp) was divided into either the former (5') or the latter half (3'). Other plasmid sequences including internal control (Renilla luciferase gene) are not shown. SacI, SalI or XhoI denotes the recognition sequence of restriction enzyme for the plasmid construction.

METHODS

Plasmid construction

All recombinant DNA experiments were appropriately designed according to institutional regulations and the approved protocol was strictly observed. 3' UTR sequences of human *RUNX1* and *RUNX3* were amplified by PCR (PrimeSTAR polymerase, Takara Bio Inc., Shiga, Japan) from genomic DNA of human embryonic fibroblasts (OUMS36, JCRB1006.1, Japanese Collection of Research Bioresources Cell Bank, Osaka, Japan). Primer sequences were : 5'-GGATCT CGCTGTAGGTCA-3' and 5'-CTGTGTACCGTGGAC TGTGG-3' for *RUNX1* 3'UTR or 5'-CTACTGACCGC CCTGGTG-3' and 5'-CCAAGCAAACGATAGTGCA A-3' for *RUNX3* 3'UTR. The amplified products were inserted into TA-cloning vector pMD20 (Takara Bio Inc.) and the cloned fragments were Sanger-sequenced. We confirmed that the cloned fragments were identical to reference *RUNX1* and *RUNX3* 3' UTR, respectively (GRCh37/hg19, UCSC genome browser). 3'UTR sequences were sub-cloned into microRNA sensor plasmids pmirGLO (Promega, Fitchburg, WI, USA) using SacI and SalI recognition motifs (Fig. 1). The 4kbp of *RUNX1* 3'UTR was further divided into 5' or 3' regions using XbaI restriction enzyme (Fig. 1). The plasmid pcDNA3-tRNA scaffold Streptavidin aptamer (tRSA plasmid#32200, Addgene, Cambridge, MA, USA) is a gift from Dr. Hidekazu Iioka. We sub-cloned tRSA sequence into another expression vector pEBMulti-Neo (Wako Pure Chemical Industries, Ltd. Osaka, Japan) using KpnI and XhoI enzymes (affinity tag plasmids). Subsequently, the 5' region of *RUNX1* 3'UTR or the full length *RUNX3* 3'UTR was inserted into SacI-SalI motifs as a bait sequence of a microRNA pull down assay.

Cell culture and plasmid transfection

The sources and culture conditions of neuroblastoma cell lines have been described elsewhere[4]. Plasmid expression vectors were delivered into cells using 4D-Nucleofector system (Lonza, Basel, Switzerland). The optimized electroporation program EH-100 (Cell line kit SF) achieved nearly 80% transfection efficiency (data not shown).

MicroRNA sensor reporter assay

MicroRNA sensor plasmids with distinct 3'UTR sequences (Fig. 1) were delivered into NGP neuroblastoma cell line or HepG2 hepatoblastoma cell line by electroporation. 24 hours after plasmid delivery, Firefly and Renilla luciferase syntheses were quantified with Dual-Luciferase reporter assay (Promega). Luminescent signals were measured by a luminometer

(AB-2280, ATTO, Tokyo, Japan).

MicroRNA pull down and quantitative RT-PCR

Biotin binding RNA aptamer (tRSA)[10,11] was used as an affinity tag to purify microRNAs/3'UTR complexes. tRSA expression plasmids with distinct 3'UTR sequences were delivered into NGP neuroblastoma cell line by electroporation. Cells which incorporated and inherited the episomal plasmids were selected by neomycin resistance cassette (0.8 mg/ml G418, ThermoFisher Scientific, Waltham, MA, USA). 7 days after plasmid delivery, cytoplasmic fractions were gently lysed (Cell Lysis Buffer M, Wako) and insoluble fractions were removed by centrifugation. The soluble fractions were used for tRSA purification with biotin-conjugated magnetic beads (Magnosphere MS300, Takara). Pulled down microRNAs were eluted through one minute's denature at 90℃. Taqman® MicroRNA Assays (ThermoFisher) to quantify specific microRNA are as follows : hsa-miR-17 (AssayID : 002308), hsa-miR-18a (AssayID : 002422), hsa-miR-19a (AssayID : 000395), hsa-miR-20a (AssayID : 000580) and hsa-miR-130a (AssayID : 000454). Each microRNA copy number was quantified using real-time PCR machine (QuantStudio3, ThermoFisher). Log_2 copy number (arbitrary base line) was calculated as 40 minus cycle of threshold.

3'UTR target inhibition and immunoblotting

To inhibit multiple N-Myc regulated microRNAs, we adopted 3'UTR target protection assay (miScript Target Protector, Qiagen, Hilden, Germany). Target protection enables to block the microRNA binding with specific mRNA (i.e. *RUNX1* or *RUNX3*) without affecting other target genes. 3'UTR target sequences selected for the assay are as follows : R1-miR18 (TTATTTTTAATTTTTCCGCA CCTTATCAATTG CAAAATGC), R1-miR20 (TTTACACACATGCAGT AGCA CTTTGGTAAGAGTTAAAGAG), R3-miR19 (AGCTGGGTGGAAACTGCTTT GCACTATCGTTT GCTTGGTG) and R3-miR20 (CAGACCGGCTCCTC CATGCA CTTTACCAGCTCAACGCATC). 200 pmol of target protectors (small RNA) were delivered into the KELLY neuroblastoma cell line by electroporation. 48 hours after RNA delivery, cells were collected and whole cell proteins were solubilized in urea solution (7M urea, 2M thiourea and 4% CHAPS, ThermoFisher). The entangled genomic DNA was sheared by sonication for 15 min (Bioruptor, Diagenode SA, Seraing, Belgium). Procedures for protein quantification and immunoblotting have been described elsewhere[12]. Primary antibodies used in this study are as follows : RUNX1 (#4336, Cell Signaling Technologies, Danvers, MA, USA), RUNX3 (R3-5G4, a gift from Dr. Kosei Ito, also available at MBL, Nagoya, Japan) and GAPDH (#5174, Cell Signaling).

Neuroblastoma differentiation assay

1 mg of pmaxGFP with or without 200 pmol of each target protector were delivered into the SK-N-FI neuroblastoma cell line by electroporation. 72 hours after RNA and plasmid delivery, morphology of GFP positive cells were observed by a fluorescence microscope (BZ-X700, KEYENCE, Osaka, Japan).

Statistical analyses

Mean values of numerical data (relative luciferase activities or Log_2 copy numbers of pulled down microRNAs) were compared using GraphPad PRISM (version 6.0, GraphPad software Inc, La Jolla, CA, USA). We adopted Fisher's least significant difference procedure for multiple comparisons between two groups. Thus, we compared two particular groups with Student's *t*-test contingent upon one way-analysis of variance rejected the null hypothesis (i.e. all the groups are same). P-value less than 0.05 was defined as statistically significant.

RESULTS

3'UTR sequences of RUNX1 and RUNX3 inhibited reporter gene translation

To investigate the post-transcriptional gene regulation of *RUNX1* and *RUNX3*, we constructed microRNA sensor plasmids to detect microRNAs in neuroblastoma cell lines. We compared the degree of reporter inhibition in between NGP and HepG2 cell lines, because the two displayed distinct protein syntheses of RUNX1 and RUNX3 (data not shown). In HepG2 (hepatoblastoma), test sequence insertion modestly inhibited the reporters activity (Fig. 2). However, the inhibition was sequence-independent, since all the different inserts displayed similar degree of inhibi-

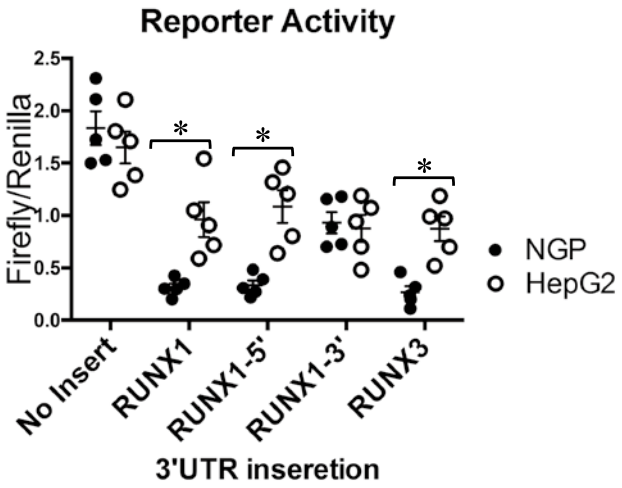

Fig. 2

Neuroblastoma specific microRNAs inhibited reporter activity through 3'UTR of *RUNX1* (5') or *RUNX3*. Each microRNA sensor plasmid was delivered into the NGP neuroblastoma or HepG2 hepatoblastoma cell line by electroporation. Scatter plot indicates the normalized reporter activity (Firefly luciferase/Renilla luciferase). Line summary denotes mean±SEM. Degree of inhibition was measured by comparing between control (no insert) and each 3'UTR insertion. Note that NGP displayed significantly stronger inhibition than those observed in HepG2 and the phenomena depended on *RUNX1* (5') or *RUNX3* 3'UTR. Asterisk indicates statistically significant difference (Student's *t*, $p<0.05$).

tion (Fig. 2). In NGP (neuroblastoma), full length 3' UTR of *RUNX1* or *RUNX3* displayed more remarkable inhibition than those observed in HepG2 (Fig. 2). On the other hand, the deletion of 5' region of *RUNX1* 3'UTR diminished the inhibition up to similar degree as observed in HepG2 (Fig. 2). These data indicated that neuroblastoma specific microRNAs inhibited the reporters activity through 3'UTR sequence of *RUNX1* (5'-2kbp) or *RUNX3*.

Computational analysis predicted bindings of N-Myc-activated microRNAs in RUNX1/RUNX3-3'UTR

Next, microRNA bindings with human *RUNX1* (5'-2 kbp) and *RUNX3* 3'UTR (2.5 kbp) were predicted using mirSVR algorithm (microrna.org)[13]. Among the multiple potential bindings of microRNAs, N-Myc-activated microRNAs were detected in particular sequences. We termed the four sequences as follows (Fig. 3) : R1miR18 (~0.5 kbp of *RUNX1* 3'UTR, predicted to bind to miR-18a/b), R1miR20 (~1.9kbp of *RUNX1* 3'UTR, predicted to bind to either miR-17, miR-20a/b or miR-106a/b), R3miR20 (~0.8 kbp of *RUNX3* 3'UTR, predicted to bind to either miR-17, miR-20a/b or miR-106a/b) and R3miR19 (~2.5kbp of *RUNX3* 3'UTR, predicted to bind to either miR-19a/b or miR-130a/b) (Fig. 3).

Physical bindings between N-Myc-activated microRNAs and RUNX1/RUNX3-3'UTR

To validate the *in silico* prediction of microRNA bindings, we performed a microRNA pull down assay using 3'UTR sequences as biochemical probes. We compared enrichments of microRNAs (miR-17, miR-18a, miR-19a, miR-20a and miR-130a) by comparing between tRSA affinity tag (no bait) and tRSA-3'UTR (*RUNX1* or *RUNX3*, Fig. 4). 5' region of *RUNX1* 3' UTR significantly enriched miR-17, miR-18a and miR-20a (Fig. 4). Meanwhile, full length *RUNX3* 3' UTR enriched miR-17, miR-19a, miR-20a and miR-130a (Fig. 4). Data indicates that the 3'UTR sequence of *RUNX1* or *RUNX3* is sufficient to bind to N-Myc-activated microRNAs in a neuroblastoma cell line.

Target protection of N-Myc-activated microRNAs induced protein synthesis of RUNX1 or RUNX3

To assess the impact of N-Myc-activated microRNAs on *RUNX1* or *RUNX3*, we utilized an oligonucleotide inhibitor, 3'UTR target protector. One specific target protector enables to block all the potential bindings of microRNAs around the target sequence, without affecting the other target genes. Transfection of anti-R1miR18 (αR1miR18) or αR1miR20 increased protein synthesis of RUNX1 in a neuroblastoma cell line, but the effect against RUNX3 or GAPDH was negligible (Fig. 5). Combination of αR1miR18 and αR1miR20 did not display further additive increase of RUNX1 protein (Fig. 5). Meanwhile, transfection of αR3miR19 or αR3miR20 increased protein synthesis of RUNX3, and the effect against RUNX1 or GAPDH was negligible (Fig. 5). Combination of αR3miR19 and αR3miR20 displayed an additive increase of RUNX3 protein (Fig. 5). These data indicate, that the 3'UTR sequences, especially around R1miR18, R1miR20, R3miR19 or R3miR20, are necessary for inhibition of protein synthesis in a neuroblastoma cell line.

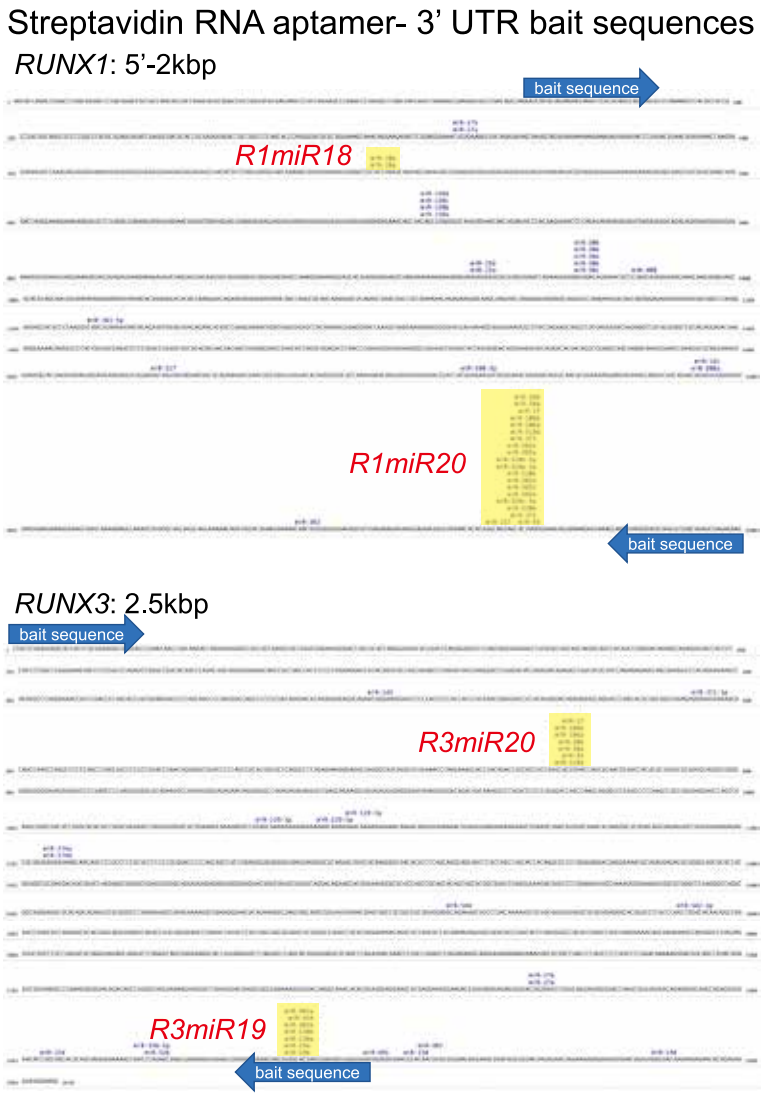

Fig. 3

mirSVR algorithm predicted bindings of N-Myc-activated microRNAs to *RUNX1* and *RUNX3*-3'UTR. Putative microRNA binding sites to *RUNX1* (5') or *RUNX3* 3'UTR are shown. Blue arrows indicate the upper and lower limits of 3'UTR bait sequence, which was fused with a streptavidin RNA aptamer for a pull down assay. Yellow highlight indicates the binding sites for N-Myc-activated microRNAs, termed as R1miR18, R1miR20, R3miR19 or R3miR20.

Target protection of N-Myc-activated microRNAs arrested cell proliferation and induced differentiation of a neuroblastoma cell line

Some neuroblastoma cell line induces neuronal differentiation by ectopic synthesis of RUNX1 or RUNX3 protein[4]. We addressed the possibility whether the 3' UTR target protector could induce the neuronal differentiation. Consistent with the above observations (Fig. 5), transfection of αR1miR18, αR1miR20, αR3miR19 or αR3miR20 halted cell proliferation of SK-N-FI cell line and induced neurite extensions (Fig. 6). These results indicate that the 3'UTR sequences (around R1miR18, R1miR20, R3miR19 or R3miR20) are necessary for continual proliferation and block of differentiation in a neuroblastoma cell line.

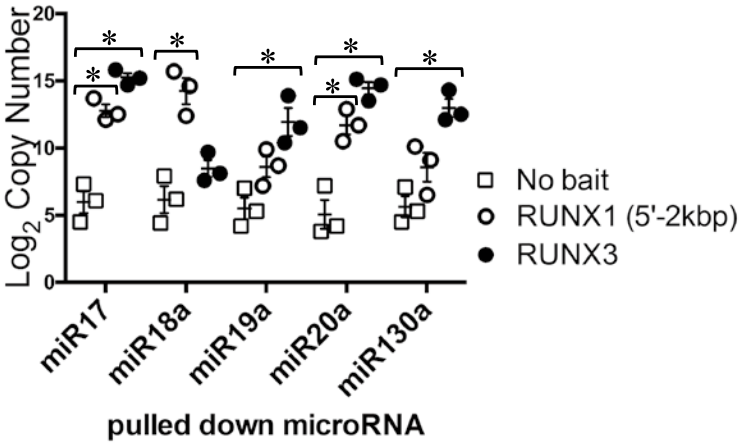

Fig. 4

N-Myc-activated microRNAs physically bind to *RUNX1* or *RUNX3*-3' UTR in a neuroblastoma cell line. Streptavidin RNA aptamer (tRSA)[10,11] expression plasmid with distinct 3'UTR sequences were delivered into the NGP neuroblastoma cell line by electroporation. Copy numbers of biotin-pulled down microRNAs were quantified by real time RT-PCR. Sequence specific binding was measured by comparing between a control (no bait) and each 3'UTR bait sequence. The scatter plot indicates Log_2 copy number (arbitrary base line) for designated microRNA. Line summary denotes mean ± SEM. Asterisk indicates statistically significant difference (Student's *t*, $p<0.05$).

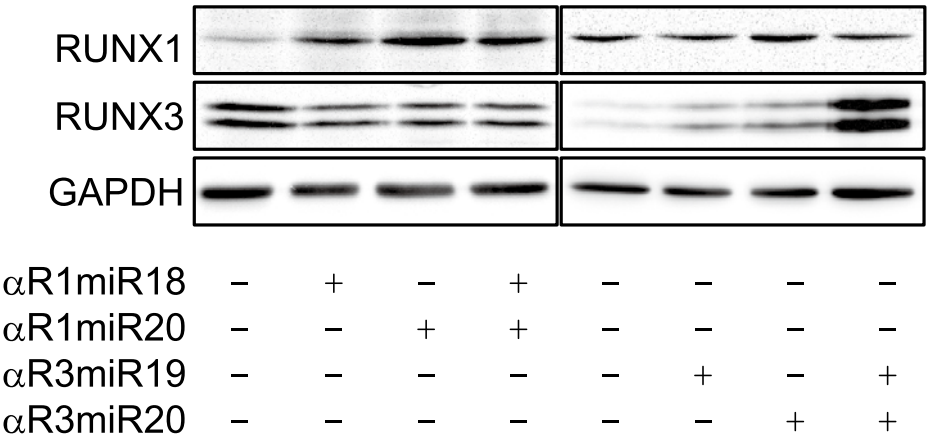

Fig. 5

Target protection of N-Myc-activated microRNAs induced protein synthesis of RUNX1 or RUNX3 in a neuroblastoma cell line. Target protectors (small RNA) were delivered into the KELLY neuroblastoma cell line by electroporation. One specific target protector enables to block all the potential bindings of microRNAs around the target sequence, without affecting the other target genes. 40 mg of whole cell protein lysate was loaded per lane. Immunoblotting against RUNX1 or RUNX3 were performed. GAPDH served as an internal control.

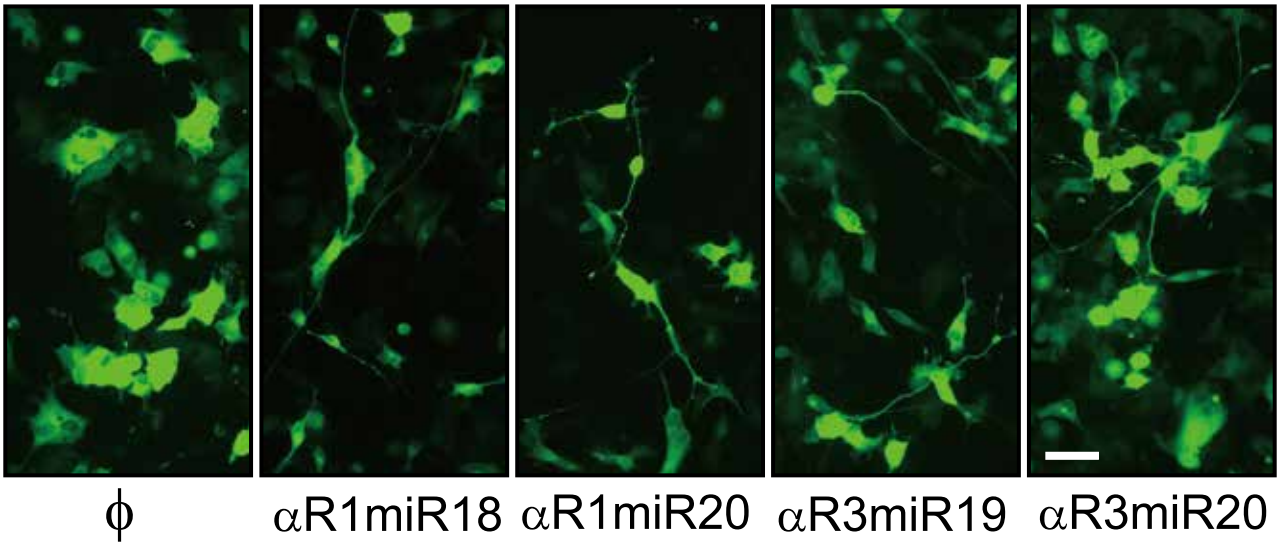

Fig. 6

Target protection of N-Myc-activated microRNAs halted proliferation and induced differentiation in a neuroblastoma cell line. GFP plasmids with or without each target protector were delivered into the SK-N-FI neuroblastoma cell line by electroporation. 3 days later, the cellular morphology was observed by a fluorescence microscope. Scale bar : 200 mm.

DISCUSSION

Post-trancriptional regulation of RUNX1/RUNX3 genes in neuroblastoma cell lines

Unlike established oncogenes and tumor suppressors, roles of *RUNX* genes in cancer are context-dependent and have been debated[14,15]. In neuroblastoma cell lines, functional RUNX1 protein is necessary for cell growth (i.e. RUNX1 could work as an oncogene) but conversely, excess amount of either RUNX1 or RUNX3 is also detrimental (i.e. RUNX1/RUNX3 as tumor suppressors)[4]. Thus, neuroblastoma is actively maintaining the levels of RUNX1 and RUNX3 proteins in a very narrow range for continual cell growth. In this study, we sought to characterize the molecular bases of such tight controls, focusing on the post-transcriptional regulation by microRNAs. One major finding is that N-Myc-activated microRNAs inhibits the protein syntheses of both RUNX1 and RUNX3, through two binding sequences in 3'UTR.

N-Myc oncoprotein and RUNX3 tumor suppressor could be linked through microRNAs

Amplification of *MYCN* locus is one of the first genetic abnormalities to be associated with poor patients' outcome in neuroblastoma[16]. Myc transcription factors globally reprogram cells to drive proliferation through a battery of target genes, including microRNAs[17~20]. In neuroblastoma, comprehensive analyses identified the N-Myc-activated microRNAs as highly expressed non-coding RNAs in unfavorable patients[21,22]. A more integrated approach revealed the framework of regulatory networks : N-Myc binds to microRNA gene promoters and the upregulated microRNAs cooperatively inhibit the target mRNAs[23]. Overall, the target mRNAs have several microRNA binding sequences, suggesting concerted inhibition by multiple N-Myc-activated microRNAs[23]. In this study, we found two potential binding sequences for N-Myc-activated microRNAs both in *RUNX1* and *RUNX3* (Fig. 3). When target protectors αR3miR19 and αR3miR20 were combined, RUNX3 protein synthesis was increased in an additive manner (Fig. 5). On the other hand, such concerted regulations by multiple microRNAs hindered us to pin down relative contributions of one particular microRNA. Although we tried to block each microRNA by an antisense LNA inhibitor (Exiqon, Vedbaek, Denmark), we failed to obtain reproducible data from functional analyses (data not shown). One possible explanation is that one 3'UTR target sequence binds to several microRNAs and the effect of a specific LNA inhibitor could

be compensated by other microRNAs. So far, preceding studies identified Myc-activated microRNAs which bind to 3'UTR of either *RUNX1* or *RUNX3*. For example, Fontana et al. reported that miR-17, miR-20a and miR-106a inhibit AML1 (RUNX1) protein synthesis in hematopoietic progenitors[7] and the binding sequence is the one we termed as R1miR20 (Fig. 3). Another interesting example is that RUNX3 is inhibited by N-Myc activated miR-4295 in glioma[24], of which the binding sequence is overlapping with our R3miR19 (Fig. 3). In addition, accumulating evidence reinforces the regulatory relationship between Myc-activated microRNAs and RUNX1 (miR-9[25~27], miR-18a[28] and miR-181a[29]) or RUNX3 (miR-20a[30], miR-93[31], miR-106a/b[32,33] and miR-130a[34~37]). In the future, functional relationship between N-Myc and RUNX1/3 should be further investigated, given the fact that the opposite regulation (i.e. RUNX3 accelerates protein degradation of N-Myc) was reported in neuroblastoma[5].

CONCLUSIONS

Neuroblastoma specific microRNAs inhibit protein syntheses of RUNX1 and RUNX3 through 3'UTR sequences. Both *RUNX1* and *RUNX3* 3'UTR have two binding sequences for N-Myc-activated microRNAs. Target protection of each sequence increases protein synthesis of either RUNX1 or RUNX3, and induces differentiation in neuroblastoma cell lines.

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