that NFκB-p65 and IKKα are early post-initiation events in human colorectal tissues, perhaps involved in tumor progression. Heterocyclic amine mutagens have been studied extensively,[31-36] but this is the first report to show that PhIP-induced colon tumors have increased nuclear NFκB-p50 and NFκB-p65 expression, and higher levels of IL1β, IL6, TNF-α and TNFR1.

In HT29 cells, LPS-induced phosphorylation of IKK was blocked following Nox1 knockdown. This suggests that NFκB activation induced by cytokines, growth factors, and other external stimuli might be mediated, at least in part, by Nox1. Even in the absence of LPS treatment, however, Nox1 knockdown lowered nuclear NFκB-p50 and NFκB-p65 protein expression, attenuated NFκB-p50 DNA binding activity, and reduced transcription of NFκB downstream targets. We were also interested in the reverse scenario, namely that loss of NFκB might attenuate Nox1 levels. However, NFκB knockdown did not alter Nox1 mRNA or protein expression in human colon cancer cells (data not presented), suggesting that alternative mechanisms regulate Nox1.

Finally, we observed that Nox1 knockdown in HT29 cells resulted in the accumulation of cells in G1, whereas the same knockdown strategy in Caco2 cells strongly induced apoptosis. These cell-specific effects are intriguing and may suggest a causal role in tumorigenesis. However, the findings should be interpreted cautiously, since it is still not entirely clear whether Nox/Duox overexpression is a cause of tumorigenesis, or whether it results from tumor formation. The data showing increased Nox1 expression in colonic mucosa several weeks before colon tumor formation (Supporting Information Fig. 3) suggest a causal role, but additional work is needed to confirm this possibility. Moreover, immunohistochemical analyses of rat colon tumors revealed increased NFκB expression in areas that were either positive or negative for Nox4 (data not presented). This is perhaps not surprising, given that multiple pathways activate NFκB,[2] and further studies appear to be warranted on other Nox/Duox family members in the context of NFκB signaling and colon cancer development. The present report is the first to show that PhIP-induced colon tumors have increased Nox/Duox expression and NFκB activation, but these events are probably not specific to heterocyclic amines, and other colon carcinogens may act in a similar fashion.

In summary, we provide here the first evidence for the involvement of Nox1, Nox4 and NFκB during PhIP-induced colon carcinogenesis, and provide further support for a role of Nox/Duox isoforms in primary human colon cancers and colon cancer cell lines. In cultured human colon cancer cells, Nox1 knockdown blocked LPS-induced phosphorylation of IKK, reduced nuclear NFκB levels and DNA binding activity, and attenuated the transcription of downstream NFκB targets. We conclude that the interplay between Nox/Duox family members and NFκB signaling during colon cancer development is worthy of further investigation.

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Functional screening using a microRNA virus library and microarrays: a new high-throughput assay to identify tumor-suppressive microRNAs

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MicroRNA (miRNA) is a class of non-coding RNAs that represses expression of target messenger RNAs posttranscriptionally. A growing body of evidence supports their roles in various normal cellular processes, as well as in pathological conditions, such as cancer. We established a functional screening assay that enables high-throughput identification of miRNAs that have a role in cancer phenotypes of interest, via the combination of pooled lentivirus vectors expressing several hundred miRNA precursors and a custom-made microarray. Self versus self-hybridization analysis using pooled polymerase chain reaction products generated highly linear and reproducible results. To test the feasibility of the assay, we focused on miRNAs that control proliferation of pancreatic cancer cells and successfully identified five miRNAs that negatively control cell proliferation, including miRNA-34a that was previously identified as a representative tumor-suppressive miRNA. The results were further validated using lentivirus vectors expressing each of the five miRNAs or synthetic miRNAs. The function-based nature of the assay enabled identification of miRNAs that were strongly linked to cell proliferation, but the relative ease and flexibility of the assay allow for future studies of cancer stem cells, metastasis and other cancer phenotypes of interest.

Introduction

MicroRNA (miRNA) is a class of evolutionarily conserved non-coding RNAs of ~19 to 22 nucleotides that modulate expression of their target genes posttranscriptionally together with the RNA-induced silencing complex. Since the first identification of *lin*-4 in *Caenorhabditis elegans*, the number of registered miRNAs is expanding and 940 miRNA genes (1100 mature miRNAs) are annotated in humans (miRBase release 15, http://www.mirbase.org/) and more than a thousand miRNA genes are estimated to be located in the human genome (1). Also, contrary to the 'evolutionarily conserved' definition of miRNAs, species-specific miRNAs have been implicated and cloned, suggesting their critical role in the integrity of higher organisms (2). A growing body of evidence suggests that miRNAs have pivotal roles in normal cellular processes (differentiation, proliferation and cell death) and stress response, as well as implicating their involvement in cancer and other pathological conditions (3,4). Indeed, expression profiling studies of miRNA have shown that miRNAs are aberrantly expressed in a variety of cancers (5). Some miRNAs are consistently upregulated or downregulated in cancers, suggesting their possible tumor-promotive or tumor-suppressive features. Moreover, miRNAs were generally downregulated in tumors compared with normal tissues, implicating a multitude of tumor-suppressive miRNAs that have not been fully recognized (5). In light of these facts, the development of functional assays for miRNAs appears to be warranted in order to better elucidate the mechanisms underlying characteristic features of cancer. Here, we have established a high-throughput functional screening assay in which hundreds of miRNAs are characterized after expression of corresponding miRNA precursors via lentivirus vectors. To test the feasibility of the assay, we screened for miRNAs that suppress proliferation of the pancreatic cancer cell line MIA PaCa-2. Five miRNAs were identified, including *microRNA-34a* (*miR-34a*) that was previously reported as one of the p53-responsive miRNAs, with strong tumor-suppressive activity in various cancers (6). Proliferation-suppressive effects of the five miRNAs identified by the new functional screening assay were individually validated by the infection of lentivirus vectors expressing each miRNA precursors. Cooperative data were obtained following transfection of synthetic miRNAs to MIA PaCa-2 cells; flow cytometry revealed that cell cycle arrest was, at least in part, an underlying mechanism for the observed phenotypic effects.

Abbreviations: CDC42, cell division cycle 42 (GTP-binding protein, 25kDa); gDNA, genomic DNA; copGFP, green fluorescent protein (GFP)-like protein from a copepod; miRNA, microRNA; PCR, polymerase chain reaction; PAK1, p21 protein (Cdc42/Rac)-activated kinase 1.

Materials and methods

Cell culture

MIA PaCa-2 cells and 293T cells (ATCC, Manassas, VA) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 50 U/ml penicillin and 50 μg/ml streptomycin (Invitrogen, Carlsbad, CA). The cells were routinely incubated at 37°C in a humidified atmosphere with 5% CO_2.

Custom-made oligonucleotide microarray

Sequences of miRNA precursors were obtained from Ensembl Genome Browser (Release 55, http://www.ensembl.org) and the UCSC genome browser (hg19, http://genome.ucsc.edu). Oligonucleotide (60mer) probes were designed for 445 miRNA precursors in a Lenti-miR miRNA precursor clone collection (System Biosciences, Mountain View, CA) using eArray software (Agilent Technologies, Santa Clara, CA). All probe sequences were BLAST searched against miRNA precursor sequences using BlastStation2 software (TM Software, Arcadia, CA). The specificity of the probes with the corresponding miRNA precursors was manually checked and probes with poor specificity were replaced with redesigned probes. The custom-made oligonucleotide microarray was designed so that each microarray contains 32 replicates (16 sense and 16 antisense) of specific probes for 445 miRNA precursors on an 8 × 15 k format (Agilent Technologies). Detailed information of the custom-made microarray we designed is available on request.

Infection of lentivirus library into cells and subsequent passages

MIA PaCa-2 cells were seeded at 4×10^5 cells in a six-well dish 1 day prior to viral transduction. A Lenti-miR Virus Library (System Biosciences) and polybrene (hexadimethrine bromide, Sigma–Aldrich, St Louis, MO) at a final concentration of 5 μg/ml were added to the culture medium. Two parallel infections of the Lenti-miR Virus Library (pooled virus library) were performed with a multiplicity of infection of ~3. Cells were incubated at 37°C in a humidified atmosphere for 24 h, after which medium containing the virus library was replaced with Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics. Half of the infected cells were transferred to a 10 cm dish and DNA was extracted from the remaining cells (P0). Afterward, cells were cultured in a 10 cm dish and passaged in the proportion of 1:8 when they reached 80–90% confluency. The cells not used for the passage were used for the extraction of DNA. Throughout the screening process, cells were monitored for green fluorescent protein (GFP)-like protein from a copepod (copGFP) by fluorescence microscopy (IX71 Inverted Microscope; Olympus, Tokyo, Japan).

Functional screening assay using the custom-made microarray

Genomic DNA (gDNA) was extracted with a DNeasy Blood and Tissue Kit (Qiagen, Germantown, MD) according to the manufacturer's instructions. miRNA precursors were recovered from gDNA by polymerase chain

reaction (PCR) with specific primers against lentivirus vectors (forward primer: 5′-GCCTGGAGACGCCATCCACGCTG-3′; reverse primer: 5′-GATGTGCGCTCTGCCCACTGAC-3′). The PCR amplicon is a composite of 445 miRNA precursors; each precursor is composed of the stem–loop sequence (defined in the miRBase, ~100 bp) and the flanking genomic regions (~200 bp upstream and downstream) of the miRNA gene, making the total size of the amplicon ~500 to 700 bp (supplementary Figure S1 is available at *Carcinogenesis* Online). Fifty microliter of PCR reaction contained the following final concentrations: 200 ng of gDNA template, 400 μmol/l each of deoxynucleoside triphosphates, 0.3 μM of each PCR primer and 1 U of KOD FX DNA polymerase (Tokyobo, Osaka, Japan). PCR was performed with the following program on a Veriti thermal cycler (Applied Biosystems, Foster City, CA): 94°C for 3 min, 25 cycles of 94°C for 35 s, 65°C for 35 s, 72°C for 1 min and a final step of 72°C for 7 min. Four independent PCR reactions using the same gDNA template were pooled and purified with the Rapid PCR Purification System (Marligen Biosciences, Rockville, MD). Hundred nanogram of purified PCR product were labeled with either Cy3- or Cy5-deoxycytidine triphosphate using a Genomic DNA Enzymatic Labeling Kit (Agilent Technologies) and purified with a Microcon YM-30 Centrifugal Filter Unit (Millipore, Billerica, MA). A pair of Cy3- and Cy5-labeled DNA was combined and hybridized to the custom-made microarray at 65°C and 20 r.p.m. for 24 h in a hybridization oven (Agilent Technologies). Washing of the microarray and data analysis were performed according to the CGH protocol version5.0 (Agilent Technologies). The log ratio of each miRNA precursor was calculated by averaging the log ratio of replicate probes for each miRNA precursor excluding the highest and the lowest values.

Production of pseudovirus particles

Cells (5×10^6 293T) (ATCC) were seeded in a 10 cm dish 1 day prior to transfection and cotransfected with packaging plasmids (pPACKH1-GAG, pPACKH1-REV and pVSV-G) and a vector plasmid containing each miRNA precursor/copGFP (System Biosciences) using Lipofectamine2000 (Invitrogen) according to the manufacturer's instructions. Cells were cultured at 32°C for 48 h in a humidified atmosphere with 5% CO_2, after which culture medium containing pseudovirus particles was collected and stored at −80°C before infection to MIA PaCa-2 cells.

Cell proliferation assay

For the validation using lentivirus vector expressing miRNA precursors, cells were infected with pseudovirus particles using 5 μg/ml of polybrene for 24 h. Cells (0.5×10^4) were subcultured into a 48-well plate after infection, and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was performed using a CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI) and a 2030 Arvo *X* 3 multilabel plate reader (PerkinElmer, Waltham, MA) at 3 and 5 days after subculturing. Cells infected with pseudovirus that only express copGFP, but no miRNA precursor (lenti-miR-control), were used as a control of the assay. Expression of miRNA in cells transduced with each miRNA clones was measured by quantitative reverse transcription–polymerase chain reaction prior to 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (supplementary method and supplementary Table S2 are available at *Carcinogenesis* Online). For the validation using synthetic mature miRNA, 1×10^4 MIA PaCa-2 cells were subcultured in a 24-well plate and transfected with HiPerfect Transfection Reagent (Qiagen) and 10 nmol/l of Pre-miR miRNA Precursor Molecules (Ambion, Austin, TX). Cells transfected with Pre-miR miRNA Precursor Molecules—Negative Control (Ambion) were used as a control of the assay. Cells were counted every day after transfection up to 5 days using a Countess Automated Cell Counter (Invitrogen) according to the manufacturer's instruction.

Flow cytometric analysis of cell cycle

2×10^5 MIA PaCa-2 cells were plated per 10 cm dish 1 day prior to transfection of synthetic mature miRNAs and incubated at 37°C in a humidified atmosphere with 5% of CO_2 until transfection. Synthetic *miR-29b*, *-34a*, *-222*, *-224*, *-532* and negative control miRNA (Pre-miR, Ambion) were transfected with HiPerfect Transfection Reagent (Qiagen) at a final concentration of 10 nmol/l. Cells were trypsinized and collected at 48 and 72 h after transfection, then fixed with 90% methanol and finally suspended in phosphate-buffered saline(−) with 50 μg/ml of propidium iodide (Sigma–Aldrich), 50 μg/ml of RNase A (Nippon Gene, Tokyo, Japan) and 0.1% fetal bovine serum (Invitrogen). Ten thousand stained cells were analyzed with a FACSCalibur flow cytometer and CellQuest Pro software (BD Biosciences, San Jose, CA) according to the manufacturer's instructions. The proportions of cells in sub-G_1, G_0/G_1, S and G_2/M phase were analyzed with ModFit *LT* software (Verity Software House, Topsham, ME). The averages and standard deviations of cell cycle distributions of MIA PaCa-2 cells transfected with each miRNA were obtained from three biological replicate samples.

Statistical analysis

Pearson product-moment correlation coefficient and standard deviation were calculated using Excel software (Microsoft, Redmond, WA).

Results

Development of a miRNA functional screening assay

To establish a functional screening assay of miRNAs, we employed a pooled lentivirus vector library expressing 445 human miRNA precursors and a custom-made oligonucleotide microarray for the detection of these precursors (Figure 1A). Each lentivirus vector expresses human miRNA precursors together with copGFP, a green fluorescent protein cloned from a copepod, which enables fluorescent microscopic monitoring of cells infected with the vectors (Figure 1B) (7). Although a pooled library is more convenient than individually assessing phenotypic changes of cells infected with each miRNA and is thus suitable for a functional screening assay, it is virtually impossible to screen miRNAs that negatively regulate phenotypes of interest as the number of cells infected with such miRNAs decrease throughout the screening process. We employed a microarray-based approach to the functional screening assay that enables quantification of changes in the copy number of each miRNA precursor to identify miRNAs that negatively as well as positively regulate phenotypes of interest. Briefly, cells infected with a pooled lentivirus vector library undergo phenotypic screening, during which miRNAs that positively or negatively regulate phenotypes of interest are enriched or excluded compared with the original library. miRNAs that generate phenotypes of interest are identified according to the ratio of change in the copy number of each miRNA precursor that is detected by the ratio of differently labeled fluorescent intensity.

In order to assess the linearity of PCR amplification of mixed miRNAs precursors, reproducibility of PCR reactions from two independent experiments was examined (Figure 2A and B). Eight independent PCR amplifications were performed, and two pooled

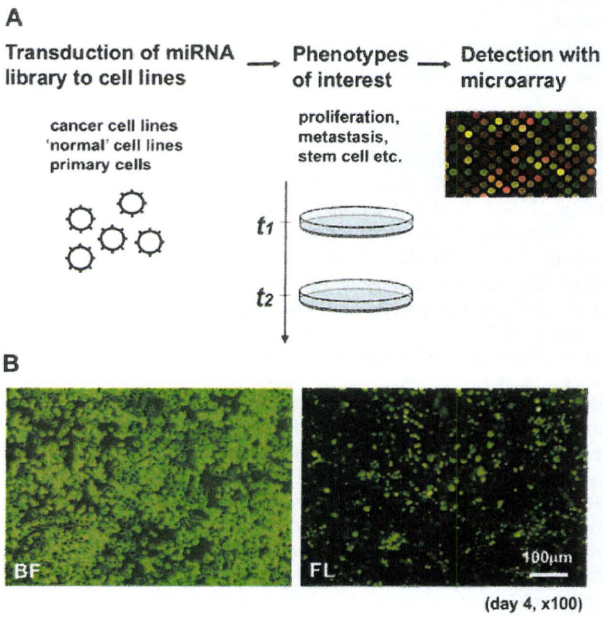

Fig. 1. (**A**) Schematic overview of the functional screening assay of miRNAs. After infection of a pooled lentivirus library (t_1), the phenotypic outcome of each miRNA will be assessed at an appropriate time point (t_2). (**B**) Phase contrast (bright field, BF) and fluorescent (FL) microscopic images of MIA PaCa-2 cells infected with a pooled lentivirus vector library showing copGFP-positive cells.

PCR products, each comprising four PCR reactions (pool I and pool II), were labeled with Cy3- and Cy5-deoxycytidine triphosphate, respectively, and hybridized to the custom-made miRNA oligonucleotide microarray (self versus self-hybridization). We compared the correlation coefficient of pool I and pool II under several PCR conditions, together with the optimized annealing temperature and the number of amplification cycles. A high correlation coefficient for the assay was obtained (R = 0.944, Figure 2B). The correlation coefficient was comparable between PCR products purified by gel electrophoresis or silica membrane (data not shown).

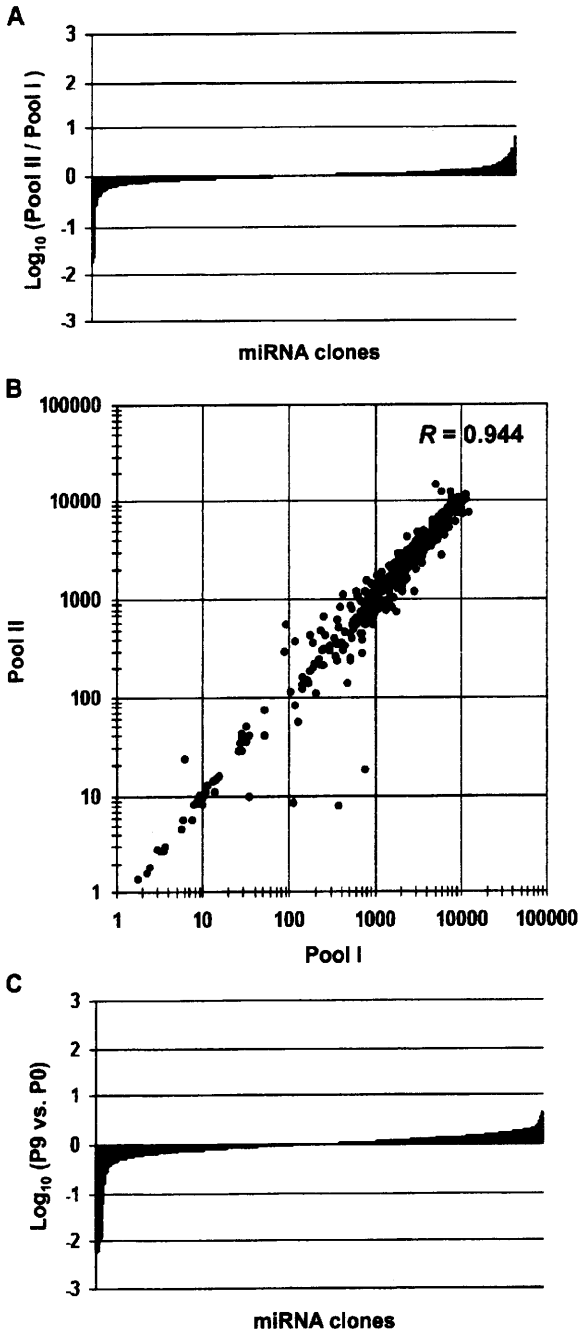

Fig. 2. Distribution of the $\log_{10}$ ratio (**A**) and scatter plot (**B**) of self versus self-microarray hybridization analysis. (**C**) Representative distribution data of the $\log_{10}$ ratio of MIA PaCa-2 cells infected with a pooled lentivirus vector library (P9 versus P0).

Identification of miRNAs that repress proliferation of pancreatic cancer cells

To test the feasibility of the functional screening assay, we focused on the identification of miRNAs that negatively regulate proliferation of human pancreatic cancer cells. MIA PaCa-2 cells were infected with a pooled lentivirus library of miRNA-expressing vectors and passaged several times, during which the expression of miRNA precursors in a majority of cells was confirmed via microscopic inspection of the expression of copGFP (Figure 1B). gDNA was extracted from cells not used for the passage and integrated miRNA precursors were amplified by PCR. Subsequently, amplified miRNA precursors were labeled with Cy3- or Cy5-deoxycytidine triphosphate and hybridized to custom-made microarrays. Any change in the relative proportion of cells expressing each miRNA precursor were measured by comparing labeled miRNAs from cells passaged nine times (P9) and cells immediately after infection (P0) (Figure 2C; supplementary Table S1 is available at *Carcinogenesis* Online). We focused on five miRNAs, namely *miR-29b*, *-34a*, *-222*, *-224* and *-532*, which consistently showed a remarkably low log ratio in two independent screening assays (Table I) and individually validated the proliferation-suppressive phenotypes either using lentivirus vector expressing miRNA precursors, which are components of the pooled virus library used in the functional screening or synthetic mature miRNAs. MIA PaCa-2 cells infected with lentivirus vectors expressing each positive miRNA significantly suppressed cell proliferations (Figure 3A). Moreover, transfection of synthetic *miR-29b*, *-34a*, *-222*, *-224* and *-532* also suppressed proliferation of MIA PaCa-2 cells in comparison with cells transfected with negative control miRNAs. (Figure 3B and C). The fold changes in the expression of the five proliferation-suppressive miRNAs showed variations according to the endogenous expression level of these miRNAs and the titers of the lentivirus vectors transduced (supplementary Table S2 is available at *Carcinogenesis* Online).

Induction of cell cycle arrest after transfection of proliferation-suppressive miRNAs

To gain further insight into the proliferation-suppressive effects of these five miRNAs, flow cytometric analysis was performed using MIA PaCa-2 cells 48 and 72 h after transfection with synthetic miRNAs (Figure 4; supplementary Figure S2 is available at *Carcinogenesis* Online). MIA PaCa-2 cells transfected with *miR-34a*, *-224* and *-532* showed an increased proportion of G_0/G_1 phase in comparison with cells transfected with negative control miRNA, whereas the proportion of G_2/M phase was increased in cells transfected with *miR-222*. No prominent change in cell cycle was observed in MIA PaCa-2 cells transfected with *miR-29b*. A marked increase in the proportion of the sub-G_1 fraction was not observed in MIA PaCa-2 cells transfected with any of the five proliferation-suppressive miRNAs.

Table I. Top five ranked miRNAs that suppress proliferation of MIA PaCa-2 cells

miRNA clones	Mean $\log_{10}$ ratio of two independent screenings (P9 versus P0)
miR-532	−1.668
miR-224	−1.639
miR-29b	−1.211
miR-34a	−1.153
miR-222	−0.970

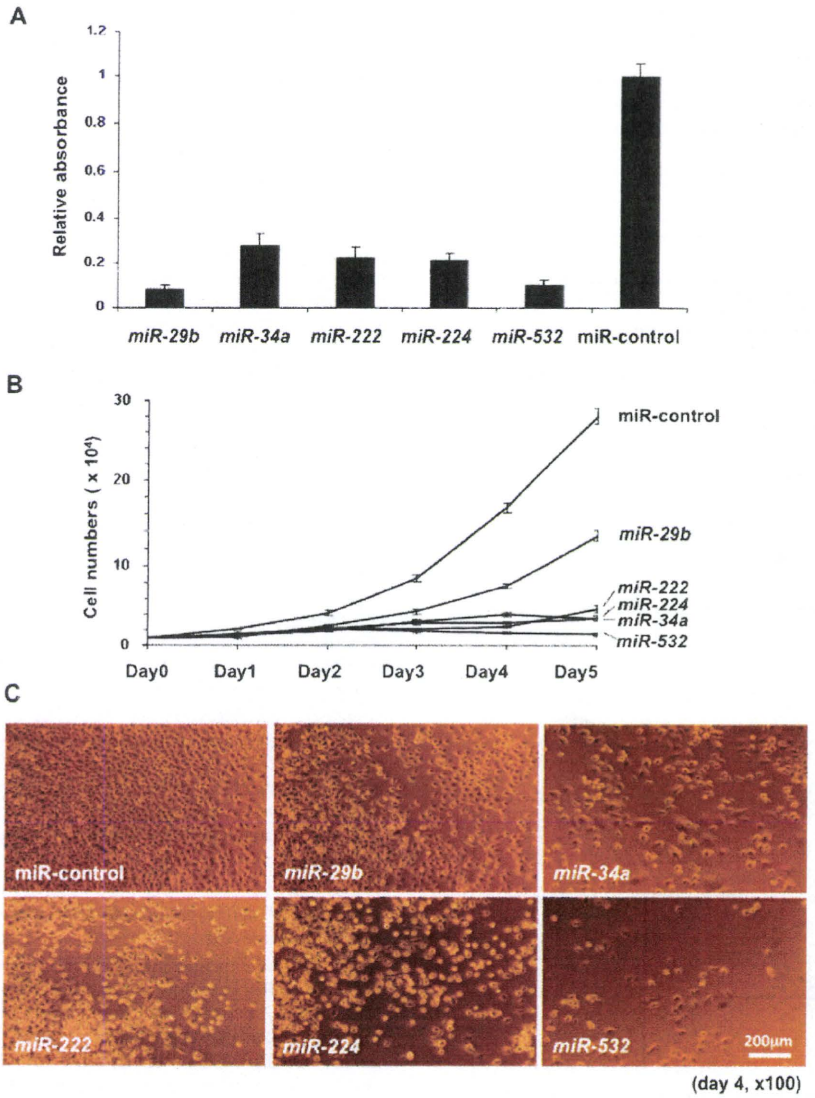

Fig. 3. Cell proliferation assays of MIA PaCa-2 cells. (**A**) 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay of MIA PaCa-2 cells 5 days after infection with lentivirus vectors expressing miRNA, (**B**) Cell proliferation curve and (**C**) Phase contrast micrographs 4 days after transfection with synthetic miRNAs.

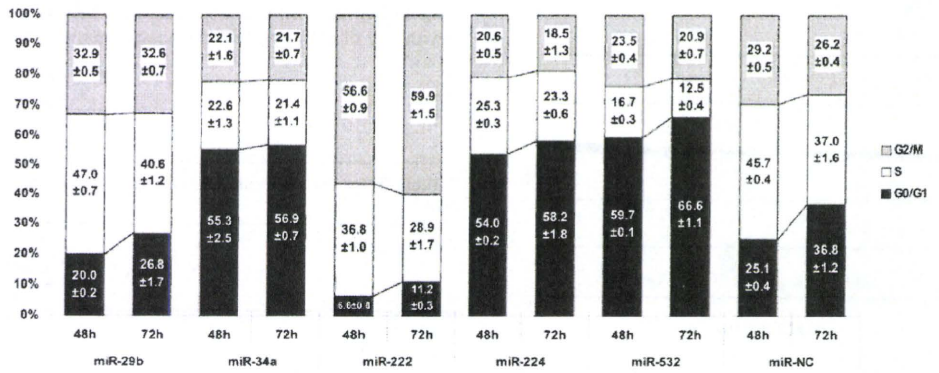

Fig. 4. Distribution of cell cycle phases of MIA PaCa-2 cells 48 and 72 h after transfection with synthetic *miR-29b*, *-34a*, *-222*, *-224*, *-532* and miR-negative control (miR-NC). The average ± standard deviations of three biological replicate samples are shown.

Discussion

A growing body of evidence supports the critical roles of miRNAs in a variety of normal cellular processes, including cell proliferation, cell death and development (3,4). Comprehensive profiling studies have revealed aberrant expression of miRNAs in various pathological conditions, suggesting that miRNAs have a role in the pathogenesis of some diseases, such as cancer. There is increasing interest in miRNA as therapeutic targets, as well as in the application of miRNA as therapeutic agents (5,8). The identification of *miR-34a*, which is transactivated by p53 and represses cell proliferation both *in vitro* and *in vivo*, prompted us to develop an assay to systematically screen for miRNAs that are directly linked to phenotypes characteristic of cancer cells (6). We combined lentivirus vectors expressing miRNA precursors and a custom-made microarray, which we used to monitor the number of lentiviral integrations based on specific sequences within the Lenti-miR virus library.

The usefulness and advantages of such a functional screening approach has recently been shown using a retrovirus vector library expressing short hairpin RNA for several thousand genes, although these short hairpin RNAs are only targeted to knockdown protein-coding genes (9–11). Voorhoeve *et al.* (12) have also constructed a genetic screening assay of miRNAs using a retrovirus vector library (miR-Lib) expressing miRNA minigenes and a barcode microarray (miR-Array). These phenotype-based approaches enabled identification of essential genes critical in cell proliferation that do not necessarily have mutated sequences or aberrant copy numbers and that do not exhibit significantly altered gene expression. Together with the aforementioned assays, the functional screening assay presented here would be a powerful complementary tool for the elucidation and identification of genes critical in establishing the phenotypic characteristics of cancers. By employing lentivirus vectors, we can perform the functional screening assay using non-dividing cells, including stem cells and neural cells, thus broadening the potential application of the assay.

The feasibility of our assay was shown by the successful identification of miRNAs that suppress proliferation of MIA PaCa-2 pancreatic cancer cell lines. We individually validated the proliferation-suppressive effect of the miRNAs that showed significant reduction in their copy numbers in our functional screening assays by transfection of synthetic miRNAs or transduction using lentivirus vectors (Figure 3). Although the fold changes in the expression of these miRNAs in comparison with the endogenous counterpart showed variations, the proliferation-suppressive effect of these miRNAs was at least evident under our experimental conditions (supplementary Table S2 is available at *Carcinogenesis* Online). Interestingly, *miR-34a*, a representative tumor-suppressive miRNA, was identified in the functional screening assay. *miR-34a* is located on chromosome 1p36, which is frequently lost in a variety of cancers (13–17). p53 is one of the factors transactivating *miR-34a*, and it also upregulates p53, suggesting a positive feedback loop formed by p53 and *miR-34a* (18,19). When introduced to cells, *miR-34a* strongly repressed proliferation of colon cancer cell lines HCT116 and RKO, and the cells underwent apoptosis or a premature-senescence phenotype depending on the experimental conditions (18,20–23). Topical application of *miR-34a* also inhibited growth of HCT116 cell lines transplanted to nude mice, implicating *miR-34a* as a potential novel therapeutic agent (18). The precise role of *miR-34a* in pancreatic cancers is still unclear, though its expression is downregulated in most pancreatic cancer cell lines (24). The present functional assay supports a tumor-suppressive role for *miR-34a* in pancreatic cancers, warranting further *in vivo* validation studies of this miRNA using mouse models of pancreatic cancers.

Besides *miR-34a*, we identified four candidate tumor-suppressive miRNAs in pancreatic cancers, namely *miR-29b*, *-222*, *-224* and *-532*. Although a detailed function analysis of these miRNAs is beyond the scope of this study, flow cytometric analysis revealed changes in the distribution of cell cycle phases of MIA PaCa-2 cells transfected with *miR-222*, *-224* and *-532*. Thus, cell cycle arrest is, at least in part, an underlying mechanism of the proliferation-suppressive effect of these miRNAs. Among the predicted targets of these miRNAs are cell division cycle 42 (GTP-binding protein, 25kDa) (CDC42) (*miR-224* and *-532*) and p21 protein (Cdc42/Rac)-activated kinase 1 (PAK1) (*miR-222*) (25–28). CDC42 is a small-guanosine triphosphatase of the Rho-subfamily that contributes to G_1–S phase progression through p70 S6 kinase-mediated induction of cyclin E expression, suggesting the possible role of *miR-224* and *-532* in G_1 arrest through translational suppression of CDC42 (29,30). PAK1 is a serine/threonine p21-actived kinase and is the downstream effector of CDC42 and Rho (31). Knockdown of PAK1 in gastric cancer cells exhibited an increased proportion of cells in G_2/M phase, indicating the possible role of *miR-222* in G_2 arrest through translational suppression of PAK1 (32). Whereas cell cycle arrest was evident in MIA PaCa-2 cells transfected with *miR-222*, *-224* and *-532*, *miR-29b* apparently suppresses cell proliferation through other mechanisms. Park *et al.* (33) reported that *miR-29b* upregulates p53 levels through suppression of p85α and CDC42. Although *TP53* is mutated in MIA PaCa-2 cells (R248W), the combination of suppression of p85α and CDC42 may synergistically confer a proliferation-suppressive phenotype independent of p53.

In conclusion, we have developed a functional screening assay of miRNAs by the combination of a pooled lentivirus library expressing miRNA precursors and a custom-made microarray. The feasibility of the assay was shown by the successful identification of miRNAs that suppress proliferation of MIA PaCa-2 pancreatic cancer cells. Flow cytometric analysis revealed that cell cycle arrest was, at least in part, the underlying mechanism of proliferation-suppressive effects of *miR-34a*, *-222*, *-224* and *-532*. The flexible nature of the assay should facilitate its use in the identification of miRNAs that are involved in a wide array of cancer phenotypes, including cancer stem cells or metastasis.

Supplementary material

Supplementary Tables S1 and S2 and Figures S1 and S2 can be found at http://carcin.oxfordjournals.org/

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Mini review

MicroRNA, SND1, and alterations in translational regulation in colon carcinogenesis

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ABSTRACT

Post-transcriptional regulation of gene expression by microRNA (miRNA) has recently attracted major interest in relation to its involvement in cancer development. miRNA is a member of small non-coding RNA, consists of 22–24 nucleotides and regulates expression of target mRNA species in a post-transcriptional manner by being incorporated with RNA-induced silencing complex (RISC). *Staphylococcal* nuclease homology domain containing 1 (SND1), a component of RISC, is frequently up-regulated in human colon cancers and also chemically induced colon cancers in animals. We here showed that SDN1 is involved in miRNA-mediated gene suppression and overexpression of SND1 in colon cancer cells causes down-regulation of APC without altering APC mRNA levels. As for the miRNA expression profile in human colon cancer, *miR-34a* was among the list of down-regulated miRNA. Expression of *miR-34a* is tightly regulated by p53, and ectopic expression of *miR-34a* in colon cancer cells causes remarkable reduction of cell proliferation and induces senescence-like phenotypes. *MiR-34a* also participates in the positive feedback loop of the p53 tumor suppressor network. This circuitry mechanism for p53 activation is of interest in understanding the tumor suppressive function of *miR-34a* in colon carcinogenesis. miRNA should also be considered as novel anti-cancer agents in tumor suppressive therapeutic applications.

1. Introduction

Colon cancers have been demonstrated to develop after multistep accumulation of genetic and epigenetic alterations, accompanying substantial changes in global gene expression profiles as a consequence [1–3]. Genetic alterations, such as chromosomal translocation, gene amplification, copy number aberrations, and point mutations, result in activation or inactivation of genes involved in cancer development [1]. Epigenetic alterations, such as hyper- or hypo-methylation of CpG sites in promoter regions of genes and modification of histone also contribute to the substantial changes of gene expression profiles in cancer cells [2,3].

Recently, post-transcriptional regulation of gene expression by microRNA (miRNA) has attracted major attention among cancer researchers in relation to its involvement in cancer development [4]. Indeed, altered expression of miRNA has been found in almost all human cancers [5–8]. miRNA, which is a member of small non-coding RNA, consists of 22–24 nucleotides, and pairs with complementary sequences located mainly in the 3′ untranslated regions of target mRNAs and regulates gene expression in a post-transcriptional manner by being incorporated with RNA-induced silencing complex (RISC) [9], and contributes significantly to the development of human carcinogenesis [5–8]. Interestingly, we frequently observe upregulation of *Staphylococcal* nuclease homology domain containing 1 (SND1) in colon carcinogenesis, even at early stages [10,11]. SND1 is one of the components of RISC and its disruption was shown to cause perturbation of small interfering RNA-induced gene silencing [12]. In this article, we describe the possible involvement of miRNA and its effector complex SND1 in colon carcinogenesis *via* post-transcriptional regulation of gene expression.

2. SND1, a component of RISC, is a regulator of gene expression

SND1, also known as Tudor-SN and p100, is a highly conserved protein from yeast to humans and is suggested to be associated with multiple steps involved in the regulation of gene expression, includ-

Abbreviations: APC, adenomatous polyposis coli; AT1R, angiotensin II type 1 receptor; HA, hemagglutinin; Luc, luciferase; miRNA, microRNA; RISC, RNA-induced silencing complex; SA-β-gal, senescence-associated β-galactosidase; SIRT1, silent information regulator 1; SND1, staphyloccal nuclease homology domain containing 1; 3′-UTR, 3′-untranslated region.

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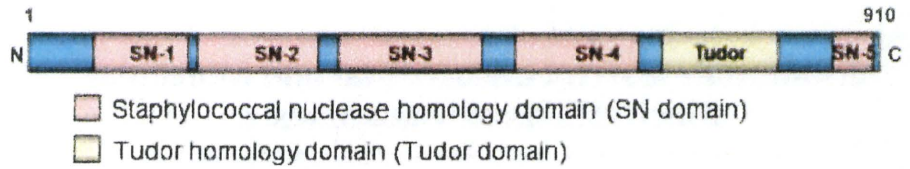

Fig. 1. Structural feature of SND1 protein. SND1 is composed of 910 amino acid residues, has 4 complete (SN-1–4) and one incomplete (SN-5) *Staphylococcal* nuclease homology domains and a Tudor homology domain.

ing transcription and pre-mRNA splicing, as well as translation and RNA interference, as detailed later. SND1 has four complete and one incomplete sets of repeats of *Staphylococcal* nuclease homology domains (SN domain), and a Tudor homology domain (Tudor domain) in its C-terminal half (Fig. 1). Whereas the biological function of these structural domains remains to be elucidated, it was recently reported that the Tudor domain is a binding platform for the peptide containing dimethylated lysines [13] with high affinity and the repeats of SN domains are required for binding to RNA [14].

SND1 was originally identified as a protein interacting with the Epstein-Barr virus nuclear antigen 2 (EBNA2), and promotes EBNA2-dependent transcription [15,16]. The protein was also reported to interact with some transcriptional regulators, including c-Myb, STAT5 and STAT6, suggesting its biological role as a transcriptional co-activator [17–19]. SND1 was also demonstrated to be involved in pre-mRNA splicing through the enhancement of spliceosome formation by interacting with snRNP proteins [20,21]. It was also reported that SND1 regulates translation of angiotensin II type I receptor (AT1R) by binding to the 3′ UTR of its mRNA [22].

Recently, Caudy et al. showed that SND1 is one of the components of RISC, and disruption of SND1 in *Caenorhabditis elegance* was demonstrated to cause defects in siRNA mediated gene silencing [12]. More recently, SND1 was further shown to be a component of *let-7* directed RISC [23]. Although the biological function of SND1 is not fully determined, these intriguing findings suggested the possible involvement of SND1 as a key regulator for gene expression at both transcriptional and post-transcriptional levels [24].

3. Possible involvement of SND1 in activation of the Wnt signaling pathway in colon carcinogenesis

We recently reported up-regulation of SND1 in human colon cancers and also in colon cancers induced in rat by chemical carcinogens, such as a food-borne carcinogen, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine, and an alkylating agent, azoxymethane [11]. The expression of *SND1* mRNA in colon cancer tissues was remarkably high, being 5-fold or even greater, compared with their normal counterparts. Interestingly, cytoplasmic accumulation of SND1/Snd1 protein was also observed in aberrant crypt foci, putative pre-cancerous lesions of the colon [11].

Stable over-expression of recombinant Snd1 in rat intestinal epithelial cells, IEC6, lead to the enhancement of cell proliferation and anchorage-independent growth activity in a soft agar colony formation assay, accompanying the subcellular translocation of E-cadherin to the cytoplasm. This suggests that up-regulation of SND1 may induce loss of contact inhibition and thereby promotes cell proliferation. Interestingly, in Snd1-transfected cells, considerable reduction of Apc protein by 25–75% was observed without significant changes in its mRNA level [11]. Exogenous and transient introduction of SND1 into human cancer cell lines, HCT 116, SW48 and HeLa cells, harboring wild type APC, induced down-regulation of the APC protein without altering its mRNA level (data not shown), and also caused up-regulation of the β-catenin protein (Fig. 2A). Furthermore, knockdown of SND1 in SW48 cells caused restoration of APC protein levels (Fig. 2B). Taking all these results together, over-expression of SND1 may induce activation of the Wnt-β-catenin signaling pathway through down-regulation of the APC protein, even at early stages of colon carcinogenesis.

4. SND1 serves as a regulator for miRNA-mediated gene silencing

As for the mechanism for the post-transcriptional regulation of gene expression by SND1, the fact that SND1 binds to APC mRNA [11] leads to the intriguing scenario that down-regulation

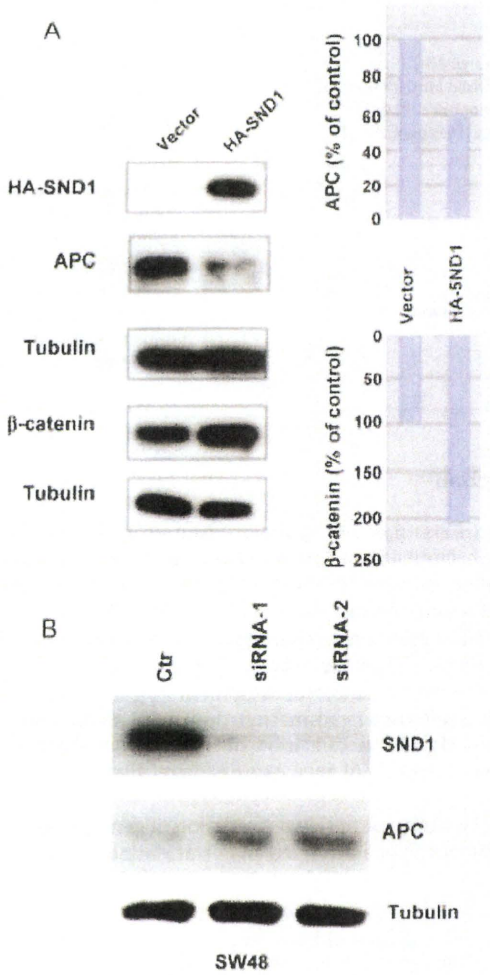

Fig. 2. SND1 is involved in the regulation of APC expression. (A) HeLa cells were transfected with either a mammalian expression plasmid containing HA-tagged SND1 (HA-SND1) cDNA or control vector (pCDA-HA) and propagated for 48 h. The protein levels of APC and β-catenin were analyzed by immunoblotting following the standard protocol. Graphs indicate the relative values of signal intensities of the bands for APC or β-catenin normalized to that of tubulin. (B) SW48 colon cancer cells were transfected with SND1 siRNAs (Qiagen) or control siRNA (ctr; Qiagen) for 48 h. After incubation, the protein levels of SND1 and APC were determined by immunoblot analysis.

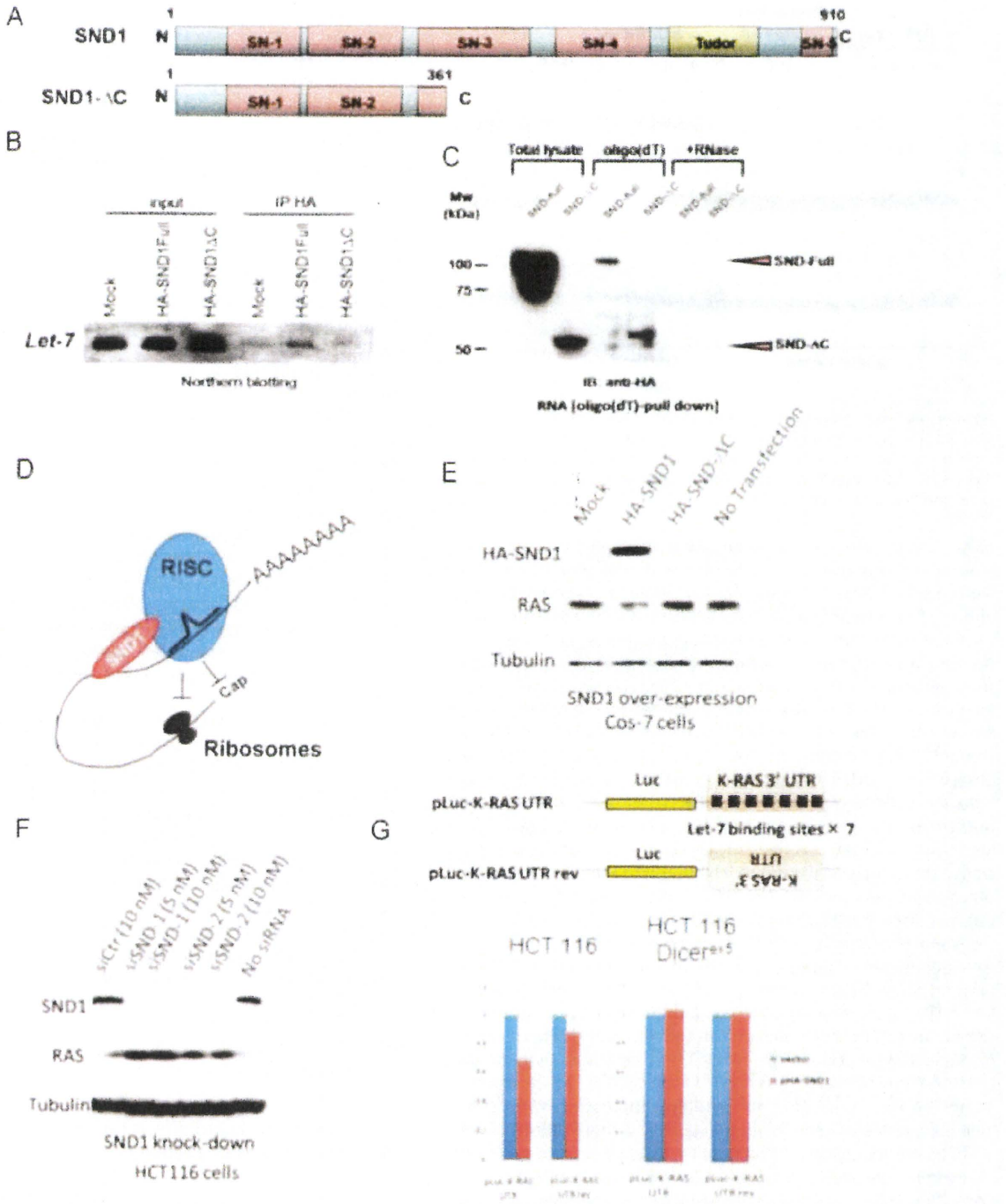

Fig. 3. Association of SND1 with miRNA-mediated gene silencing. (A) Structural features of the full length and ΔC form of SND1 deleting the C-terminal half. (B) Interaction of SND1 with *let-7* miRNA. Cell extracts of Tet-on HeLa cells (Clontech), expressing the full length or ΔC form of HA-SND1, were subjected to immunoprecipitation using anti-HA antibody. RNAs bound to either the full length or ΔC form of HA-SND1 were extracted by Trizol, and subjected to Northern blot analysis after size fractionation in polyacrylamide gel. *Let-7* miRNA sequence was hybridized with a labeled *let-7* anti-sense probe. (C) Physical interaction of SND1 with mRNA. Cytoplasmic lysates extracted from HeLa cells expressing the full length or ΔC form of HA-SND1 were mixed with oligo(dT) cellulose beads, and poly(A)$^+$ RNA–protein complexes were collected. After several washes of the oligo(dT) precipitants, pulled-down proteins were eluted from the beads by adding Laemmli's SDS-sample buffer, and HA-SND1 (SND-Full and SDN-ΔC) was determined by immunoblot analysis using an anti-HA antibody. Arrow heads indicate the full length and ΔC form of HA-SND1, respectively. (D) A proposed model of the ternary complex with SND1, RISC and mRNA in gene silencing. SND1 interacts directly with mRNA through its N-terminal region, and the association with RISC is mediated by the C-terminal half of the protein. The ternary complex of SND1 is considered to participate in repressing the expression of target genes *via* inhibition of either translation initiation complex or loading of ribosomes. (E) COS-7 cells were transiently transfected either with pHA-SND1 (full length), pHA-SND-ΔC (ΔC form), or control vector (pCDNA-HA). Protein levels of RAS and HA-SND1 were analyzed by immunoblot analysis, and tubulin was used as a loading control. Substantial reduction of RAS protein was observed by exogenous induction of HA-SND1, but not either by HA-SND ΔC or Mock transfection. (F) HCT 116 cells were transfected with siRNA targeting SND1 (siSND-1 and siSND-2) or control siRNA. Cell lysates were collected 48 h after transfection, and subjected to immunoblot analysis. (G) Effect of SND1 and the K-RAS 3′-UTR on enzymatic activity of *firefly* luciferase reporter protein. 3′ UTR of RAS mRNA or its reverse direction (negative control) was fused to the 3′ end of *firefly* luciferase reporter gene. HCT 116 and HCT 116 Dicer^{ex5} were co-transfected with the reporter plasmid and HA-SND1. After incubation for 48 h, cell extracts were prepared and luciferase activity was measured by Dual Luciferase Assay Kit (Promega). The activity of *firefly* luciferase for each sample was normalized by *Renilla* luciferase activity as detailed elsewhere.

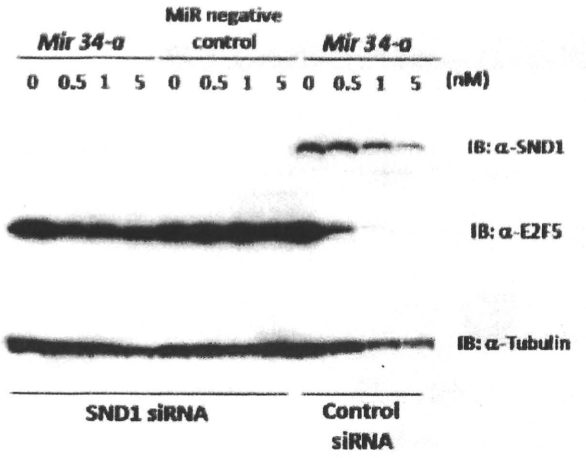

Fig. 4. Reduction of *miR-34a*-mediated repression of E2F-5 by SND1 knockdown. HCT 116 cells were introduced with either SND1 siRNA (left 8 lanes) or control siRNA (right 4 lanes) in combination with *miR-34a* (left 4 lanes and right 4 lanes) or miR-negative control (miR-NC; middle 4 lanes), incubated for 48 h, and cell extracts were subjected to immunoblot analysis.

of APC could be mediated by miRNAs. To support this idea, SND1 was indeed demonstrated to interact with endogenous miRNA by Northern blot analysis using a probe against *let-7* miRNA (Fig. 3A and B). The interaction of the ΔC form of SND1 deleting the C-terminal half of SND1 with *let-7* was not detectable (Fig. 3A and B), indicating that the C-terminal region of SND1 is required for the interaction with miRNA and the RISC complex. Furthermore, an oligo(dT) pull-down experiment using HeLa cell lysates demonstrated the interaction of SND1 with poly(A)$^+$ RNA is mediated through the N-terminal region of SND1 (Fig. 3C). Based on these observations, SND1 may serve as a platform for a molecular bridge between mRNA and RISC by incorporating the mRNA-specific miRNA species as depicted in Fig. 3D. In fact, over-expression of SND1 in COS-7 cells reduced the expression of RAS protein, the transcript of which is known to be targeted by *let-7* [23]. On the other hand, knockdown of SND1 by siRNA induced the substantial increase of RAS protein (Fig. 3E and F). In order to confirm the suppressive effect of SND1 on gene expression occurs in a *let-7*-dependent manner, we generated *firefly* luciferase reporter plasmids fused either with the 3′ UTR of RAS mRNA (pLuc-K-RAS UTR), containing several recognition sites of *let-7*, or with its reverse direction (negative control; pLuc-K-RAS UTRrev), and co-introduced with pHA-SND1 into HCT 116 and Dicer hypomorphic cells derived from HCT 116 (HCT 116 Dicer^{ex5}) [25]. As demonstrated in Fig. 3G, SND1 substantially suppresses expression of luciferase reporter gene containing 3′-UTR of *RAS* mRNA, but not of that with its opposite direction. Furthermore, not even a little change was detected in HCT 116 Dicer^{ex5} cells, even with Luc-K-RAS UTR (Fig. 3G), strongly suggesting the requirement of mature miRNA for this effect. Taking all these results together, SND1 was suggested to regulate gene expression in a *let-7*-dependent manner.

Further to this, SND1-mediated gene suppression was also observed in another gene, E2F5. The expression of E2F5, one of the target genes for *miR-34a*, is considerably repressed in the colon cancer cell line RKO by ectopic expression of *miR-34a* in a dose-dependent manner as depicted in Fig. 4 (see right-handed 4 lanes). Concomitant knock-down of SND1 by siRNA substantially suppressed the gene silencing activity of *miR-34a* against E2F5 (Fig. 4, left-handed 4 lanes). Although these observations suggested the contribution of miRNAs for the regulation of gene expression, it remains to be elucidated whether SND1 is specifically involved in the regulation of colon cancer-related genes, including APC, and how SND1 is associated with colon cancer development. Identification of mRNAs and miRNAs, being preferentially interacted with SND1, will provide new molecular insights into abnormalities of translational regulation of gene expression at early stages of colon carcinogenesis.

5. *MiR-34a*, a p53-regulated tumor suppressive miRNA, controls proliferation of colon cancers cells

Comprehensive analysis of miRNA expression in human colon cancers has revealed that a substantial fraction of miRNAs were either down-regulated or up-regulated in colon cancer tissues compared to their normal counterparts. Among the list of dys-regulated miRNA species in colon cancers, *miR-34a*, which we previously demonstrated to work as a strong repressor for cell proliferation [26], is significantly down-regulated in colon cancers. *miR-34a* is a member of the *miR-34* gene family, located at chromosome 1p36, and is composed of two exons. A nucleotide sequence for the mature form of the *miR-34a* is entirely embedded within the second exon [27]. The expression of *miR-34* family, including *miR-34a*, is tightly regulated by p53 [26–31], and the *miR-34* family negatively regulates cell proliferation *via* induction of apoptosis [27–29], cell cycle arrest [29,30], or senescence [26,31].

We previously demonstrated that introduction of *miR-34a* into colon cancer cells showed a remarkable reduction of cell numbers when compared to negative control miRNA. Cells that were transfected with *miR-34a* manifested characteristic morphological changes, namely large cellular/nuclear sizes and positive staining for senescence-associated β-galactosidase (SA-β-gal), suggesting the induction of senescence-like phenotypes in colon cancer cells (Fig. 5A).

To gain further insight into the biological role of *miR-34a*, we conducted global gene expression analysis using two colon cancer cell lines, HCT 116 (wild-type p53) and RKO (wild-type p53), transfected with either *miR-34a* or negative control miRNA. mRNAs, whose expression was commonly altered 2-fold or greater by *miR-34a* in both HCT 116 and RKO cells were extracted as detailed elsewhere [26]. *miR-34a* positively regulates the expression of some p53 target genes, including p21 and sestrin 1 [26,32]. In contrast, E2F family members and their target genes were down-regulated. This result suggested that one of the key roles of *miR-34a* in the anti-proliferation of cancer cells is the activation of the p53 network with the concomitant reduction of positive regulators for the cell cycle under the p53 wild type situation. In this regard, the direct repression of CDK4/6 and E2F3 by *miR-34a* was clearly demonstrated [26,30,31,33]. This result also suggested the positive feedback regulation between p53 and *miR-34a* [26].

6. SIRT1 participates in the positive feedback loop of the p53 tumor suppressor network

Recently, silent information regulator 1 (SIRT1) was identified as another target for *miR-34a* and demonstrated to regulate the induction of p53-dependent apoptosis [34]. SIRT1, a class III histone deacetylase, is known to be a negative regulator of p53 through the modulation of acetylation at K382 of p53 and thereby associated with multiple cellular processes, including apoptosis and cellular senescence [35–38]. We also observed the physical interaction of *miR-34a* and SIRT1 mRNA in HCT 116 cells (unpublished data), and the acetylated form of p53 accumulated in the nucleus by the exogenous introduction of *miR-34a*. Restoration of SIRT1 expression by introducing an SIRT1 open-reading frame (ORF) into *miR-34a*-transfeted cells abolished p53 activation caused by *miR-34a* (unpublished data). MDM2/MDMX, negative regulators of p53, were also demonstrated to be targets of *miR-34a* [39], and serve as key molecules for the negative regulatory circuitry of p53 in either

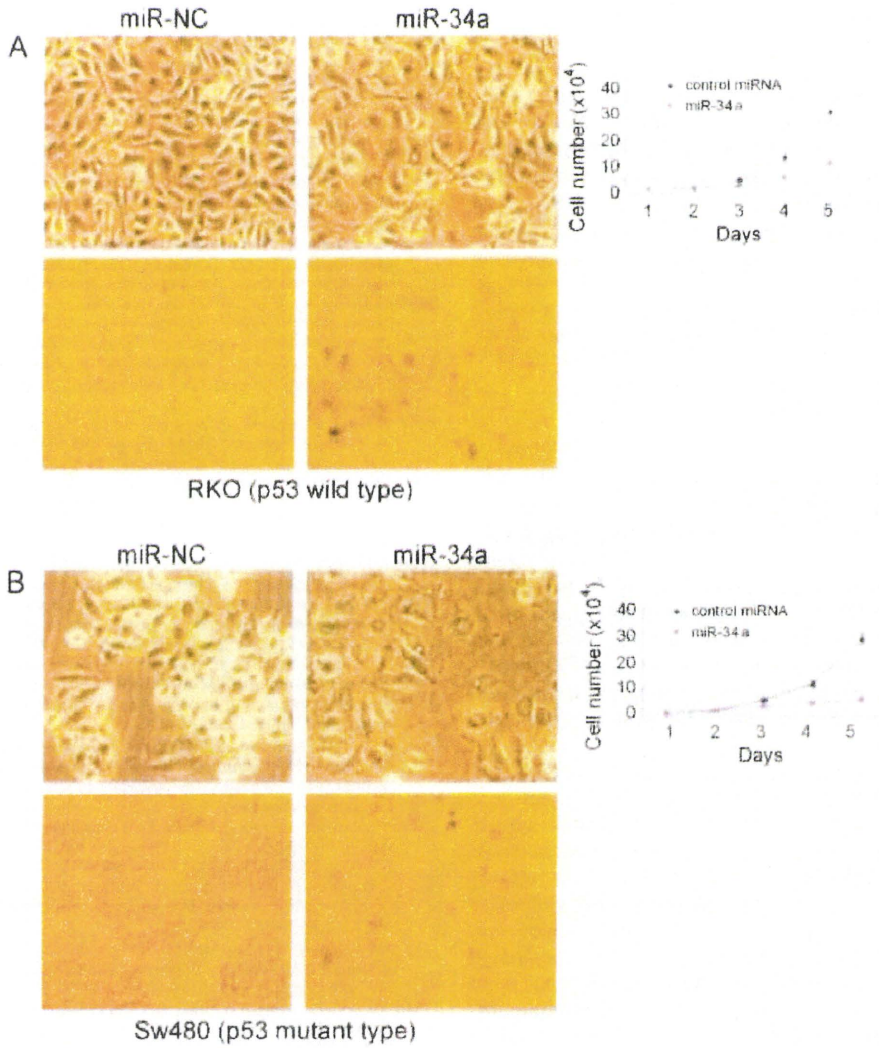

Fig. 5. Induction of senescence-like growth arrest by *miR-34a*. (A) RKO cells (wild-type p53) were transfected with *miR-34a* or miR-NC, and propagated for 4 days, and cells were subjected to SA-β-gal staining. The right upper panel indicates growth suppression of RKO cells by *miR-34a*. (B) Induction of senescence-like growth arrest was also observed in p53-mutant SW480 cells. Exogenous introduction of *miR-34a* was carried out as described above.

a direct or indirect fashion. These circuitry mechanisms for p53 activation are important for understanding the biological role of *miR-34a* in carcinogenesis. Importantly, the anti-proliferative activity of *miR-34a*, accompanying the senescence-like growth arrest, was also observed in SW480 colon cancer cells with the mutant p53 (Fig. 5B). This suggests that *miR-34a* may also participate in the p53-independent tumor-suppressor networks. Since the suppression of SIRT1 by *miR-34a* was observed even in the p53 mutant cell lines (unpublished data), it would be of interest to address whether SIRT1 could be involved in the induction of senescence-like growth arrest under the p53 mutant situation. Alternatively, other p53-independent tumor suppressor network activated by *miR-34a* should be involved, and the mechanism be clarified in the future.

7. Therapeutic application of tumor suppressive miRNA as a novel anti-cancer tool

Currently, development of RNAi drugs against various types of human disorders is being extensively investigated. For instance, systemic administration of *miR-26a* in a mouse model of Myc-induced hepatocellular carcinoma using adeno-associated virus (AAV) causes inhibition of cancer cell proliferation, induction of tumor-specific apoptosis, and drastic protection from disease progression without measurably manifestation of liver toxicity in normal hepatocytes [40]. Minimal adverse effect on normal hepatocytes by *miR-26a* could be based on the specific pattern of *miR-26a* expression, namely expression of *miR-26a* being suppressed specifically in liver cancers [40]. Further to this, inhibition of liver specific *miR-122* using an LNA-modified anti-miRNA oligonucleotide was recently shown to be implicated in cholesterol and lipid metabolism [41] and in hepatitis virus replication [42–44]. Systemic delivery of miR-16 is also reported to inhibit the growth of metastatic prostate tumors by suppressing multiple cell-cycle genes [45].

As for *miR-34a*, subcutaneous administration of *miR-34a*/atelocollagen complexes in mice caused significant suppression of xenograft tumor growth of both HCT 1116 and RKO colon cancer cells compared with the administration of a control miRNA/atelocollagen complex [26]. Tumor tissues treated with *miR-34a* showed a considerable degree of necrosis. In addition, ectopic expression of *miR-34a* in glioma and medulloblastoma cells clearly inhibits cell proliferation, G1/S cell cycle progression, and cell survival and cell migration [46]. Furthermore *miR-34a* expres-

sion was also demonstrated to inhibit *in vivo* glioma xenograft growth [46].

Although the tumor suppressive effect of *miR-34a in vivo* is still currently somewhat limited, repeated injection of *miR-34a*/atelocollagen complexes may achieve more efficient and significant regression of xenograft tumors and could also be applicable for *in vivo* tumors. Alternatively, identification of miRNA species that possess much higher tumor suppressive activity *in vivo* should be performed. Functional screening system *in vivo* should be of great help to achieve this goal. Toward this end, we recently succeeded to construct a drop-out functional screening system, as detailed in our recent publication [47], for identification of tumor suppressive miRNAs, which could be applicable for tumor treatment *in vivo*.

8. Conclusion

Translational regulation of gene expression, especially that by miRNA, has recently attracted major interest among cancer researchers. Because of the nature of miRNA regulating multiple target genes *via* the diverse manner of recognition of target sequences at 3′-UTR of genes, phenotypes caused by the exogenous introduction of miRNA are sometimes drastic even an *in vivo* setting in the cases of, for example, *miR-26a*, *miR-34a* and *miR-122*. Although the efficient delivery of miRNA to the target tissues/organs is a crucial subject to be solved, possible application of miRNA not only for therapeutic agents, but also for diagnostic biomarkers, is of great interest to be explored.

In addition to the translational researches' point of view, the biological nature of miRNA is quite intriguing, and it opens up a wide new field in cancer research. Although each miRNA targets and regulates functions of multiple genes in a post-transcriptional manner, it seems to suppress a set of genes involved in certain signaling pathways, such as cell cycle, apoptosis, cell proliferation, differentiation, and so on [48,49]. The p53 tumor suppressor network is, for example, regulated by several miRNAs, including *miR-34a* [50]. *Let-7* regulates the Ras signaling pathway [51,52], and the PI3K/AKT/PTEN regulatory circuit is regulated by several miRNAs, such as, *miR-21* [53,54], miR-17-92 cluster [55], *miR-126* [56], and *miR-214* [57]. The feature of an oncogene-tumor suppressor gene network as one of the hallmarks of cancer [58] now requires incorporation of miRNA as an essential component to explain the integrated signal circuit of the cell.

Conflicts of interest

None declared.

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【総論　マイクロRNAと癌】

MicroRNAs and Cancer

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Key words
転写後制御、cancer, microRNA,
functional screening

要　約

癌では，正常組織に比べて様々な遺伝子の発現変化が認められ，これらの変化が癌細胞としての特性の賦与に重要な役割を果たしていることから，癌を「遺伝子発現病」として捉えることができる。最近，遺伝子発現の転写後制御に関わるマイクロRNA（miRNA）の重要性が注目されている。miRNAは，標的とする遺伝子（mRNA）の3'非翻訳領域の部分的に相補的な配列と相互作用することにより，mRNAの翻訳阻害や分解の促進を介して，遺伝子の発現調節に関与していると考えられている。miRNAは，細胞分化や細胞増殖，細胞死などの制御を介して多様な生物学的現象に関わり，その発現異常は癌などの様々な病態の成立に関与することが分かってきた。また，一つのmiRNAは複数のmRNAを標的とし発現を調整していることから，細胞の多様性にも重要な役割を果たしていると考えられる。miRNAの発現制御のメカニズムや機能を解明することは，癌細胞を初めとする様々な細胞の特性を明らかにするためにも必要である。

1．癌は「遺伝子発現病」

癌は，さまざまな癌化関連遺伝子の変異や遺伝子内の欠失，組み換えなどにより遺伝子が質的に変化したり，コピー数の変化，或いはエピジェネティックな変異による遺伝子発現の量的変化などが多段階的に蓄積することにより発生する。従来，癌はDNA（遺伝子）に傷ができることによって発生することから「癌は遺伝子の病気」と言われてきたが，同時に，遺伝子の質的或いは量的な異常が蓄積することにより引き起こされる「遺伝子の発現異常がもたらす『遺伝子発現病』」と捉えることが出来る。癌における遺伝子の発現異常のメカニズムとしてはこれまで，転写因子の活性変化や，遺伝子発現制御領域におけるCpG配列のメチル化異常や，ヒストン修飾の異常などのエピジェネティックな変化による不活化（サイレンシング）など，DNAからRNAに転写されるレベルでの異常（転写制御異常）を中心にとらえられてきたが，最近になり，DNAからRNAに転写された後のRNAレベルの段階，或いは，RNAから蛋白質に翻訳される段階での制御異常（転写後制御）と癌化との関連が注目されている。マイクロRNA（miRNA）は，遺伝子発現の転写後制御に関わる重要な因子として，様々な病態との関連において近年注目されている。

2．マイクロRNA（miRNA）とは

miRNAは約22塩基からなる機能性non-coding RNAの一種で，ヒトゲノム中には1,000を超えるmiRNA遺伝子の存在が示唆されている。miRNAは，通常の遺伝子と同様にRNAポリメラーゼIIにより転写され（primary miRNA; pri-miRNA），核内及び細胞質内でプロセッシングを受け，最終的に約22塩基長の成熟型miRNAとなる。成熟型miRNAはAGO2を主要な構成因子とするタンパク複合体（RISC: RNA-induced silencing complex）に取り込まれ，標的とするmRNAの3'非翻訳領域の部分的に相補的な配列と相互作用することによ

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り，標的mRNAの翻訳阻害や分解促進を介して，遺伝子の発現制御に関与していると考えられている[1]。

ゲノム上にコードされた遺伝情報はmRNAに転写され，さらに蛋白質へと翻訳される（セントラルドグマ）。遺伝子発現の変化は細胞特性の変化を引き起こし，癌などの様々な病態の成立に寄与すると考えられることから，遺伝子発現異常の解析に関しては，通常，転写レベルでの発現制御機構について解析を行うことが多い。一方，miRNAは各臓器に特徴的な発現様式を示し，一つのmiRNAは複数（100以上～）のmRNAを標的とすることが知られている。これまでの多くの研究成果により，miRNAは細胞分化や細胞増殖，細胞死（アポトーシス）等の制御を介して，多様な生物学的現象に関わっていることが明らかになり，またその発現異常は癌などの疾患の成立にも関与することが分かってきた。興味深いことに，miRNAはヒト癌において高頻度に染色体の増幅や欠失が認められる領域に局在することから，いわゆる，癌遺伝子あるいは癌抑制遺伝子的な機能を有するmiRNAが存在し，癌化に重要な役割を果たしている可能性が考えられている[2]。

3．miRNAの発現異常と癌

miRNAの発現変化は，その標的となる遺伝子（mRNA）の発現異常を引き起こすことになる。事実，癌では正常組織に比較して特徴的な遺伝子の発現プロファイルを示すことが知られているが，miRNAの発現に関して癌部と非癌部とではそのプロファイルに大きな違いがあることが分かっている[3]。特に，癌部では全般的にmiRNAの発現が低下していることや，この分子基盤として，miRNA遺伝子→pri-miRNA（核内）→pre-miRNA（細胞質内）→mature miRNA（細胞質内）というmiRNAの生合成過程において，主要ながん抑制遺伝子であるp53の機能不全が重要な役割を果たしていることも明らかにされた[4]。miRNAの発現プロファイルは，mRNAの発現プロファイルと比較してもより臓器特異性の高い発現様式を示すことが示唆されている。

4．DNA損傷応答におけるmiRNAの役割

発がんのプロセスにおいて，様々なDNA傷害性ストレスに曝露された細胞に生じたDNAの損傷は，DNAの複製に対して強い阻害作用を有し，細胞周期停止（増殖停止）や細胞死を誘導することが分かってきた。細胞の「DNA損傷応答（DDR）」と呼ばれる反応である。DNA損傷応答（DDR）は，細胞の悪性転化の制御機構という観点からも重要な細胞応答の一つと考えられている。この細胞傷害応答においても，miRNAが重要な役割を果たしていることが分かってきた。大腸がん細胞株をアドリアマイシンで処理した際に，再現性良く発現誘導されるmiRNAの一つとして我々が見出したmiR-34aは，p53依存的に発現誘導される代表的なmiRNAで，大腸がん細胞株に導入すると強烈に細胞増殖を抑制し，細胞老化様の形質を誘導することが分かってきた[5]。さらに，誘導されたmiR-34aはヒストン脱アセチル化酵素の一つであるSIRT1 mRNAを標的にし，p53のアセチル化状態を変化させることでp53の安定性を促進する。即ち，miR-34aはDNA損傷に対する主要な監視機構であるp53ネットワークのpositive feedback機構として作用することも分かった[6]。

最近我々は，がん抑制的機能を有するmiRNAを網羅的かつ効率的に同定するシステムとして，レンチウィルスのmiRNAライブラリーを用いた「機能スクリーニング法」（ドロップアウトスクリーニング法）を開発した。この方法の詳細については本特集の中で泉谷・土屋により紹介されているが，この機能スクリーニング法により同定されるがん抑制的なmiRNAの中にも，DNA損傷により発現誘導されるものが多く含まれることが分かってきた。細胞傷害性ストレスに対する細胞側の応答機能として，miRNAの果たす役割の重要性を示すものである。

特集　miRNA研究の最近の進歩

機能スクリーニング系による がん抑制的miRNAの単離

泉谷昌志　土屋直人　中釜　斉

miRNAは細胞分化・増殖などの正常の生命現象のみならず，がんをはじめとする疾患との関連についても注目されている。さらには，miRNAを用いたあるいは標的とした治療への応用も試みられている。その一方で，数百あるmiRNAのうちから特に疾患と関連したものを同定する作業は必ずしも容易ではなく，miRNA発現解析のみでは十分な手がかりを得られない場合も多い。われわれは，miRNA発現解析と相補的なアプローチとして，miRNAを細胞へ導入し，形質に与える影響を半定量的に検出する「miRNA機能スクリーニング系」を構築し，数百のmiRNAから新規の大腸がん抑制的miRNAを同定することができたので，スクリーニングの概要と合わせて紹介したい。

1　miRNAとがん

miRNAは22ヌクレオチド程度の小分子RNAであり，タンパクをコードしない非コードRNAのうちで最もよく知られたものである。標的遺伝子の翻訳抑制を介して，細胞分化・増殖・細胞死などのさまざまな生命現象に関与していることが知られているが，これらの異常と疾患との関連についても報告されるようになってきた。このうちで現在最も注目されているものの一つは，「がん」におけるmiRNAの機能異常である。ヒトのがん検体を用いたmiRNA発現解析は精力的に行われており，がん組織においては非腫瘍部と比較して特異的なmiRNA発現プロファイルを示すことが報告されている[1]。さらには，miRNAの発現はがんの生じる臓器により特異的なプロファイルを示すことも知られており，miRNAが細胞分化に果たす役割を示していると考えられる。

がんにおけるmiRNAの役割は大きく，1）腫瘍促進的なもの（oncogenic miRNAs）と2）腫瘍抑制的なもの（tumor-suppressive miRNAs）に分けることができる。前者の代表的なものはmiR-21，miR17-92クラスターなどであり，miR-21は大腸・胃・肺・乳がんなどで，miR17-92クラスターは悪性リンパ腫や肺がんなどで高発現がみられ，それぞれ*PTEN*，*E2F1*などを標的とすることにより腫瘍促進的に作用しているものとされている[2,3]。一方で，後者の代表的なものにはmiR-34やlet-7などが知られている。miR-34は*p53*により活性化され，またそれ自身も*p53*の発現を亢進させ，代表的な腫瘍抑制遺伝子である*p53*と密接に関連して作用している[4-9]。let-7は主にがん遺伝子RASの発現を抑制することにより腫瘍抑制的に作用するものと考えられており，肺がんではその発現が低下している[10]。これらのmiRNAの腫瘍促進的または抑制的作用の詳細な機序については，現在のところmiRNAの標的分子同定が必ずしも容易ではないことから，今後ともさらな

Identification of tumor-suppressive miRNAs using high-throughput functional screening assay
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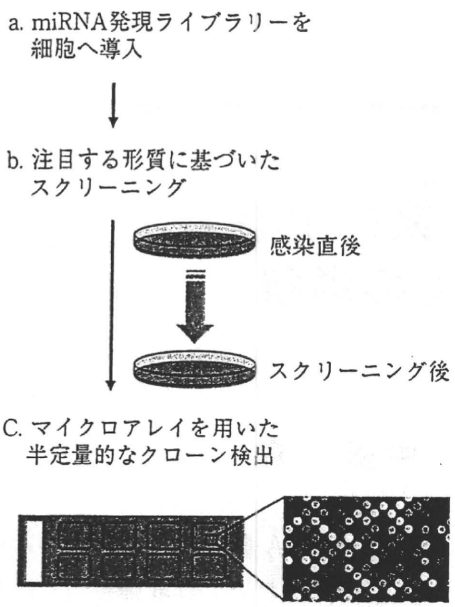

図1 機能スクリーニングの概略

る検討が必要である。

前述の通り，がんの観点からmiRNAは腫瘍促進的なものと抑制的なものに大別されるが，がん組織のmiRNAプロファイリングの結果によれば，腫瘍部では非腫瘍部と比較して発現の低下しているmiRNAが数多く存在していることから，どちらかというとmiRNAには腫瘍抑制的なものが多いことが推測される。最近は，がんとの関連が示されたmiRNAを医療へと応用することへの関心も高まっており，がん組織や体液におけるmiRNAプロファイリングを応用した新規診断法や，がん促進的または抑制的なmiRNAを標的とした新規治療法の開発への期待が高まっている。診断マーカーの探索では発現異常の解析を行えばよいが，治療への応用に関しては標的となりうる候補miRNAの同定が不可欠となる。すなわち，がんの病態そのものに直結したmiRNAを同定することが肝要となる。

2 miRNA機能スクリーニング系の構築

このように，miRNAは正常の生命現象のみならず，がんをはじめとする幅広い疾患との関わりが報告されているが，実際の病態と関連するmiRNAの同定は必ずしも容易ではない。前述の通り，miRNAマイクロアレイを用いたがん組織のmiRNA発現プロファイリングは広く行われているが，一方でいくつかの課題点もある。一つには，miRNAの発現異常は非腫瘍組織の数倍～数分の一程度と軽度のことが多く，発現異常を手がかりにしてmiRNAを絞り込むのに困難を伴うことがある。さらには，発現異常を示すmiRNAが必ずしも病態と直結し，よい治療標的となるとも限らない。

このようなmiRNAの発現プロファイリングと相補的なアプローチとして，「機能スクリーニング系」がある。すなわち，代表的なmiRNA登録データベースであるmiRBase(Release 15, http://www.mirbase.org)に現在登録されている成熟miRNAはヒトで1,000以上，マウスで700以上あるが，これらを細胞に導入し，ある特定の注目する細胞形質に与える効果を半定量的に検出しようとする系である。

われわれは，miRNA発現レンチウイルスライブラリーならびにカスタム作製マイクロアレイを用いてmiRNA機能スクリーニング系を構築した。実際に，ヒト大腸がん細胞株を用いてその増殖を制御するmiRNAを同定可能であったので，その概要について紹介する(図1)。miRNA機能スクリーニングは大きく分けて以下の3段階からなる：1) miRNA発現ウイルスライブラリーの導入，2) 注目する形質によるスクリーニング，3) カスタム作製マイクロアレイを用いた半定量的な検出である。以下，各段階について解説する。

1．miRNA発現ウイルスライブラリーの導入

機能を調べたい細胞へmiRNAを導入し発現させる。miRNA導入方法には，1) ウイルスベクターによるmiRNA前駆体の発現，2) 合成miRNAのトランスフェクションによる導入，の主に2種類があり，それぞれに長所と短所があるが，本スクリーニング系においてはカスタムマイクロアレイを用いた検出過程があることから，前者のmiRNA前駆体発現ウイルスベクターを用いる。数百のクローンを混合したプールライブラリーが市販されており(System Biosciences)，われわれもこれを利用しているが，ウイルスベクターの骨格を利用してライブラリーに含まれないmiRNAの発現ベクターを自作することも可能である。ラ

イブラリーの細胞への導入に当たっては，MOI (multiplicity of infection)や感染させる細胞数などの実験条件の十分な検討が必要である。

2．注目する形質によるスクリーニング

miRNAライブラリーを細胞へ導入した後，注目する形質に影響を与えるmiRNAを発現するクローン数の変化を検出できるような実験系を構成し，スクリーニングを行う。例えば，細胞増殖に影響を与えるmiRNAをスクリーニングする場合は，ライブラリーを導入した細胞株を一定期間継代することにより，関連するクローンの増加または減少を検討する。また，細胞浸潤に影響を与えるものの同定においては，ライブラリー導入後にcell invasion assayを行う。さらには*in vitro*だけでなく，ライブラリーを導入した細胞を動物個体に移植することにより，*in vivo*におけるスクリーニングを行うことも可能である。

3．カスタム作製マイクロアレイを用いた半定量的な検出

2のスクリーニング過程で生じた，miRNA前駆体を発現する各ウイルスベクターのクローン数の変化を，カスタムマイクロアレイを用いて半定量的に検出する。レンチウイルスベクターは，miRNAのstem-loop配列(およそ100 pb程度)ならびにその上下流200～300 bp程度のゲノム配列を含む合計500～700 bp程度を発現するが，これを検出するオリゴヌクレオチドプローブを搭載したマイクロアレイをカスタム作製して用いる。ライブラリー導入直後(t_1)ならびにスクリーニング後(t_2)の細胞より抽出したDNAからPCRにより前駆体部分のみ増幅し，これらをそれぞれCy5ならびにCy3で蛍光ラベル化し，カスタムマイクロアレイ上で競合的にハイブリダイゼーションする。アレイを洗浄後に，レーザースキャナでスキャンし蛍光強度比を算出することで，各miRNAの導入によるコピー数の変化を半定量的に検出できる。例えば，あるmiRNAを発現するmiRNA前駆体のコピー数がt_1よりもt_2で減少していれば，形質を負に制御することになり，t_2で増加していれば正に制御することを意味する。

3 機能スクリーニング系による大腸がん抑制的miRNAの単離 —実験を開始する前に

上述のように，機能スクリーニング系を用いれば，がんの病態変化の誘導に関連したmiRNA分子を短期間で効率よく単離することが可能となる。一方，この方法で同定したmiRNA分子は，解析した腫瘍組織およびその正常組織において発現が認められず，単に*in vitro*で細胞株の表現型に影響を与えるのみで，実際の病態変化には無関係な分子を釣り上げる危険性も考慮する必要がある。いわゆるexperimental artifactである。このリスクを回避し，真にがんの発生・伸展と深く関連するmiRNA分子を同定するためには，機能スクリーニング系と網羅的なゲノム解析との組み合わせは有効である。したがって，どのような機能を有するmiRNAを単離するか，実験開始前にその条件を検討し，複数のスクリーニング法を利用した戦略を考えなければならない。

4 大腸がん抑制的miRNAの条件設定

ひとつの例として，大腸がん抑制因子として機能するmiRNAのスクリーニング法について述べる。大腸がんの発がん過程では，ジェネティックおよびエピジェネティック変異が，がん遺伝子およびがん抑制遺伝子へと多段階的に蓄積することが知られている。このような遺伝子変異は，がん細胞におけるmRNA，さらにはmiRNAの発現プロファイルに大きな影響を与える。大腸発がん過程における最も重要ながん抑制遺伝子は*APC*と*TP53*であることはよく知られている。即ち，われわれが単離を試みる大腸がん抑制的miRNA分子の機能としては，APC変異によって活性化されたWnt経路の遮断機能を有するもの，もしくはp53-がん抑制ネットワークを活性化する分子などが考えられる。

実験を開始する前に，単離すべきmiRNAのクライテリアを定める。

(1) 細胞増殖抑制機能を有する

(2) 正常大腸組織で発現している