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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

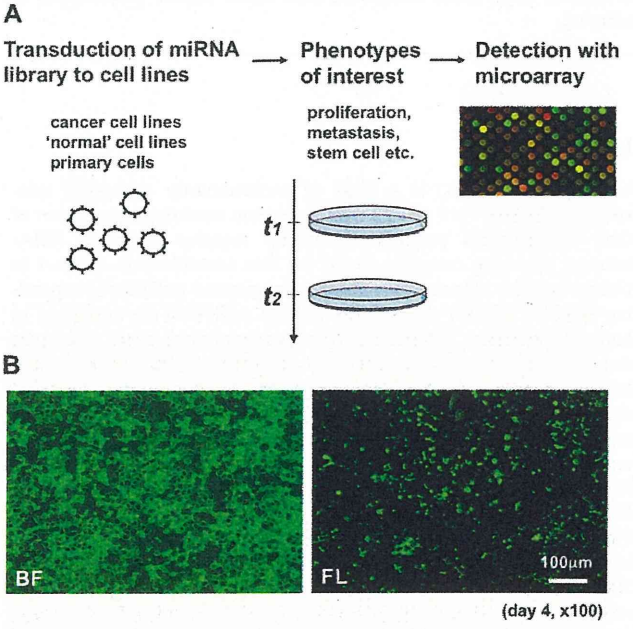

Fig. 1. (**A**) Schematic overview of the functional screening assay of miRNAs. After infection of a pooled lentivirus library (t_1), the phenotypic outcome of each miRNA will be assessed at an appropriate time point (t_2). (**B**) Phase contrast (bright field, BF) and fluorescent (FL) microscopic images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

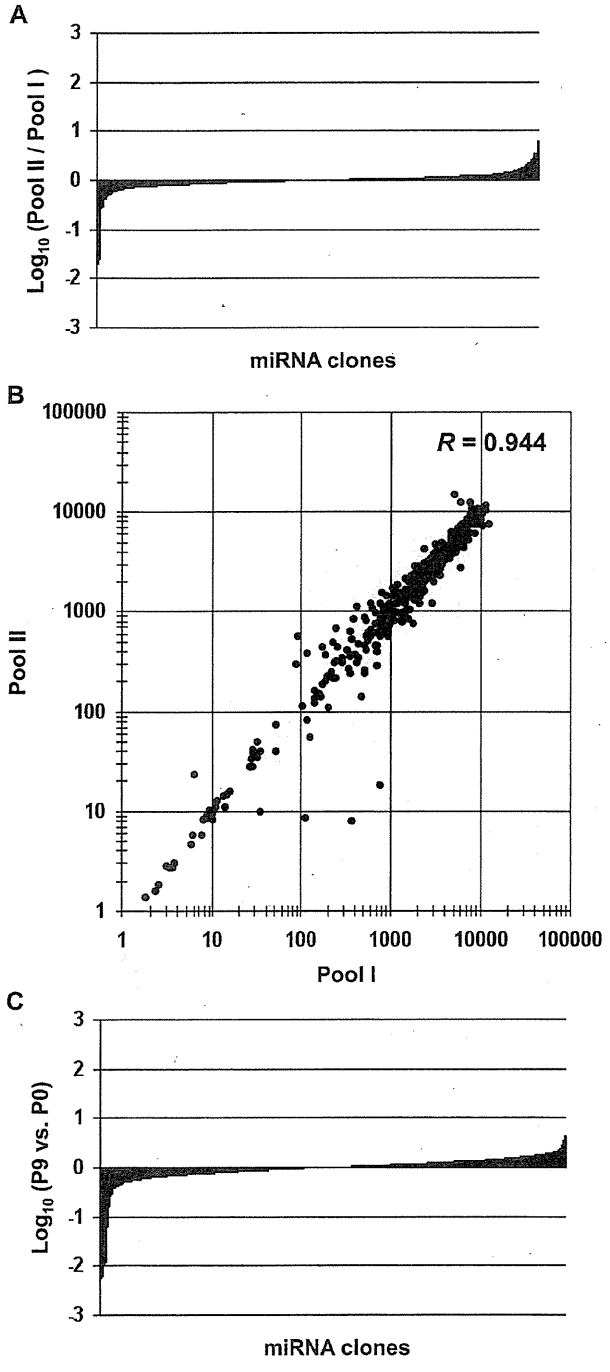

Fig. 2. Distribution of the $\log_{10}$ ratio (**A**) and scatter plot (**B**) of self versus self-microarray hybridization analysis. (**C**) Representative distribution data of the $\log_{10}$ ratio of MIA PaCa-2 cells infected with a pooled lentivirus vector library (P9 versus P0).

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

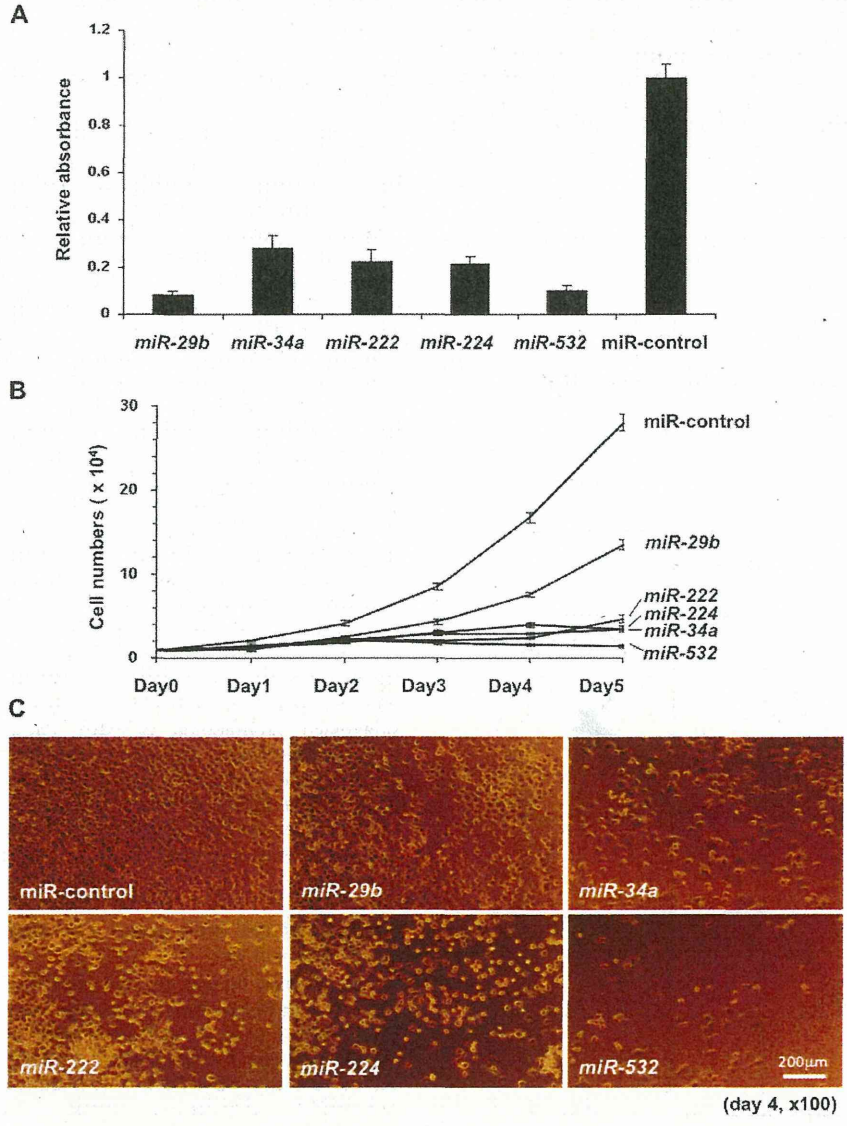

Fig. 3. Cell proliferation assays of MIA PaCa-2 cells. (**A**) 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay of MIA PaCa-2 cells 5 days after infection with lentivirus vectors expressing miRNA, (**B**) Cell proliferation curve and (**C**) Phase contrast micrographs 4 days after transfection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

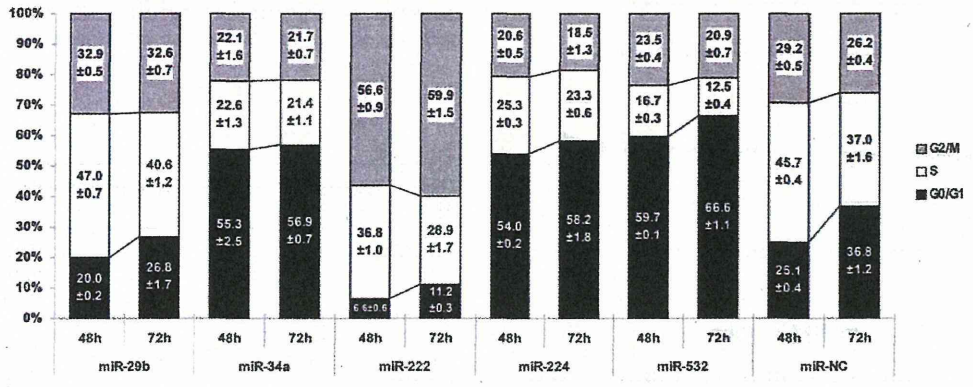

Fig. 4. Distribution of cell cycle phases of MIA PaCa-2 cells 48 and 72 h after transfection with synthetic *miR-29b*, *-34a*, *-222*, *-224*, *-532* and miR-negative control (miR-NC). The average ± standard deviations of three biological replicate samples are shown.

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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Clinical Study

PPARγ Ligand as a Promising Candidate for Colorectal Cancer Chemoprevention: A Pilot Study

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Activating synthetic ligands for peroxisome proliferator-activated receptor gamma (PPARγ), such as pioglitazone, are commonly used to treat persons with diabetes mellitus with improvement of insulin resistance. Several reports have clearly demonstrated that PPARγ ligands could inhibit colorectal cancer cell growth and induce apoptosis. Meanwhile, aberrant crypt foci (ACF) have come to be established as a biomarker of the risk of CRC in azoxymethane-treated mice and rats. In humans, ACF can be detected using magnifying colonoscopy. Previously, CRC and adenoma were used as a target for chemopreventive agents, but it needs a long time to evaluate, however, ACF can be a surrogate marker of CRC even for a brief period. In this clinical study, we investigated the chemopreventive effect of pioglitazone on the development of human ACF as a surrogate marker of CRC. Twenty-nine patients were divided into two groups, 20 were in the endoscopically normal control group and 9 were in the pioglitazone (15 mg/day) group, and ACF and adenoma were examined before and after 1-month treatment. The number of ACF was significantly decreased (5.8 ± 1.1 to 3.3 ± 2.3) after 1 month of pioglitazone treatment, however, there was no significant change in the number of crypts/ACF or in the number and size of adenomas. Pioglitazone may have a clinical application as a cancer-preventive drug. This investigation is just a pilot study, therefore, further clinical studies are needed to show that the PPARγ ligand may be a promising candidate as a chemopreventive agent for colorectal carcinogenesis.

1. Introduction

Peroxisome proliferator-activated receptor gamma (PPARγ) is expressed in adipose tissue and plays a central role in adipocyte differentiation and insulin sensitivity. Activated synthetic ligands for PPARγ are widely used as treatment for type 2 diabetes mellitus (DM) in order to improve insulin resistance. PPARγ is also overexpressed in many tumors [1–5]. Several studies have reported that treatment of cancer cells with PPARγ ligands induces cell differentiation and apoptosis, suggesting their potential application as chemopreventive agents against carcinogenesis [4, 6, 7]. Recent studies have suggested that PPARγ has an inhibitory effect on cancer cell growth [8–10] and might inhibit cell growth and induce apoptosis in adenocarcinomas [9, 11–13], as well as affect tubulin formation *in vitro* [14]. Initial efforts have focused on activation with PPARγ ligands, as these have been shown to induce G1 cell cycle arrest in a variety of tumor cell lines [15, 16]. Su et al. reported that PPARγ agonist inhibits both initiation and progression of colon tumors in the AOM-mouse model study [17]. We have reported previously that PPARγ ligands suppress colonic epithelial cell turnover and colon carcinogenesis through inhibition of the beta-catenin/T cell factor pathway [18], and PPARγ ligands may be potential chemopreventive agents in an azoxymethane-induced colorectal carcinogenesis model and $Apc^{Min/+}$ mice

Table 1: Clinical characteristics of study participants.

	Treatment group		
	Control	Pioglitazone	P value
N	20	9	
Age (years)	63.9 ± 10.0	61.8 ± 5.7	> .05
Waist Circumference (cm)	96.5 ± 13.6	91.8 ± 4.0	> .05
BMI (kg/m^2)	24.4 ± 3.7	23.4 ± 3.1	> .05
VFA (cm^2)	130.0 ± 61.2	146.1 ± 40.9	> .05
SFA (cm^2)	151.5 ± 60.5	141.8 ± 53.5	> .05

Data are expressed as mean ± SD.

model [19, 20]. Colorectal cancer (CRC) is potentially one of the most preventable malignancies [21, 22]. However, the results of clinical trials with PPARγ ligands in CRC have shown only modest results. This implies that focusing on PPARγ as a specific antitumoral target is not likely to be successful, because PPARγ ligands are not an active agent for the treatment of metastatic CRC or liposarcoma [23, 24]. Therefore, we have evaluated chemopreventive effects of PPARγ ligand on the formation of the human aberrant crypt foci (ACF), which is an early stage of colorectal carcinogenesis. ACF were first discovered in mice treated with azoxymethane [25] and have become established as a biomarker of the risk of CRC in azoxymethane-treated mice and rats [26]. In humans, ACF can be detected using magnifying colonoscopy [27]. Previously, CRC and adenoma were used as a target for assessing the efficacy of potential chemopreventive agents; however, this model can only be evaluated over a long period of time. In contrast, the therapeutic efficacy of a product can be evaluated in ACF within a comparatively brief period. We report hire on the results of a study that evaluated the chemopreventive effect of PPARγ ligand by using ACF as a surrogate marker of CRC.

2. Methods

2.1. Magnifying Colonoscopy for Identification of ACF. Bowel preparation for the colonoscopy was carried out using polyethylene glycol solution. A Fujinon EC-490ZW5/M colonoscope was used to perform the magnifying colonoscopy (Fujinon Toshiba ES Systems Co., Ltd, Tokyo, Japan). Total colonoscopy was performed before imaging of rectal ACF. The exclusion criteria included: presence of contraindications to colonoscopy; current or past nonsteroidal anti-inflammatory drug use including aspirin; or family history of CRC; or history of adenoma, carcinoma, familial adenomatous polyposis, inflammatory bowel disease, or radiation colitis. Subjects with a history of colectomy, gastrectomy, or colorectal polypectomy were also excluded. Colorectal adenomas were diagnosed from pathological findings. Subsequently, 0.25% methylene blue was applied to the mucosa of the lower rectal region extending from the middle Houston's valve to the dentate line using spray catheter. ACF were described as lesions consisting of large, thick crypts in methylene blue-stained specimens of the colon (Figure 1). All ACF were recorded photographically and evaluated by two independent endoscopists who were unaware of the subjects' clinical histories. All the patients were divided into two groups, 20 were in the endoscopically normal control group and 9 were in the pioglitazone (PPARγ ligand) group, (15 mg/day); ACF and adenoma were examined before and after 1 month of treatment.

2.2. Measurement of the Visceral and Subcutaneous Fat Areas. Body mass index (BMI) was calculated using the following equation: body weight (kg)/[height (m)^2]. Intra-abdominal adipose tissue was assessed, as previously described, by measuring the visceral fat area (VFA), subcutaneous fat area (SFA), and waist circumference from computed tomographic (CT) images at the level of the umbilicus. All CT scans were carried out with the subjects in the supine position. The borders of the intra-abdominal cavity were outlined on the CT images, and the VFA was quantified using Fat Scan software (N2 System Corporation, Kobe, Japan).

2.3. Statistical Analysis. Data are expressed as mean ± standard deviation (SD), unless otherwise indicated. The relationships between the number of ACF and relevant covariates were examined by univariate regression analysis and determined using the Stat View software (SAS Institute Inc., Cary, NC, USA).

3. Results and Discussion

The clinical characteristics of the study participants are shown in Table 1. There were no significant differences between the groups in terms of their mean age, waist circumference, BMI, VFA, and SFA. The typical colonoscopic features of ACF are shown in Figure 1. The number of ACF was significantly decreased after 1 month's treatment with PPARγ ligand compared with the controls who received no treatment (P = .0226), however, there was no change in the number of crypts/ACF (Table 2) and in ACF size (data not shown). Similarly, after one month of treatment there was no change in the number and size of adenoma (Table 2).

In the present study, pioglitazone treatment decreased the number of ACF, however, the number of crypts/ACF remained unchanged. These results suggest that pioglitazone affects ACF incidence rather than growth. The lack of change in the number and size of the adenomas may have been because the duration of pioglitazone administration was too short to be effective in this respect.

The limitations of this pilot study were its small size, its short duration, and the absence of histological evaluation. Additional research in a large number of subjects is needed to elucidate the clinical effect and benefits of pioglitazone in colorectal carcinogenesis. Chemopreventive trials, the use of medications to prevent disease, have now been carried out extensively in colorectal tumors, for example, supplemental fibers [28], calcium supplementation [29], aspirin [30], nonsteroidal anti-inflammatory drugs (NSAIDs), and selective cyclooxygenase (COX)-2 inhibitors [31, 32], have all been evaluated. Higher doses and longer durations of use of

(a) (b) (c)

FIGURE 1: Typical features of ACF on magnifying colonoscopy with methylene blue staining.

TABLE 2: Effect of PPARγ ligand for human ACF and adenoma.

			Pre-treatment	Post-treatment	*P* value
Number of ACF	Control		5.4 ± 4.0	5.6 ± 5.8	> .05
	Pioglitazone		5.8 ± 1.1	3.3 ± 2.3	.0226
Number of Crypts/ACF	Control		16.8 ± 5.2	17.8 ± 6.4	> .05
	Pioglitazone		14.3 ± 5.9	13.6 ± 6.7	> .05
Number of adenoma	Control		2.0 ± 1.0	1.8 ± 0.8	> .05
	Pioglitazone		2.2 ± 1.5	2.3 ± 2.1	> .05
Mean size of adenoma (mm)	Control		6.9 ± 3.2	7.0 ± 3.3	> .05
	Pioglitazone		5.7 ± 2.8	5.8 ± 2.8	> .05
Location of maximum adenoma	Control	Right side	8	8	
		Left side	12	12	
	Pioglitazone	Right side	4	4	
		Left side	5	5	

Data are expressed as mean ± SD.

NSAIDs and COX-2 inhibitors seem to be associated with greater protection from CRC and adenoma. However, these agents are associated with significant cardiovascular events and/or gastrointestinal harms [33]. Thus, the balance of benefits to risk does not favor chemoprevention by these agents in average-risk individuals. In conclusions, our preliminary results from this pilot study suggest that pioglitazone may have a preventive potential for human ACF and have a good safety profile in this patient population. Further clinical study is required to demonstrate that the PPARγ ligand may be a promising candidate as a chemopreventive agent for colorectal carcinogenesis.

Abbreviations

ACF: Aberrant crypt foci
CRC: Colorectal cancer
PPARγ: Peroxisome proliferator-activated receptor gamma.

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