miRNAs Associated with Mitogen-Activated Protein Kinase in Human Pancreatic Cancer

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Abstract

Aberrant expression of miRNAs is associated with phenotypes of various cancers, including pancreatic cancer. However, the mechanism of the aberrant expression is largely unknown. Activation of the mitogen-activated protein kinase (MAPK) signaling pathway plays a crucial role in gene expression related to the malignant phenotype of pancreatic cancer. Hence, we studied the role of MAPK in the aberrant expression of miRNAs in pancreatic cancer cells. The alterations in expression of 183 miRNAs induced by activation or inactivation of MAPK were assayed in cultured pancreatic cancer cells and HEK293 cells by means of the quantitative real-time PCR method. We found that four miRNAs, namely, miR-7-3, miR-34a, miR-181d, and miR-193b, were preferentially associated with MAPK activity. Among these miRNAs, miR-7-3 was upregulated by active MAPK, whereas the others were downregulated. Promoter assays indicated that the promoter activities of the host genes of miR-7-3 and miR-34a were both downregulated by alteration in MAPK activity. Exogenous overexpression of the MAPK-associated miRNAs had the effect of inhibition of the proliferation of cultured pancreatic cancer cells; miR-193b was found to exhibit the most remarkable inhibition. A search for target genes of miR-193b led to identification of CCND1, NTSE, PLAU, STARD7, STMNI, and YWHAZ as the targets. Translational suppression of these genes by miR-193b was confirmed by reporter assay. These results indicate that activation of MAPK may play a significant role in aberrant expression of miRNAs and their associated phenotypes in pancreatic cancer.

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Introduction

Pancreatic cancer is one of the major leading causes of cancer death (1, 2). Despite advancements in diagnostic and therapeutic techniques, the 5-year survival rate of patients with pancreatic cancer remains less than 10% (3). The very poor prognosis highlights an urgent need to develop effective diagnostic and therapeutic measures. It is particularly important to understand the molecular pathogenesis of cancer because such knowledge is essential for developing a molecularly oriented method of diagnosis and/or targeted therapy. Pancreatic ductal adenocarcinoma, a common type of pancreatic cancer, is characterized by frequent gain-of-function mutations in the KRAS gene, which encodes a GDP/GTP-binding protein that transmits stimulatory signals to several downstream cascades, including the mitogen-activated protein kinase (MAPK) pathway (4, 5). Pancreatic cancer without the KRAS mutation, although it is rare, often has a gain-of-function mutation in BRAF, a member of the MAPK pathway (6). Moreover, pancreatic cancer cells frequently lose the expression of dual-specificity phosphatase 6 (DUSP6), a phosphatase that negatively regulates MAPK activity, which is supposed to be associated with constitutive activation of MAPK in the cells (7). Active MAPK translocates into the nucleus, activates transcription factors, and induces expression of a variety of genes (5). We previously screened downstream target genes of MAPK in pancreatic cancer cells and found that active MAPK actually induces genes implicated in proliferation, survival, and maintenance of cancer cells (8). These observations indicate that activated MAPK signaling may play a crucial role in aberrant expression of genes that are primarily implicated in malignant phenotypes of pancreatic cancer.

miRNAs are endogenous small noncoding RNAs that regulate the expression of target genes by interfering with transcription and/or translation (9, 10). miRNAs play important roles in physiologic development as well as in pathologic states, including cancer (11). Many cancer cells show aberrant expression of miRNAs, and this aberrant expression has been suggested to play important roles in the development and progression of cancers, including pancreatic cancer (12–15). Genetic amplifications or
deletions have been suggested to cause the aberrant expression of miRNA (16). However, such genetic structural alterations may not always be the case. We hypothesize that the MAPK signaling pathway plays significant roles in aberrant expression of miRNAs involved in pancreatic cancer. In this study, we explored miRNAs associated with MAPK activity in pancreatic cancer cells.

**Materials and Methods**

**Cell culture**

Human pancreatic cancer cell lines, MIA PaCa-2, PCI-35, PK-8, Panc-1, and AsPC-1, as well as the human kidney epithelial cell line HEK293 were obtained and cultured as previously described (8).

**Inhibition or activation of MAPK**

The pancreatic cancer cells were seeded in 100-mm culture dishes at 1 × 10⁶ cells per dish with an appropriate culture medium containing 10% FBS and cultured under 5% CO₂ with appropriate humidity. After 24 hours, the medium was replaced with a medium containing U0126, an inhibitor of MAPK kinase (MAP2K), dissolved in dimethyl sulfoxide (DMSO) at a final concentration of 10 μmol/L, or replaced with a medium containing the same volume of DMSO as a control. After 24 hours, RNA extraction and protein assay were carried out. HEK293 cells were seeded in a 6-well culture plate at 5 × 10³ cells per well in minimal essential medium with 10% FBS and incubated in 5% CO₂ with appropriate humidity. After 24 hours, the cells were transfected with 8 μg of pcDNA3.1-V5/His only; 4 μg of pcDNA3.1-V5/His and 4 μg of pcDNA3.1-active MAP2K1 (MAP2K1D44--51/S218E/S222E)-V5/His; 4 μg of pcDNA3.1-DUSP6-V5/His and 4 μg of pcDNA3.1-active MAP2K1-V5/His; or 4 μg of pcDNA3.1-V5/His and 4 μg of pcDNA3.1-DUSP6-V5/His using Lipofectamine PLUS (Life Technologies) according to the manufacturer’s instructions. Forty-eight hours after the transfection, expression of each miRNA was assayed by quantitative real-time PCR assay because it enabled sensitive and specific quantification of mature miRNAs. These 183 miRNAs were almost all of assayable miRNAs by using the kit when we started this study (January 2006), as listed in Supplementary Table S1. The end products of the TaqMan assay were cloned into a pCR vector (Stratagene) and were used as standards for quantification in the real-time PCR assay. RNU-6, RNU-24, and RNU-48 were used as an endogenous control for expression analysis. An miRNA precursor assay (Qiagen GmbH) to determine expression of MIR7-1, MIR7-2, and MIR7-3, which are possible precursors of miR-7, was carried out with detection of SNORD48 as a control according to the manufacturer’s instructions.

**Cell proliferation assay with transfection of oligonucleotides**

Cells were seeded at 6 × 10³ per well in 96-well plates and transfected with precursor molecules, antisense molecules, or control molecules of miRNAs (Life Technologies) at 30 nmol/L using siPORT NeoFX (Life Technologies) according to the manufacturer’s instructions. Forty-eight hours after the transfection, expression of each miRNA was assayed by quantitative real-time PCR using the Cell-to-CT Kit (Life Technologies). A colorimetric cell proliferation assay employing 0.05% MTT (Sigma) was carried out as described previously (7).

**Promoter assay**

Promoter regions spanning 1,980 base pairs (bp) upstream of EST db286351 harboring MIR34a (17) and 1,921 bp upstream of PGSF1 harboring MIR7-3 (miRBase: http://microrna.sanger.ac.uk/sequences/index.shtml) were amplified using a KOD-plus DNA Polymerase Kit (Toyobo Co. Ltd.) with a paired appropriate primers listed in Supplementary Table S2 and human genomic DNA as a template in the following condition; initial denaturation for 2 minutes at 94°C, 35 cycles of reactions comprising 30 seconds at 94°C, 30 seconds at 58°C, and 30 seconds.
at 68°C, and a final extension for 2 minutes at 68°C. The amplified product was purified with a High-Pure DNA Purification Kit (Roche Diagnostics), digested with appropriate restriction enzymes, depending on 5’ sequences of the primers, and cloned into the reporter vector, pGL3-Basic (Promega Corporation). DNA sequences of the cloned fragments were confirmed by using Bigdyne terminator (Life Technologies) and 3130x Genetic Analyzer (Life Technologies). MIA PaCa-2 cells were seeded at 5 × 10⁴ cells per well in a 6-well plate. The cells were transfected 24 hours after the seeding with 500 ng of the constructed pGL3-promoter vectors and 50 or 10 ng of phRL-TK vector (Promega) using Lipofectamine PLUS (Life Technologies) according to the manufacturer’s instructions. Three hours after the transfection, the cells were treated with U0126 or DMSO, as described above, and maintained for 24 hours. HEK293 cells were seeded at 5 × 10⁵ cells per well in a 6-well plate and transfected with each of the constructed pGL3-promoter vectors, a phRL-TK vector, and various combinations of pcDNA 3.1-active MAP2K-V5/His, pcDNA 3.1-DUSP6-V5/His, and pcDNA 3.1-V5/His vectors as described above. The transfected cells were maintained for 48 hours and then subjected to the reporter assay using the Dual-Luciferase Reporter Assay System (Promega) and the Luminoskan Ascent luminometer (Thermo Fisher Scientific Inc.) according to the manufacturers’ instructions.

Microarray

Transcriptome microarray analysis was carried out using the 44K Whole Human Genome Microarray (Agilent Technologies) of a one-color experiment system according to the manufacturer’s instructions. Hybridized samples were total RNAs extracted from MIA PaCa-2 cells transfected with the precursor molecules of miR-193b or that of negative

Figure 1. Alteration of activity of MAPK and expression of miRNA. A, immunoblots showing expression of active/phosphorylated MAPK and MAPK1/2 in cultured pancreatic cancer cells, MIA PaCa-2 and PCI-35, treated with U0126, the specific inhibitor for MAP2K, or DMSO. Expression of beta actin (ACTB) is included as a loading control. B, altered expression of miRNAs greater than 1.2-fold or less than 0.8-fold with statistical significance in pancreatic cancer cells, MIA PaCa-2 (closed bar) and PCI-35 (open bar), treated with U0126 compared with those treated with DMSO determined using quantitative real-time PCR. C, immunoblot showing expression of active/phosphorylated MAPK, MAPK1/2, and DUSP6 in HEK293 cells transfected with pcDNA-active MAP2K-V5/His; pcDNA-active MAP2K-V5/His and pcDNA-DUSP6-V5/His; pcDNA-DUSP6-V5/His; and pcDNA-V5/His. Expression of beta actin (ACTB) is included as a loading control. D, altered expression of miRNAs in HEK293 cells transfected with the active MAP2K vector compared with that transfected with the DUSP6 vector determined using quantitative real-time PCR. Closed bars indicate miRNAs exhibiting inverse responses relative to the altered expression in pancreatic cancer cells with downregulation of MAPK.
control (Life Technologies). Scanning and data processing was done by Hokkaido System Science Co., Ltd. Data analysis was done by using GeneSpring GX 10.0 software (Agilent Technologies). Altered expression of candidate genes was validated by quantitative real-time PCR employing the TaqMan system (Life Technologies) according to the manufacturer’s instructions.

Translational repression assay

Portions of the 3′-untranslated regions (3′-UTR) harboring seed sequences of candidate genes were amplified with paired primers listed in Supplementary Table S2 and pooled cDNAs obtained from human cDNA libraries (Stratagene) as templates using the KOD-plus DNA Polymerase Kit (Toyobo Co. Ltd.) in the following condition; initial denaturation for 2 minutes at 94°C, 35 cycles of reactions comprising 30 seconds at 94°C, 30 seconds at appropriate annealing temperature, and 90 seconds at 68°C, and final extension for 5 minutes at 68°C. The amplified product was purified with a HighPure DNA Purification Kit (Roche Diagnostics), digested with XbaI or SpeI depending on 5′ sequences of the primers, and cloned into pGL3 control vector (Promega) at XbaI site immediately after Luciferase coding sequence. Mutant vectors harboring substituted sequences of the core seed sequence, 5′-GGCCAGT(T/A/G)-3′, with a mutated sequence, 5′-AAAAAGT(T/A/G)-3′, in 3′-UTR were generated by using mutant primers listed in Supplementary Table S2 and QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies). DNA sequences of the cloned fragments were confirmed by using Bigdye terminator and 3130x Genetic Analyzer. HEK293 cells were seeded at 8 × 10⁴ cells per well in a 12-well plate and transfected with 400 ng of the constructed pGL3-control vectors, 50 ng of phRL-TK, and the precursor molecule of miR-193b or the control molecule (Life Technologies) at a final concentration of 30 nmol/L using Lipofectamine 2000 (Life Technologies) according to the manufacture’s instruction. The transfected cells were maintained in an appropriate culture medium for 24 hours and then subjected to the reporter assay.

Statistical analysis

Every experiment was conducted at least twice. Statistical analyses were done using Microsoft Office Excel 2007 (Microsoft Corp.). *P* values less than 0.05 were considered statistically significant.

Results

miRNAs associated with MAPK activity

To identify the miRNAs associated with MAPK activity in pancreatic cancer cells, we assayed the alterations of expression of a total of 183 miRNAs in MIA PaCa-2 and PCI-35, cultured pancreatic cancer cell lines with constitutively active MAPK, which were treated with U0126, an inhibitor of MAP2K to inhibit MAPK activity (Fig. 1A). The expression was determined using the quantitative real-time PCR method (Supplementary Table S1). The miRNAs exhibiting altered expression by more than 1.2-fold or less than 0.8-fold with significant statistical differences as a result of inhibition of MAPK in accordance with the 2 pancreatic cancer cell lines compared with those with mock (DMSO) treated were chosen as candidate miRNAs associated with MAPK activity. The 11 candidate miRNAs detected were miR-7, miR-23b, miR-34a, miR-149,
Figure 3. Associations between exogenously altered expressions of miRNAs and proliferation of pancreatic cancer cells. A, altered expression of miRNAs induced by transfection of antisense (anti-) or precursor (pre-) oligonucleotides for miRNAs determined by quantitative real-time PCR. B and C, proliferation of pancreatic cancer cells transfected with precursor (pre-) or antisense (anti-) oligonucleotides determined using the colorimetric proliferation assay.

\( ^*, \ P < 0.05 \) as determined by the unpaired Student t test.
miR-181a, miR-181b, miR-181d, miR-193b, miR-432#, miR-502, and miR-503 (Fig. 1B). Expression of these miRNAs was assayed in HEK293 cells transfected with a vector expressing the constitutive active MAP2K to induce active MAPK because the cells show low level of endogenous MAPK activity (Fig. 1C). The miRNAs revealing inverse alterations of expression relative to the alterations in pancreatic cancer cells induced by treatment with U0126 were determined to be tight MAPK-responsive miRNAs. These were 4 miRNAs, namely, miR-7, miR-34a, miR-181d, and miR-193b (Fig. 1D). The association of these miRNAs with MAPK activity was further validated in other pancreatic cancer cell lines showing different levels of MAPK activation, namely, PK-8, Panc-1, and ASPC-1 (Fig. 2). These results indicated that MAPK activation induced upregulation of miR-7, whereas down-regulation of miR-34a, miR-181d, and miR-193b in pancreatic cancer cells.

**Functional significance of miRNAs associated with MAPK activity**

To determine the functional significance of these 4 miRNAs associated with MAPK activity in pancreatic cancer cells, we examined whether cellular proliferation is altered by exogenous overexpression or underexpression of these miRNAs. Transfection of precursors of the 4 miRNAs induced overexpression of miRNAs and resulted in inhibition of cellular proliferation in all of the miRNAs (Fig. 3). Among these 4 miRNAs, miR-193b showed the most remarkable inhibition of proliferation (Fig. 3B). On the other hand, transfection of antisense oligonucleotides against miRNAs induced little suppression of the endogenous expression of miRNAs and did not show obvious alteration of proliferation (Fig. 3C). This apparent lacking of responses by anti-miRNAs may be due to low levels of endogenous expression of the target miRNAs. Hence, these results at least indicated that exogenous overexpression of MAPK-associated miRNAs induces inhibition of proliferation of pancreatic cancer cells.

**Promoter activity of miRNAs associated with MAPK activity**

To characterize the mechanism of regulation of miRNA expression by MAPK, we hypothesized that transcription of miRNAs is directly regulated by MAPK. Therefore, we examined the association between the promoter activities of host genes of intragenic miRNAs, namely, miR-7 and miR-34a, and MAPK activity. According to miRBase (http://www.mirbase.org/index.shtml), there are 3 distinct endogenous genes known as MIR7-1, MIR7-2, and MIR7-3, in which miR-7 is potentially transcribed in the human genome. To determine which endogenous gene is associated with MAPK activity, endogenous precursor transcripts of these MIR7 genes were assayed in cells treated with MAPK inhibitor. It was found that MIR7-3 revealed high endogenous expression and showed strong responses to alterations of MAPK activity (Fig. 4). According to published reports and miRbase, MIR7-3 and MIR34a are harbored in PG5F1 and EST db286351, respectively (Fig. 5A and B; ref. 17). We cloned the promoter regions of these host genes into reporter vectors and confirmed that the cloned promoter regions both exhibit promoter activities (Fig. 5C and D). To know response of the alteration of MAPK activity, the promoter activities were assayed in pancreatic cancer cells with respect to inhibition of MAPK and then in HEK293, with respect to activation of MAPK. Unexpectedly, the results indicated that both inhibition and activation of MAPK induce the downregulation of the promoter activities (Fig. 5E and F).

**Identification of target genes of miRNAs**

We proceeded to determination of target genes of miR-193b because overexpression of miR-193b significantly inhibits the proliferation of pancreatic cancer cells. MIA PaCa-2 exogenously overexpressing miR-193b was subjected to microarray-based transcriptome assay to screen genes of altered expression (Fig. 6A). Expression profiles were deposited to Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE25215. We identified 53 genes that were...
downregulated more than 2.5-fold by the overexpression of miR-193b among 44K human genes and transcripts. These 53 genes were compared with 142 candidate target genes obtained by the search using the TargetScan program (http://www.targetscan.org; refs. 18–20). This resulted in the identification of 25 genes followed by further narrowing to 15 genes that harbor highly conserved seed sequences for miR-193b. We validated inhibition of expression of these 15 candidate genes in cells exogenously overexpressing miR-193b (Fig. 6B). Then we chose 6 evidently expressed genes, namely, CCND1, NT5E, PLAU, STARD7, STNM1, and YWHAZ, among the 15 genes for further validation of translational suppression by miR-193b. 3′-UTR of these candidate genes harboring the core seed sequence for miR-193b, 5′-GGCCAGT(T/A/G)-3′, was cloned into immediate downstream of the coding region of a reporter gene in a reporter vector, to know whether the 3′-UTR causes suppression of expression of the reporter gene by interacting with miR-193b. As a result, expression of the reporter gene with the 3′-UTRs of candidate genes was
found to be variably, but significantly, suppressed by miR-193b (Fig. 6C). We also constructed reporter vectors harboring 3'UTR with a mutated seed sequence, in which the core seed sequence 5'-GGCCAGT(T/A/G)-3' was substituted with a mutated sequence, 5'-AAAAAGT(T/A/G)-3', to test a specificity of the core seed sequence for the suppressive function of miR-193b. We observed that these mutated vectors showed canceled or reduced suppressive activities of miR-193b (Fig. 6C).

In addition, to know functional evidences of target genes of the MAPK-associated miRNAs, we did a meta-analysis using TargetScan and g:Profiler (http://biit.cs.ut.ee/gprofiler/gcocoa.cgi) for obtaining a gene list according to functional categories termed by Gene Ontology (http://www.geneontology.org/; Supplementary Table S3).

**Discussion**

In this study, we identified miRNAs associated with MAPK activity in pancreatic cancer cells. Active MAPK induced upregulation of miR-7, whereas downregulation of miR-34a, miR-181d, and miR-193b. The exogenous overexpression of any of these miRNAs induces inhibition of proliferation of cultured pancreatic cancer cells. The highest inhibiting activity was noted for miR-193b. Promoter activities of the host genes of miR-7-3 and miR-34a were suppressed by alteration of MAPK activity. Moreover, we identified target genes of miR-193b as CCND1, NT5E, PLAU, STARD7, STMN1, and YWHAZ. These results indicate that MAPK may contribute to regulation of miRNAs and phenotypes of pancreatic cancer.
miR-7 has been known to target EGFR and RAF1 and to induce cell-cycle arrest and apoptosis in a human lung cancer cell line (21). EGFR encodes epidermal growth factor receptor (EGFR), a membrane-bound tyrosine kinase receptor and one of the major upstream regulators of MAPK. RAF1 encodes v-raf-1 murine leukemia viral oncogene homolog 1 (RAF1), a serine/threonine protein kinase belonging to the MAPK pathway. Our study showed that endogenous expression of miR-7 in pancreatic cancer cells was positively associated with MAPK activity. This finding suggests that EGFR–MAPK–miR-7 may form a negative feedback loop to regulate the activity of the pathway. Exogenous overexpression of miR-7 suppresses the proliferation of the pancreatic cancer cells we employed that had the gain-of-function mutations in KRAS. This effect may be associated with targeted suppression of RAF1, which is an issue for further study.

miR-34a is a highly conserved miRNA and is known to be a downstream target of tumor protein 53 (TP53), a tumor suppressor (17, 22). miR-34a is upregulated by TP53 and to target and interfere with cyclins and cyclin-dependent kinases. Because this process attenuates cellular proliferation, miR-34a is regarded as a tumor-suppressive miRNA (22). Expression of miR-34a is commonly in a low level in cancer cells (12, 17). The low expression is supposed to be associated with loss of 1p36, where miR-34a resides, or a loss of function of TP53 (17, 23). Our study showed that miR-34a was inversely associated with MAPK activity, in which MAPK activation suppressed miR-34a expression, and its overexpression inhibited proliferation of pancreatic cancer cells. The cells we employed for this study, MIA PaCa-2 and PCI-35, are known to have dysfunctional TP53 (24, 25), which may synergistically cause low expression of miR-34a in these cells along with active MAPK. Nevertheless, activation of MAPK may counteract TP53 function in controlling of expression of miR-34a, which can contribute to attenuate tumor-suppressive function of TP53 pathway. Our study indicated that miR-181d was suppressed by active MAPK, and its exogenous overexpression induced inhibition of proliferation of pancreatic cancer cells. Although miR-181d has not yet been investigated in oncologic research, it is shown to play a role in lipid deposition and proliferation of hepatocyte (26). Our result suggests that miR-181d may play an antioncogenic role in pancreatic cancer.

miR-193b has recently emerged as one of cancer-associated miRNAs (27, 28). In breast cancer cells, miR-193b is downregulated and the downregulation is associated with activation of urokinase-type plasminogen activator (PLAU), which results in promotion of invasion and metastasis (27). In melanoma cells, miR-193b is also downregulated, and exogenous overexpression of miR-193b inhibits cellular proliferation potentially by interfering with CCND1, one of the important cyclins that promote the process of cellular proliferation, which is overexpressed in many cancers (28). Our study showed that miR-193b was suppressed by MAPK in pancreatic cancer cells, and its exogenous overexpression remarkably inhibited proliferation of the cells. We identified CCND1, NT5E, PLAU, STARD7, STMN1, and YWHAZ as target genes of miR-193b, in which CCND1 and PLAU are already identified as described above. NT5E encodes ecto-5’-nucleotidase, which predomnately catalyzes the conversion of AMP to adenosine (29). Ecto-5’-nucleotidase has been shown to be implicated in cancer phenotypes involving tumor growth, angiogenesis, and cytoprotection by accumulation of adenosine (30). STARD7 encodes protein containing START domain that can bind to lipids (31). It is overexpressed in trophoblastic tumor and hepatocellular carcinoma, in which it is supposed to play promoting roles in their malignant phenotypes (32, 33). STMN-1 encodes stathmin/oncoprotein-18 that is overexpressed in breast cancer, hepatocellular carcinoma, sarcoma, and lung adenocarcinoma (34–37). Stathmin/oncoprotein-18 has been found to be associated with MAPK and promotes cell-cycle progression (38). YWHAZ encodes 14-3-3 zeta, a member of the 14-3-3 family of proteins, that is overexpressed and supposed to play a role in proliferation and malignant phenotypes in breast and head and neck cancers (39, 40). Although detailed functions of these target genes in pancreatic cancer cells are issues of further study, our current study indicates that suppression of miR-193b by MAPK plays a role in expression of these target genes and proliferation of pancreatic cancer cells, which suggests that miR-193b may function as a tumor-suppressive miRNA in pancreatic cancer.

Expression of miRNA is mediated by RNA polymerase II (41). This protein requires a promoter to initiate transcription; hence, the promoter must play a role in controlling of expression of miRNAs. miRNAs embedded in known genes are called intragenic miRNAs. Genes harboring miRNAs are called host genes, and the promoter of the host gene is supposed to control the transcription of miRNA (42). miRNAs not embedded in known genes are called intergenic miRNAs, and promoters for these intergenic miRNAs are largely unknown (42). Therefore, to investigate the mechanism of transcription of miRNAs under control of MAPK activity, we tested promoter activities of host genes of miR-7 and miR-34a. These respective promoter activities were supposed to show opposite responses to MAPK activity, that is, activation of MAPK would upregulate the promoter activity of host gene of miR-7 but downregulate that of miR-34a. However, our experiments employing pancreatic cancer cells, in which MAPK was inactivated, and HEK293 cells, in which MAPK was activated, unexpectedly showed that both inactivation and activation of MAPK nevertheless induces downregulation of the promoter activities. This result seems to indicate that the promoter activities of the host genes we tested were not directly associated with miRNA expression responding to MAPK activity. The possible explanation for this result could be that either the promoters of the host genes are not associated with the modulation of these miRNA by MAPK or the modulation motifs of the studied miRNAs by MAPK are not located in the host gene promoter region. In regard to the expression machinery of miRNA, primary miRNA transcribed as a part of a host gene or an independent transcript is subsequently processed into

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precursor miRNA and mature miRNA (41). This process involves multiple reactions, including the Drosophila reaction, nuclear export, the dicer reaction, and the helicase reaction (41). We may need to investigate these posttranscriptional processes to clarify the mechanism of association between miRNA expression and MAPK activity. We did not determine promoter activities of miR-181d and miR-193b because these were intergenic miRNAs that were not associated with any known transcribed genes.

We may need to note that we used U0126, an inhibitor of MAP2K, to induce suppression of active MAPK in pancreatic cancer cells and transfection of the constitutive active form of MAP2K to induce activation of MAPK in HEK293 cells. Because both of these treatments primarily targeted MAP2K, they might generate an indirect effect, besides controlling of MAPK activity strictly, which may indicate a possibility that the modulations of miRNAs noticed would not be only specific for MAPK activity.

Potential targets of the MAPK-associated miRNAs listed in the Supplementary Table S3 span quite a broad range of genes with diverse functions. However obviously, most of them seem to be pertained in phenotypes of cancers by regulation of exogenous as well as endogenous stimulations, metabolic, and catalytic controls, as well as nuclear signaling in cells. And this list also implies that MAPK-associated genes can span quite a number of genes just by controlling a few miRNAs. Hence, MAPK could play an important role in gene regulation not only by direct regulation of target transcription factors but also by indirect regulation via miRNAs, which is crucial for understanding various malignant phenotypes of pancreatic cancer.

miRNAs associated with MAPK activity have the potential to be used to treat pancreatic cancer. The 4 miRNAs we identified were found to be capable of inducing inhibition of proliferation of pancreatic cancer cells. Although direct delivery of miRNA in cancer cells in vivo may be a difficult task, the miRNAs as well as their target molecules are attractive candidates for development of novel therapeutic intervention for pancreatic cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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