MEK Inhibitor Suppresses Expression of the miR-17-92 Cluster with G₁-Phase Arrest in HT-29 Human Colon Cancer Cells and MIA PaCa-2 Pancreatic Cancer Cells

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Abstract. *Background: MicroRNAs (miRNAs) are small non-coding RNAs, and the deregulated expression of miRNAs is associated with tumor development. Among these, the miR-17-92 cluster, including six mature miRNAs, is known as an oncogenic miRNA cluster because expression of the miR-17-92 cluster is frequently elevated in a variety of malignant tumors. Materials and Methods: We investigated whether a mitogen-activated protein kinase kinase (MEK) inhibitor, PD0325901, suppresses expression of the miR-17-92 cluster in HT-29 human colon cancer cells and MIA PaCa-2 pancreatic cancer cells. Results: PD0325901 inhibited cell growth with G₁-phase arrest and suppressed expression of the miR-17-92 cluster. Furthermore, phosphatase and tensin homolog (PTEN), which is a target molecule of the miR-17-92 cluster, was up-regulated by PD0325901. The exogenous expression of miR-17 slightly, but significantly reduced G₁-phase arrest by PD0325901. Conclusion: These results raise the possibility that a MEK inhibitor causes G₁-phase arrest, at least partially, through suppression of the miR-17-92 cluster.*

MicroRNAs (miRNAs) are small non-coding RNAs (approximately 22 nucleotides in length) that inhibit the protein translation of target mRNAs to fine-tune the expression of target proteins to control normal development and cell homeostasis. Deregulated expression of miRNAs can be associated with various diseases, including cancer (1). Clustering of miRNA genes is frequently observed in

genomes, and the expression of miRNAs in a cluster is co-regulated, thereby playing a role in a common molecular process (2). Among miRNA clusters, the miR-17-92 cluster, including the six mature miRNAs, miR-17, miR-18a, miR-19a, miR-19b, miR-20a and miR-92a, is a representative oncogenic miRNA cluster and it has been named oncomir-1 (3). Expression of the miR-17-92 cluster is frequently high in a variety of malignant tumors, and although the oncogenic roles of the miR-17-92 cluster in tumor development have been investigated, they are not fully understood (4-6).

Oncogenes, including *KRAS* and *BRAF*, are activated by gain-of-function mutations in many human tumors. It has been reported that mitogen-activated protein kinase kinase (MEK) inhibitor inhibited cell growth in many tumor cell lines harboring *BRAF* mutations and in some tumor cell lines harboring *KRAS* mutations (7). The molecular mechanism of cell growth inhibition by MEK inhibitor consists of multiple events, including up-regulation of tumor-suppressive molecules p15 and p27 (8, 9) and down-regulation of oncogenic molecule cyclin D1 (10).

We investigated whether MEK inhibitor suppresses expression of the oncogenic miR-17-92 cluster in HT-29 human colon cancer cells harboring a *BRAF* mutation and MIA PaCa-2 pancreatic cancer cells harboring a *KRAS* mutation. Both cell lines are known to express the miR-17-92 cluster (11, 12).

Materials and Methods

Reagents. MEK inhibitor PD0325901 was purchased from Cayman Chemical (Ann Arbor, MI, USA) and dissolved in dimethyl sulfoxide (DMSO).

Cell culture. The HT-29 human colon cancer cell line was purchased as a cell line of the NCI-60 from the National Cancer Institute Developmental Therapeutics Program (Bethesda, MD, USA). The MIA PaCa-2 human pancreatic cancer cell line was purchased from Health Science Research Resources Bank (Sennan, Japan). HT-29

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cells and MIA PaCa-2 cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 4 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂.

Cell growth assay. Cells were seeded at a density of 1×10³ cells in a 96-well plate and maintained for 24 h prior to treatment with different concentrations of PD0325901. After 72-h treatment, cell proliferation was determined using a WST-8 assay with a Cell Counting Kit-8 (Dojindo Molecular Technology, Kumamoto, Japan).

Cell-cycle analysis. Cells were seeded at a density of 3×10⁴ cells in a six-well plate and maintained for 24 h prior to treatment with PD0325901 or DMSO. After 24-h and 48-h treatment, cells were harvested from the culture dishes. After washing with phosphate-buffered saline (PBS), the cells were suspended in PBS containing 0.1% Triton X-100, treated with RNase A, and the nuclei were stained with 100 µM propidium iodide. The DNA content was measured using a FACSCalibur flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA) and the data were analyzed with Cell Quest software (Becton-Dickinson) and the ModFit LT V2.0 software package (Verity Software, Topsham, ME, USA).

Western blotting analysis. Cells were lysed in lysis buffer containing 50 mM Tris-HCl (pH 7.5) and 1% sodium dodecyl sulfate. The protein extract was then boiled for 5 min and loaded onto a 7.5%, 10%, or 12.5% polyacrylamide gel, subjected to electrophoresis and transferred to a polyvinylidene difluoride membrane. The following antibodies were used as primary antibodies: mouse monoclonal anti-c-MYC (Santa Cruz Biotechnology, Inc., Dallas, TX, USA), rabbit monoclonal anti-phosphatase and tensin homolog (PTEN) (Cell Signaling Technology, Inc, Danvers, MA, USA), and mouse monoclonal anti-β-actin (Sigma-Aldrich, St. Louis, MO, USA). Enhanced chemiluminescence (GE Healthcare Bio-Sciences, Pittsburgh, USA) and Immobilon Western (EMD Millipore, Billerica, MA, USA) were used for detection.

miRNA expression analysis. Analysis of miRNA expression was performed using TaqMan miRNA assays (Applied Biosystems, Foster City, CA, USA) to evaluate *miR-17*, *miR-18a*, *miR-19a*, *miR-19b*, *miR-20a*, and *miR-92a*. cDNA was purified from 10 ng total RNA using specific primers in accordance with the TaqMan miRNA assay protocol. Quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) was performed in accordance with the manufacturer's protocol. Every product was measured using a 7300 Real-time RT-PCR system (Applied Biosystems). The expression level of each miRNA was normalized using *miR-16* or *small nucleolar RNA, C/D box 48 (RNU48)*.

miRNA mimic transfection. A mirVana miRNA mimic specific for *miR-17* and mirVana miRNA Mimic Negative Control #1 were purchased from Ambion (Austin, TX, USA). HT-29 cells and MIA PaCa-2 cells were seeded at a density of 3×10⁴ cells in six-well plates and cultured for 24 h. The cells were then transfected with *miR-17* mimic or miRNA Mimic Negative Control #1 using Lipofectamine RNAiMax (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's protocol. Twenty-four hours after transfection, the cells were treated with PD0325901 or DMSO and maintained for a further 24 h. The cells were then harvested, and cell-cycle analysis was performed as described above.

Statistical analysis. Statistical analysis of the data was performed using Student's *t*-test for comparison between treatments and controls. A value of *p*<0.05 was considered significant.

Results

MEK inhibitor inhibits cell growth with G₁-phase arrest of HT-29 human colon cancer cells and MIA PaCa-2 pancreatic cancer cells. Here, we investigated the effects of PD0325901 on *BRAF*-mutated HT-29 human colon cancer cells. After treatment with different concentrations of PD0325901 for 72 h, PD0325901 dose-dependently inhibited cell growth (Figure 1A). In *KRAS*-mutated MIA PaCa-2 human pancreatic cancer cells, PD0325901 also inhibited cell growth (Figure 1B). We then performed cell-cycle analysis using flow cytometry. Treatment with PD0325901 for 24 h or 48 h significantly increased the G₁-phase population of HT-29 cells in a dose-dependent manner (Figure 2A). PD0325901 also significantly increased the G₁-phase population of MIA PaCa-2 cells in a dose-dependent manner (Figure 2B). These data demonstrate that PD0325901 inhibited cell growth with G₁-phase arrest in both HT-29 cells and MIA PaCa-2 cells.

PD0325901 suppresses expression of the miR-17-92 cluster and c-MYC, and up-regulates PTEN, one of the target molecules of the miR-17-92 cluster. We investigated the effects of PD0325901 on expression of the oncogenic miR-17-92 cluster. After treatment with 16 µM PD0325901 for 24 h and 48 h, the expression of all miRNAs in the miR-17-92 cluster was suppressed in HT-29 cells (Figure 3A). In MIA PaCa-2 cells, the suppression of expression of the miR-17-92 cluster was also observed after treatment of PD0325901 (Figure 3B). It has been reported that expression levels of the miR-17-92 cluster are up-regulated by c-MYC through transcriptional regulation (3); therefore, we evaluated the effect of PD0325901 on the expression of c-MYC. As a result, the expression of c-MYC was down-regulated by treatment with PD0325901 for 24 h in both cell lines (Figure 4A and B). PTEN is a known target molecule of the miR-17-92 cluster (13); therefore, we evaluated the effect of PD0325901 on the expression of PTEN. As a result, PTEN expression was up-regulated in HT-29 cells by treatment with PD0325901 for 24 h and 48 h (Figure 4C) and in MIA PaCa-2 cells treated for 48 h (Figure 4D).

Overexpression of miR-17 decreases G₁-phase arrest induced by PD0325901. *miR-17* in the miR-17-92 cluster is known to play a critical role in resistance to MEK inhibitor (14). Therefore, we investigated whether overexpression of *miR-17* suppressed G₁-phase arrest by PD0325901. After transfection with the *miR-17* mimic, HT-29 cells were incubated with PD0325901 for 24 h. As shown in Figure 5A, overexpression of the *miR-17* mimic reduced G₁-phase arrest of HT-29 cells induced by PD0325901. In addition, overexpression of the

Figure 1. PD0325901, a mitogen-activated protein kinase kinase inhibitor, inhibits cell growth. HT-29 (A) and MIA PaCa-2 (B) cells were treated with different concentrations of PD0325901. After 72 h treatment, cell growth was determined using a WST-8 assay. The data obtained with DMSO were taken as 100%. Data are the means $\pm$ SD (n=3). **p<0.01, compared to the DMSO-treated control.

Figure 2. PD0325901 increases G₁-phase cell population. HT-29 (A) and MIA PaCa-2 (B) cells were treated with different concentrations of PD0325901. After 24 h and 48 h treatment, the cell-cycle distribution was analyzed by flow cytometry. The percentages of cells in G₁, S, and G₂/M phases of the cell cycle were analyzed using the Modfit LT software. Data are the means $\pm$ SD (n=3). *p<0.05 and **p<0.01, compared to the dimethyl sulfoxide-treated control, indicated as 0 nM.

Figure 3. PD0325901 suppresses expression of the miR-17-92 cluster. HT-29 (A) and MIA PaCa-2 (B) cells were treated with and without 16 μ M PD0325901. After 24 h and 48 h treatment, expression of the miR-17-92 cluster was measured by real-time reverse transcription polymerase chain reaction. miR-16 in HT-29 cells (A) and small nucleolar RNA, C/D box 48 (RNU48) in MIA PaCa-2 cells (B) were used as internal controls. Values are fold-change in the expression of each microRNA of the miR-17-92 cluster/internal control compared with the untreated control. Data are the means $\pm$ SD (n=3). *p<0.05 and **p<0.01 compared to the untreated control.

Figure 4. PD0325901 down-regulates c-MYC and up-regulates phosphatase and tensin homolog (PTEN). HT-29 (A, C) and MIA PaCa-2 (B, D) cells were treated with and without 16 μ M PD0325901. After 24 h and 48 h treatment, expression levels of c-MYC and PTEN were measured by western blotting analysis. β -Actin was used as an internal control.

Figure 5. Overexpression of miR-17 reduces the G₁-phase arrest induced by PD0325901. HT-29 (A) and MIA PaCa-2 (B) cells were transfected with a miR-17 mimic or negative control, and then treated with or without PD0325901. After 24 h treatment, the cell-cycle distribution was analyzed by flow cytometry. The percentages of cells in G₁ and S phases of the cell cycle were analyzed using Modfit LT software. Data are the means $\pm$ SD (n=3). **p<0.01, compared to the respective control.

miR-17 mimic also reduced the G₁-phase population not treated with PD0325901. In MIA PaCa-2 cells, overexpression of the *miR-17* mimic also reduced G₁-phase arrest induced by PD0325901 (Figure 5B). On the other hand, overexpression of the *miR-17* mimic did not reduce the G₁-phase population of MIA PaCa-2 cells not treated with PD0325901. These results suggest that suppression of *miR-17* contributes to G₁-phase arrest by PD0325901.

Discussion

The deregulated expression of oncogenic miRNAs is associated with tumor development; therefore, we investigated the effects of a MEK inhibitor on the expression levels of the miR-17-92 cluster, a well-known oncogenic miRNA cluster. As a result, we found that MEK inhibitor PD0325901 suppressed the expression of the miR-17-92 cluster with G₁-phase arrest, down-regulation of c-MYC expression and up-regulation of PTEN expression in human colon cancer HT-29 cells and pancreatic cancer MIA PaCa-2 cells.

It was recently reported that histone deacetylase inhibitors suppressed expression of the miR-17-92 cluster in colon cancer cells (11) and hepatocarcinoma cells (15). However, as far as we are aware, this is the first report that a MEK inhibitor suppressed expression of the miR-17-92 cluster.

We reported previously that another MEK inhibitor, JTP-70902, a derivative of trametinib, suppressed c-MYC expression with G₁-phase arrest in HT-29 cells (8). c-MYC suppression by a MEK inhibitor is explained by the finding that extracellular signal-regulated kinase phosphorylates c-MYC and enhances its stability (16, 17). It has been reported that c-MYC induces expression of the miR-17-92 cluster through transcriptional regulation (3); therefore, we investigated the effect of MEK inhibitor PD0325901 on expression of the miR-17-92 cluster. As a result, PD0325901 suppressed expression of the miR-17-92 cluster with the suppression of c-MYC in HT-29 cells and MIA PaCa-2 cells.

PTEN is the most well-known target molecule of the miR-17-92 cluster (4-6, 13), and up-regulation of PTEN by PD0325901 was observed in both cell lines, as expected. However, knockdown of *PTEN* did not clearly reduce G₁-phase arrest induced by PD0325901 (data not shown). miRNAs inhibit the protein translation of target mRNAs by binding to sites on the target mRNA. Because the base-pair complementarity between the target mRNA and its corresponding miRNA is relatively low, each miRNA may have many different target mRNAs (18). Therefore, not only PTEN but also other target molecules of the miR-17-92 cluster may contribute to G₁-phase arrest through suppression of the miR-17-92 cluster.

Recently, it was reported that PD0325901 suppressed expression of *miR-25*, which is a member of the miR-106b-25 cluster, a paralog of the miR-17-92 cluster (5), with up-regulation of PTEN expression in human melanoma M14 cells harboring a *BRAF* mutation (19). On the other hand, it was also demonstrated that both the miR-17-92 and miR-106b-25 clusters were up-regulated by RAS/RAF signaling using 'Rasless' fibroblasts (20). These results suggest that activation of the mitogen-activated protein kinase pathway induces expression of oncogenic miRNA clusters, including the miR-17-92 and miR-106b-25 clusters, and *vice versa*, a MEK inhibitor might suppress expression of these oncogenic miRNA clusters.

MEK inhibitor PD0325901 is known to up-regulate p15 and p27 (8, 9) and to down-regulate cyclin D1 and c-MYC (8, 10), but suppression of the miR-17-92 cluster by a MEK inhibitor might also, at least partially, contribute to its tumor growth inhibition.

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