Anti-tumor effect of miR-1291 in colon cancer cells

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Abstract

Background: Cancer stem cells (CSCs) are drug-tolerant and cause distant metastasis and recurrence in various cancers, including colorectal cancer (CRC). Thus, CSC-targeted therapy may be an effective curative approach in CRC. MiR-1291 has an anti-tumor effect in carcinoma of kidney, esophagus, pancreas, and prostate. However, there is no report about the effect of miR-1291 on CRC.

Methods: In this study, we took CSC marker DCLK-1 as a target gene, and screened promising miRNAs that may suppress DCLK-1 by using TargetScan Human. We performed luciferase reporter assay, quantitative real-time PCR analysis, and Western blot analysis to verify the interaction between DCLK-1 and miR-1291 in CRC cells. We also confirmed the function of miR-1291 on cancer stemness by identifying the expression of Bmi1 and CD133 in CRC cells by quantitative real-time PCR analysis, Western blot analysis, and flow cytometric analysis, as well as performing sphere formation assay. We also explored the effect of miR-1291 on cell proliferation, invasion, and wound-healing, colony formation, and cell cycle regulation.

Results: We found a 7-base seed sequence of miR-1291 that matches the 3' UTR sequence of DCLK-1 using TargetScan Human. A luciferase reporter assay showed that miR-1291 directly bound the 3' UTR sequence of DCLK-1 and suppressed its expression at both the mRNA and protein levels. In addition, miR-1291 suppressed CSC markers Bmi1 and CD133 as well as sphere formation ability in CRC cells. Moreover, miR-1291 significantly suppressed the proliferation, invasion, wound-healing, and colony formation capability of colon cancer cell lines. MiR-1291 caused altered expression of the cell cycle-regulatory proteins representatively, CDK inhibitors p21^{WAF1/CIP1} and p27^{KIP1}.

Conclusions: Taken together, these findings indicate that miR-1291 has an anti-tumor effect by modulating multiple functions, including, cancer stemness, cell cycle, and invasiveness. Our data suggest that miR-1291 could be a promising nucleic acid medicine against CRC.

Background

Colorectal cancer (CRC) is the third most widespread cancer and the second most deadly cancer in the world. Approximately 1.8 million new cases of CRC and 881,000 related deaths were estimated in 2018 [1, 2]. In the past few decades, improved treatment options have become available, including surgery, radiotherapy, chemotherapy, and molecular-targeted therapy for advanced CRC [3–6]. However, the 5-year survival rate of CRC is < 65% due to cancer relapse [2].

Cancer stem cells (CSCs) possess characteristics associated with stem cells and they are hypothesized to exist as a top class of hierarchy within solid tumors or hematological cancers [7, 8]. Such cells are also believed to have distinct capability for self-renewal, unlimited proliferation, reduced capacity to undergo apoptosis, and multi-potential differentiation [9, 10].

Conventional anticancer drugs and radiotherapy may reduce tumor bulk, but CSCs can still survive, as they confer resistance to these therapies and cause distant metastases and recurrence in various cancers [11–13]. Thus, the development of CSC-targeted therapy may be an effective approach for overcoming the shortage of current therapies and completely cure CRC patients [14–16].

Several CSC markers for CRC have been found, including CD133, Bmi1, and Lgr5 [17–19]. Recently, doublecortin-like kinase 1 (DCLK-1) was demonstrated to be a CSC marker in CRC [20, 21]. DCLK-1 belongs to the protein kinase superfamily and the doublecortin family, and is over-expressed in several human malignancies, including colon, pancreas, kidney, and prostate cancer [22–25]. Excision of DCLK-1-positive CSCs results in regression of the intestinal tumor without apparent impairment of normal tissue, which indicates that DCLK-1 may be a novel target for CSC-targeted therapy [21].

MicroRNAs (miRNAs) are short (18–25 nucleotides) internally originated non-coding RNAs that mainly bind to the 3'-untranslated region (3' UTR) of target mRNAs, contributing to mRNA cleavage or translational suppression [26, 27]. MiRNAs play an important role in many biological progresses, including tumor growth, apoptosis, invasion, and survival [28], which are closely related to oncogenesis and tumor progression. Recent studies have shown that the pathological mechanisms underlying CRC depend on a variety of signaling pathways, including Wnt/ β -catenin, EGFR, TGF- β , TP53, and epithelial-to-mesenchymal transition, and miRNAs play a pivotal role in regulating these pathways [29–31]. For example, miR-4689 has an anti-tumor effect on mutant KRAS CRC by inhibiting the EGFR pathway [32]. In addition, miR-34a can inhibit cell proliferation and increase the expression of p21^{WAF1/CIP1} in HCT116 and RKO colon cancer cells [33]. Through *in silico* analysis and *in vitro* selection, we focused on miR-1291 as a possible upstream modulator for DCLK-1. In recent years, miR-1291 has been demonstrated to have anti-tumor effects in carcinoma of the kidney, esophagus, pancreas, and prostate [34–37]. However, to the best of our knowledge, miR-1291 in CRC has not been reported. Therefore, we investigated the anti-tumor effect of miR-1291 in CRC cells in an effort to improve our understanding of the potential mechanisms of miR-1291 in CRC.

Methods

Bioinformatics

TargetScan Human (http://www.targetscan.org/vert_72/) was used to identify the target miRNAs of DCLK-1. And we extracted candidate miRNAs via Ingenuity Pathway Analysis' microRNA Target Filter.

Cell lines and cell culture

Human CRC cell lines DLD-1, HT29, HCT116, human pancreatic adenocarcinoma cell line Panc-1, and human non-tumor cell line HEK293 were purchased from the American Type Culture Collection (Rockville, MD, USA). These cell lines were authenticated by morphological inspection, short tandem repeat profiling, and mycoplasma testing. DLD-1, HT29, and Panc-1 cells were cultured in RPMI 1640 medium, and HCT116 cells were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal

bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were cultured in the humidified incubator at 37°C and 5% CO₂.

Transduction of the degron reporter

The degron sequence of ornithine decarboxylase (ODC) is recognized directly by proteasomes, which leads to the immediate destruction of the involved protein. The retroviral expression vector pQCXIN-ZsGreen-cODC, containing green fluorescence ZsGreen-labeled degron ODC (Gdeg), was kindly provided by Dr. Frank Pajonk (UCLA's Jonsson Comprehensive Cancer Center, CA, USA). The vector was transfected into Platinum retroviral packaging cells, and the retrovirus collected from the supernatant was used to infect pancreatic cancer Panc-1 cells. Stable transfectants were selected with G418 solution (Roche, Germany). ZsGreen⁺ cells were sorted by the flow cytometry (Cell Sorter SH800, SONY, Japan).

MiRNA and plasmid transfection

Mimic-hsa-miR-1291 (miR-1291):

sense (5'-UGGCCCUGACUGAAGACCAGCAGU-3') and antisense (5'-ACUGCUGGUCUUCAGUCAGGGCCA-3'), and negative control miR (NC): sense (5'-AUCCGCGCGAUAGUACGUA-3') and antisense (5'-UACGUACUAUCGCGCGGAU-3'), mimic-hsa-miR-34a-5p (miR-34a): sense (5'-UGGCAGUGUCUUAGCUGGUUGU-3') and antisense (5'-ACAACCAGCUAAGACACUGCCA-3') were synthesized by Gene Design (Osaka, Japan). Cells were transfected with miRNAs and plasmids using Lipofectamine2000 (Thermo Fisher Scientific, Madison, USA) or Lipofectamine RNAiMAX (Thermo Fisher Scientific).

pmirGLO plasmid vector construction

The 3' UTR of DCLK-1 mRNA was amplified by PCR using the following primer sequences (amplified product size 211 bp): Forward 5'-GCTCGCTAGCCTCGAGCTAGTGTACTGAGCCTGCGG-3', Reverse 5'-ATGCCTGCAGGTCGACTGACTGGTCACATTCCACTG-3'. The amplified products were subcloned and ligated into the multicloning site between Sal I and Xho I in the pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega, Madison, WI, USA) using the In-Fusion HD Cloning Kit (Clontech, Mountain View, CA). The entire sequence (insert and vector) was confirmed by Sanger sequencing.

Luciferase reporter assay

Cells were seeded in 96-well plates at a density of 10,000 cells per well and co-transfected with 50 ng of the pmirGLO plasmid vector and 50 nM of either miR-NC or miR-1291. After 24 hours of transfection, cells were assayed for both firefly and renilla luciferase using the Dual-Luciferase Reporter Assay System (Promega).

RNA isolation

Total RNA, including miRNA, was isolated from cell lines using the miRNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Total RNA concentration and purity were measured using a NanoDrop one spectrophotometer (Thermo Fisher Scientific).

Quantitative real-time PCR analysis of miRNA

We used the TaqMan MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific) to synthesize the complementary DNA from miRNA according to the manufacturer's protocol. TaqMan Universal PCR Master Mix, No AmpErase UNG (Thermo Fisher Scientific), and a LightCycler 480 Real-Time PCR system (Roche Diagnostics, Mannheim, Germany) was used to quantify miRNA, and RNU6B was used as the endogenous control. Relative expression was quantified with the $2^{-\Delta\Delta C_t}$ method.

Quantitative real-time PCR analysis of messenger RNA

A High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) was used to synthesize the complementary DNA from mRNA according to the manufacturer's protocol. qRT-PCR was amplified using oligonucleotide primers and the LightCycler 480 Real-Time PCR system (Roche). The amplification products were detected using the THUNDERBIRD SYBR qPCR Mix (TOYOBO, Osaka, Japan), and the level of target gene expression was calculated. The expression of the target gene was normalized to endogenous GAPDH expression. Relative expression was quantified by the $2^{-\Delta\Delta C_t}$ method. The PCR primers are listed in Table S1.

MiRNA expression

TaqMan miRNA analysis (Applied Biosystems) was used to measure miRNA expression. The reverse transcription reaction was performed with the TaqMan MicroRNA RT Kit (Applied Biosystems) according to the manufacturer's protocol. Quantitative real-time PCR was performed using the 7900 HT Sequence Detection System (Applied Biosystems). Amplification data were normalized to endogenous RNU6B expression. The relative expression level was quantified by the $2^{-\Delta\Delta C_t}$ method.

Proliferation assay

Cells were seeded in 96-well plates at a density of 4000-8000 per well and were transfected with miR-NC or miR-1291 at a final concentration of 30 nM the second day after seeding. Twenty-four, 48, and 72 hours after transfection, 10 μ l of Cell Counting Kit-8 (DOJINDO Molecular technologies, Inc., Kumamoto, Japan) was added to each well, and the 96-well plates were shaded for 2 hours. After that, the absorbance was detected by Multiskan Go (Thermo Fisher Scientific) to determine cell number.

Matrigel invasion assay

Cells were seeded in BD BioCoat Matrigel Invasion Chambers (BD Biosciences, San Jose, CA, USA) at a density of 50,000-100,000 cells per chamber. The cells were transfected with the miRNAs at a final concentration of 50 nM. After 48-72 h of transfection, invaded cells were stained with hematoxylin.

Wound healing assay

Cells were seeded in ibidi culture 2-well inserts (ibidi, Gräfelfing, Germany) in 24-well plates at a confluent density. The inserts were removed after 24 hours to create wounds. The miRNAs were transfected at a final concentration of 30 nM. The areas of the wounds were measured at 0-48 hours using ImageJ software.

Colony formation assay

Cells were transfected with miR-NC or miR-1291 at a final concentration of 30 nM for 8 hours and then seeded in 6-well plates at a density of 500 cells per well. After 10 days, the cells were stained by crystal violet and counted.

Cell cycle assay

Cells were starved in serum-free medium for 48 hours. Twenty-four hours before the end of starvation, miR-NC or miR-1291 was transfected at a final concentration of 30 nM (Fig. S1). Cells were collected at the indicated times (0, 12, 24, 48 hours), fixed in 70% ethanol for 30 minutes at 4°C. After fixation, cells were washed twice with PBS and incubated with RNase (Sigma Aldrich, St. Louis, MO, USA) for 20 minutes at 37°C. Cells were treated with PI (Dojindo) for 20 minutes on ice and analyzed by flow cytometry (Spectral Analyzer SA3800, Sony Biotechnology, Inc., Tokyo, Japan).

Western blot analysis

Cells were seeded in 6-well plates at a density of 100,000-200,000 per well and then transfected with miR-NC or miR-1291 at a final concentration of 30 nM the next day. After 48 and 72 hours, cells were lysed with RIPA buffer (0.05 M Tris-HCl pH 7.6, 0.15 M NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% SDS) with 1% proteinase inhibitor cocktail (Nacalai Tesque, Kyoto, Japan). The protein samples were electrophoresed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride transfer membrane (PVDF, Bio-Rad, Hercules, CA, USA). The membranes were incubated with primary antibodies, including anti- β -actin ((13E5) Rabbit mAb #4970, Cell Signaling Technology, Danvers, MA, USA), and anti-DCLK-1 (ab31704, Abcam), anti-Bmi1 (10832-1-AP, Thermo Fisher Scientific), anti-p21^{WAF1/CIP1} (ab80633, Abcam, Cambridge, UK), anti-p27^{KIP1} (sc-528, Santa Cruz Biotechnology, Dallas, TX, USA), anti-CDC25A (#3652, Cell Signaling Technology), anti-CDC25B (#9525, Cell Signaling Technology), anti-CDC25C ((5H9) Rabbit mAb #4688, Cell Signaling Technology), anti-CDK4 (MAB8879, Merck Millipore, Burlington, MA, USA), anti-CDK6 (SAB4300596, Sigma-Aldrich), anti-Cyclin D1 (#2922, Cell Signaling Technology), anti-Cyclin E1 (sc-247, Santa Cruz Biotechnology), anti-Rb (ab24, Abcam). HRP anti-mouse IgG antibody (NA931, GE Health Care, Little Chalfont, UK) and anti-rabbit IgG antibody (NA934, GE Health Care) were used as secondary antibodies. The bands were visualized by the ECL Detection System (GE Health Care).

Sphere formation assay

Single cells were seeded 24 hours after transfection of miR-negative control or miR-1291 in 96-Well Clear Ultra Low Attachment Microplates (Corning Inc., USA) at the density of 1000 cells per well. And the cells were cultured in DMEM/F-12 serum-free medium (Invitrogen, USA) supplemented with 20 ng/ml epithelial growth factor, 10 ng/ml basic fibroblast growth factor-2 (PeproTech, USA), and 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were cultured in the humidified incubator at 37°C and 5% CO₂. The number of spheres ≥40 µm was counted 4 days after seeding.

Flow cytometric analysis for CD133 marker expression

Suspensions of HCT116 single cells were stained with antibodies against human CD133 (APC-conjugated, No. 130-113-106, Miltenyi Biotec, Bergisch Gladbach, Germany). Dead cells were excluded by utilizing forward and side scatter. One million cells were incubated with antibodies on ice for 20 minutes in the dark, centrifuged, and washed twice with PBS containing 2% FBS. Spectral Analyzer (SA3800, Sony Biotechnology, Inc.) was used for flow cytometric analysis.

Statistical analysis

Data are presented as the mean ± SEM. Statistical analyses were performed using GraphPad Prism 5 (San Diego, CA, USA) and Microsoft Excel. The statistical differences between the miR-NC and miR-1291 groups were analyzed by student's t-test (two-tailed). Comparisons among more than two groups were performed by one-way analysis of variance (ANOVA). $P < 0.05$ was considered significant.

Results

Screening of candidate miRNAs

Firstly, we identified 1749 miRNAs that target to DCLK-1 in TargetScan Human. Using Ingenuity Pathway Analysis' microRNA Target Filter, we screened candidate miRNAs whose target genes correlate with Notch Signaling, Wnt/β-catenin Signaling or Wnt/Ca²⁺ Signaling pathway. Eventually thirty candidate miRNAs were selected for cell viability assessment (Fig. 1a). For the assessment, we made a CSC model by transducing ornithine decarboxylase (ODC)-degron to pancreatic cancer Panc-1 cells [38] to test the effects of these miRNAs on stem (degron (+)) cells and non-stem (degron (-)) cells. MiR-34a, which reached phase I clinical trial as a therapy for human solid tumor [39], was used as a therapeutic control in this experiment. Among these 30 miRNAs, miR-1291 (the 16th miRNA) obviously suppressed both the stem (degron (+)) and non-stem (degron (-)) Panc-1 cells viability compared to either negative control miR (NC) or even positive control miR-34a (Fig. 1b). This result is consistent with the report that miR-1291 presented anti-tumor function in pancreatic cancer [36]. However, there is no previous report about miR-1291's effect in CRC, therefore, in this study we tried to clarify the function of miR-1291 in CRC.

MiR-1291 directly targeted DCLK-1

Using miRNA target prediction algorithm TargetScan Human, we identified a target site in the 3' UTR of DCLK-1 mRNA that is complementary to the seed sequence of miR-1291 (Fig. 2a). MiR-1291 significantly suppressed the expression of DCLK-1 at both the mRNA and protein levels (Fig. 2b, c). Using plasmid containing the 3' UTR sequence of DCLK-1, miR-1291 significantly inhibited luciferase activity in the luciferase reporter assay in DLD-1, HT29, and HCT116 cells, indicating a direct interaction between the DCLK-1 3' UTR and miR-1291 (Fig. 2d). These findings indicate that CSC marker DCLK-1 is a direct target of miR-1291.

MiR-1291 suppressed CRC stem-like properties

We assessed stemness of CRC cell after treatment of miR-1291. We demonstrated that other CSC markers in addition to DCLK-1, including Bmi1 and CD133, were significantly down-regulated by miR-1291 overexpression at mRNA level (Fig. 3a, b). MiR-1291 treatment also down-regulated the protein level of Bmi1 by Western blot analysis (Fig. 3c). Moreover, the ratio of CD133 positive cells decreased with treatment of miR-1291 by flow cytometric analysis (Fig. 3d). Furthermore, miR-1291 treatment significantly decreased the ability of sphere formation in HCT116 cells (Fig. 3e). These findings suggest that miR-1291 may be involved in the regulation of stem cell properties through direct inhibition of DCLK-1.

Expression of miR-1291 in CRC cells

We evaluated the expression of miR-1291 in nine CRC cell lines and non-tumor human HEK293 cells by qRT-PCR. The expression of miR-1291 was apparently lower in five of the nine CRC cell lines compared to HEK293 cells (Fig. S2).

MiR-1291 overexpression after transfection

Either 4 hours or 24 hours after transfection of miRNAs, miR-1291 transfection group presented 1000 to 5000-fold higher miR-1291 expression than miR-NC transfection group in DLD-1, HT29, and HCT116 cells (Fig. 4a).

MiR-1291 inhibited cell proliferation

To confirm the anti-tumor effect of miR-1291 in CRC cell growth, we detected the absorbance of cells transfected with miR-NC or miR-1291 as determined by Cell Counting Kit-8. The miR-1291 transfection group presented a significant low absorbance compared to the miR-NC transfection group in DLD-1, HT29, and HCT116 cells after 48 and 72 hours of transfection (Fig. 4b). miR-1291 significantly suppressed the proliferation ability of the three cell lines.

MiR-1291 inhibited cell invasion

miR-NC or miR-1291 was transfected into CRC cells to evaluate the effect on invasion ability. The cells invading through Matrigel were stained with hematoxylin 48 hours for DLD-1 or 72 hours for HT29 and

HCT116 after transfection and then counted. The invasion ability of miR-1291-transfected cells was significantly inhibited compared to miR-NC-transfected cells in the three cell lines tested (Fig. 4c).

MiR-1291 inhibited cell migration ability

We evaluated the effect of miR-1291 on the wound-healing ability of CRC cells. The wound area of the cells was measured at the same location every 24 hours after miR transfection. The migration ability of DLD-1, HT29, and HCT116 cells was significantly suppressed in the miR-1291 group compared to the miR-NC group either at 24 hours or 48 hours or both (Fig. 5a).

MiR-1291 inhibited colony formation ability

To confirm the function of miR-1291 in cell colony formation ability, we observed and evaluated the colony formation of the CRC cells which were transfected with miR-NC or miR-1291. The colonies were stained with crystal violet 10 days after transfection, and we counted the number of the colonies. MiR-1291 significantly inhibited the colony formation potential compared to miR-NC in DLD-1, HT29, and HCT116 cells (Fig. 5b).

Effect of miR-1291 on cell cycle regulation

To determine the mechanism underlying the growth inhibitory effect of miR-1291, we performed cell cycle analysis in DLD-1 and HT29. Twelve hours refed with FBS, cells in G1 phase were significantly increased in miR-1291 treated cultures of DLD-1 cells (60.81% vs 67.89%, Fig. 6a). On the other hand, cell population in each cell cycle phase was not apparently changed in HT29 cells throughout the time points examined (Fig. 6a, Fig. S3).

Altered expression of cell cycle components when treated with miR-1291

Cells were examined for the change in cell cycle regulatory protein expression after transfection of miR-1291. In DLD-1 cells, the expression of CDK inhibitors p21^{WAF1/CIP1} and p27^{KIP1} were up-regulated and CDK4 and CDC25A level decreased at 48 hours after transfection with treatment of miR-1291 and these changes maintained till 72 hours (Fig. 6b). In HT29 cells, on the other hand, the expression of p21^{WAF1/CIP1} and p27^{KIP1} protein slightly increased at 48 hours but returned back to the basal levels of negative control cultures at 72 hours. Instead, Cyclin E1, CDK4, and CDK6 subsequently decreased at 72 hours with treatment of miR-1291 (Fig. 6c).

Discussion

DCLK-1 is over-expressed in subsets of cancers and has an oncogenic function [40, 41]. In the Caki-2 renal cancer cell line, knocking down DCLK-1 contributes to inhibition of cell proliferation, invasion, and wound-healing [42]. In 2013, DCLK-1 was reported as a marker distinguishing tumor stem cells from intestinal normal stem cells [21]. Accumulating evidence supports the involvement of DCLK-1 in the stemness of CRC [43, 44]. In epigenetic regulation of DCLK-1, miR-137 suppresses the ability of cell

growth by inhibiting the expression of DCLK-1 in the SW480 CRC cell line [45]. In the present study, we considered a CSC marker DCLK-1 as the target molecule, and extracted 30 miRNAs that possibly inhibit DCLK-1 and correlate with cancer stemness signal pathways including Notch Signaling, Wnt/ β -catenin Signaling or Wnt/ Ca^{2+} Signaling pathway [43, 46, 47]. Among the candidate miRNAs, miR-1291 presented the notable effect of suppressing cell viability in the ODC-degron transduced Panc-1 cells which is previously reported as cancer stem-like cells [38]. Although Zs-green⁺ cells were more resistant to miR-1291 treatment compared with Zs-green⁻ non-stem cells, its growth inhibitory effect was even stronger than that given by positive control miR-34a.

Studies showed that miR-1291 had anti-tumor effects in multiple cancers including renal cancer, esophagus cancer, pancreatic cancer, and prostate cancer [34–37]. As the target for miR-1291, several molecules have been identified. These include SLC2A1/GLUT1 in A498 and 786-O renal cancer cells [34], MUC1 in human esophagus cancer EC9706 and EC-1 cells [35], the forkhead box protein A2-anterior gradient 2 (FOXA2-AGR2) pathway in PANC-1 pancreatic cancer cells [36]. Furthermore, miR-1291 has been shown to inhibit cell growth and tumorigenesis in prostate cancer by binding to MED1 [37]. However, to the best of our knowledge, there is no previous report of miR-1291 in CRC which is one of the widespread cancers in the world.

We verified that miR-1291 directly bound to the 3' UTR of the DCLK-1 mRNA sequence, leading to decreased expression of DCLK-1 at both the mRNA and protein levels. In addition to DCLK-1, miR-1291 lowered Bmi1 and CD133 expression, which are also representative CSC markers in CRC. Moreover, we confirmed that miR-1291 inhibited sphere formation ability of the CRC cells. These results support the notion that miR-1291 suppresses the cancer stemness through direct targeting DCLK-1, suggesting that miR-1291 may be a novel CSC-targeted therapeutic strategy for CRC.

We also found that the replacement therapy using mimic-miR-1291 played various anti-tumor roles in CRC cells, suppressing the cell proliferation, invasion, cell mobility, and colony-forming abilities. These findings suggest that miR-1291 could serve as an inhibitor in CRC tumors.

One of the major effects given by miR-1291 was drastic change in the cell cycle components. Time course study after serum starvation for DLD-1 showed the delay in G1-S transition at 12 hours. Concordantly, Western blot analysis showed increase in CDK inhibitors p21^{WAF1/CIP1} and p27^{KIP1} which bind to and block the G1-S accelerators Cyclin D1-CDK4/6 complex and Cyclin E1-CDK2 complex, leading to restraint from the G1 to S phase [48–50]. We also found that CDK4 and CDC25A were decreased. Since CDC25A is a crucial mediator that positively regulates the Cyclin D1-CDK4/6 complex and Cyclin E1-CDK2 complex [51], downregulation of CDC25A could be another possible explanation for G1 phase arrest and these schemes are simply summarized in Fig. S4. Although we could not find significant dysregulation of cell cycle in the serum-starved HT29 cells, marked reduction of G1-S facilitating modulators Cyclin E1, CDK4, and CDK6 was observed at the later time point 72 hours. In addition, CDC25B that positively acts in the G2-M transition [52, 53] was also down-regulated at 72 hours.

Collectively these results suggest that miR-1291 could cause dysregulation of cell cycle control even though its effectual action point and timing may differ by cell types.

In terms of the association of cell cycle regulators with DCLK-1, Chandrakesan *et al.* reported that DCLK-1-positive cells had higher expression of p21^{WAF1/CIP1} and p27^{KIP1} and maintained quiescence in normal small intestine [54]. However, the current study demonstrated that miR-1291 inhibited DCLK-1 and up-regulated p21^{WAF1/CIP1} and p27^{KIP1}, which caused G1 phase arrest. These findings indicate an opposite role of DCLK-1 in the cell cycle between normal and cancer cells. Further studies are required to define how DCLK-1 is associated with the cell cycle in CRC.

Conclusions

In conclusion, miR-1291 presented a strong anti-tumor effect on CRC cells and played an important role in both delaying the cell cycle and suppressing cancer stemness. To the best of our knowledge, this is the first study that demonstrates the function of miR-1291 in CRC. Considering the anti-tumor effect of miR-1291 in the broad range of cancer type [34–37], this microRNA may be one of the candidates for the next generation nucleic acid medicine using the practical DDS systems [30, 31, 55, 56].

Declarations

Ethics approval and consent to participate: This study was performed in accordance with the Declaration of Helsinki and the study was approved by the Ethics Board of Osaka University (approval No. 13377-5; Osaka, Japan).

Consent for publication: This study includes no records and samples from patients. Consent for publication is not applicable.

Availability of data and materials: This study does not use specific database and materials.

Competing interests: The authors have no competing interests to declare.

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for data collection.

Authors' contributions: All authors have read and approved the manuscript. Conceptualization, H.Y. and M.M.; Supervision, H.Y., M.M., H.T., H.H.; methodology, X.W., Y.Y., N.T., N.H., S.T.; validation, M.H., M.O.; investigation, J.W., S.B., H.H., X.W. R.I.; software, K.T.; writing-original draft, J.W.; review and editing, A.I., H.Y., T.H., N.N.

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Abbreviations

Abbreviation	Definition
CSC	cancer stem cell
CRC	colorectal cancer
DCLK-1	doublecortin-like kinase 1
miRNA	microRNA
DMEM	dulbecco's modified Eagle's medium
FBS	fetal bovine serum
ODC	ornithine decarboxylase
Gdeg	ZsGreen-degron ODC
miR-1291	mimic-hsa-miR-1291
NC	negative control miR
miR-34a	mimic-hsa-miR-34a-5p
ANOVA	one-way analysis of variance
FOXA2-AGR2	forkhead box protein A2-anterior gradient 2

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Figures

Figure 1

a

b

Figure 1

Screening of candidate miRNAs. (a) There were 1749 miRNAs target to DCLK-1 in TargetScan Human. Among the 1749 miRNAs, 30 miRNAs whose target genes related to Notch Signaling, Wnt/β-catenin Signaling or Wnt/Ca²⁺ Signaling pathway were extracted for cell viability experiment. (b) Ornithine decarboxylase (ODC)-degron transduced pancreatic cancer Panc-1 cells were used as a CSC model to test the effects of these miRNAs on stem (degron (+)) cells and non-stem (degron (-)) cells. Cell viability was

evaluated by Cell Counting Kit-8 at 72 hours after transfection. Cell viability by each treatment was normalized to that of control cells without transfection. MiR-34a, a putative Anti-OncomiR was used as a positive control in this experiment. At 72 hours after transfection, among these 30 miRNAs, miR-1291 (the 16th miRNA) significantly inhibited the cell viability in both the stem (degron (+)) and non-stem (degron (-)) cell groups compared to either miR-NC group or positive control miR-34a.

Figure 2

Figure 2

MiR-1291 directly targeted the 3' UTR of DCLK-1. (a) MiR-1291 predictively binds the 3' UTR of DCLK-1 based on a 7-base seed sequence (underlined). (b) The effects of miR-1291 on the expression of DCLK-1 were assessed by quantitative real-time PCR. MiR-1291 significantly inhibited the expression of DCLK-1 mRNA in HCT116 cells. GAPDH was utilized as an endogenous control. (c) Western blot showed that miR-1291 obviously decreased DCLK-1 protein expression after 48 and 72 hours of transfection. β -actin was used as a loading control. (d) In DLD-1, HT29, and HCT116 cells, miR-1291 significantly suppressed the luciferase activity of the plasmid containing the binding site in the 3' UTR of DCLK-1.

Figure 3

Figure 3

MiR-1291 suppressed the stemness of CRC cells. (a, b) Quantitative real-time PCR was performed to evaluate the stemness of CRC cells. The expression of stem cell markers Bmi1 and CD133 was significantly decreased by miR-1291. GAPDH was utilized as an endogenous control. (c) MiR-1291 suppressed Bmi1 protein expression after 48 and 72 hours of transfection compared to the negative control miR (NC). β -actin was used as a loading control. (d) Flow cytometry showed that the ratio of stem cell surface marker CD133 was decreased by miR-1291 compared to the negative control miR (NC). (e) The sphere formation ability was significantly suppressed in miR-1291 transfected HCT116 cells compared to the negative control miR (NC). The number of spheres $\geq 40 \mu\text{m}$ was counted 4 days after seeding. Representative images are shown on the left. In a, b, and e, the experiments were performed more than three times, and data are presented as mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 4

Figure 4

Inhibition of colorectal cancer cell malignancy by miR-1291. (a) miR-1291 exhibited 1000-fold higher overexpression than negative control miR (NC) after 4 or 24 hours of transfection. RNU6B was utilized as an endogenous control. (b) MiR-1291 significantly inhibited the proliferation of DLD-1, HT29, and HCT116 cells compared to negative control miR (NC). The absorbance at 450 nm was measured to determine cell number. (c) Invasion ability was significantly inhibited in DLD-1 cells at 48 hours, HT29 cells at 72 hours,

and HCT116 cells at 72 hours after transfection of miR-1291. Representative images are shown on the left. All experiments were performed more than three times. All data are presented as mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 5

Figure 5

The effects of miR-1291 on cell migration and colony-forming ability in colorectal cancer cells. (a) Wound healing assay in DLD-1, HT29, and HCT116 cells treated with negative control miR (NC) or miR-1291. The

wound area was measured at the indicated times by ImageJ software. (b) The colony-forming ability was suppressed in DLD-1 and HT29 cells, but not in HCT116, 10 days after transfection with miR-1291. All experiments were performed more than three times. All data are presented as mean $\pm$ SEM. *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 6

Figure 6

MiR-1291 suppressed the cell cycle progression from G1 to S phase. (a) Cell cycle analysis by flow cytometry after treatment with negative control miR (NC) or miR-1291. MiR-1291 increased the percentage of cells in G1 phase and decreased the percentage of cells in G2/M phase in DLD-1 but not in HT29 cells compared to NC. (b, c) The expression of cell cycle-related proteins was evaluated by Western blot analysis. Treatment of miR-1291 up-regulated the expression of p21WAF1/CIP1 and p27KIP1 in both DLD-1 and HT29 cells, and down-regulated the expression of CDC25A and CDK4 in DLD-1 cells and Cyclin E1, CDK4, and CDK6 in HT29 cells. β -actin was used as a loading control.

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